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**DERMAL AND EPIDERMAL CELL FUNCTIONS
IN THE GROWTH AND REGENERATION OF HAIR FOLLICLES AND
OTHER SKIN APPENDAGES**

AHMAD GHARZI

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A thesis presented for the degree of Doctor of Philosophy

University of Durham

May 1998

30 SEP 1998

I hereby declare that this thesis is entirely the result of my own work. It has not been presented in part or in whole previously for any degree in this or any other university.

Ahmad Gharzi

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Abbreviations

ASMA	Alpha smooth muscle actin
BDMA	n-Benzyl dimethylamine
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxy-uridine
CAM	Chorioallantoic membrane
DDSA	Dodecyl succinic anhydride
DMSO	Dimethyl sulphoxide
DP	Dermal papilla
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFG	Epidermal growth factor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluro isothiocyanate
GAG	Glycosaminoglycan
GE	Germinative epidermal
IGF	Insulin-like growth factor
KGF	Keratinocyte growth factor
LDS	Lower dermal sheath
LCAM	Liver cell adhesion molecules
LEF1	lymphoid enhancer factor 1
MEM	Minimum essential medium
MHC	Major histocompatibility complex
NCAM	Neural cell adhesion molecules
ORS	Outer root sheath
PBS	Phosphate buffered saline
SF	Skin fibroblast
Shh	Sonic hedgehog
TA	Transient amplifying
TGF	Transforming growth factor
UDS	Upper dermal sheath

Abstract

Epithelial-mesenchymal interactions are central to the development of skin and skin appendages in vertebrates. These interactions continue throughout adult life and underpin the cyclic growth and loss of hair in mammals. While the molecular basis of such interactions are being gradually uncovered, at the cellular level many questions remain unanswered. For example, the localisation and role of hair follicle epithelial stem cells remains a subject of debate, as does the function of the dermal sheath component. In embryonic appendage development there is strong evidence for common signalling mechanisms, but the degree to which epithelial-mesenchymal communication diverges in different adult appendages remains as yet undiscovered.

I have studied the replicative abilities of germinative epidermal (GE) cells of the rat vibrissa follicle by single and repeated plucking of fibres from individual follicles. In both cases, the cellular events following fibre removal were scrutinised at intervals up to 9 days using histology, and cell proliferation and cell death markers. Follicles that were repeatedly plucked had their growing hairs measured at regular intervals.

By analysing cell proliferation patterns I found that the new regenerated epidermal matrix came from residual GE cells left in the follicle base - after both single and repeated depilations. Indeed plucking appeared to cause an initial inhibition of proliferative activity in the follicle upper outer root sheath, the other candidate region for supplying a new matrix. Length measurements of the regenerated hairs demonstrated that the repeatedly plucked follicles produced total cumulative lengths of fibre between 60 and 265% longer than expected, as determined by measuring the original club fibre lengths. *In vivo* amputation of plucked follicles demonstrated that the residual GE cells have the ability to regenerate a new matrix and new fibre without any contribution from cells from the upper region of the follicle.

These studies, along with *in vitro* observations of prolonged replicative abilities of bulb cells suggest that the GE cells have a proliferative capacity which is beyond that of one cycle. This raises the possibility that GE cells are not transient amplifying cells with limited proliferative potential and strongly suggests that the duration of anagen cannot be attributed to the replicative limitations of the GE cells.

The behaviour and interactive abilities of dermal cells isolated from three different skin appendages (rat vibrissa follicle, rat claw unit, and pigeon feather follicle) were characterised by cell culture, immunohistochemistry and dermal-epidermal recombinations. Dermal cells from all the above appendages demonstrated common aggregation properties in culture and all expressed α -smooth muscle actin. When recombined with epidermal cells and implanted onto host rats, dermal sheath cells from the lower part of vibrissa follicles produced a robust skin-like structure with a normal

and continuous basement membrane at the dermal-epidermal interface. Rat vibrissa follicle papillae were able to induce formation of new skin appendages in heterotopic sites, but this ability was not found with claw dermal cells. Moreover, the feather papilla was unable to initiate any interactions in association with vibrissa epidermis when implanted onto athymic rats. These results showed that while the adult hair follicle dermal cells display a high level of interactive capacity, this is possibly not universal in all skin appendages.

Chapter 1

Introduction

1.1 Skin

Skin is a remarkable and versatile organ. It covers the whole body and serves as a protective shield against physical and chemical attack. It holds in the body's fluids and maintains its integrity by keeping out foreign substances and microorganisms (Montagna, 1965). About 15% of the body weight in mammals is skin. As well as its more obvious protective role the skin has a number of other important functions which are related to its position being at the interface between the organism and the external environment. It serves as a sense organ, temperature regulator, a metabolic organ involved in the metabolism and storage of fat, and a reservoir for food and water. It also acts as a facilitator of the two-way passage of gases through it, the seat of origin of vitamin D (which has a role in calcium and phosphate metabolism), and as a principle organ for sexual attraction (Ashley, 1978).

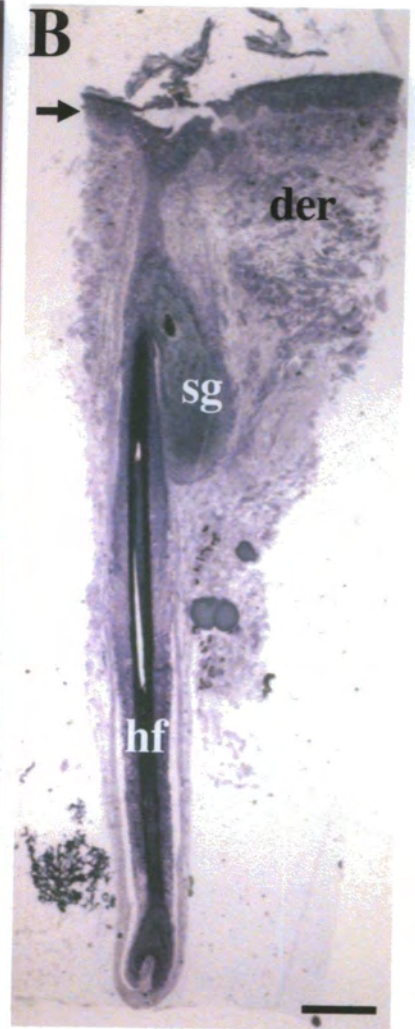
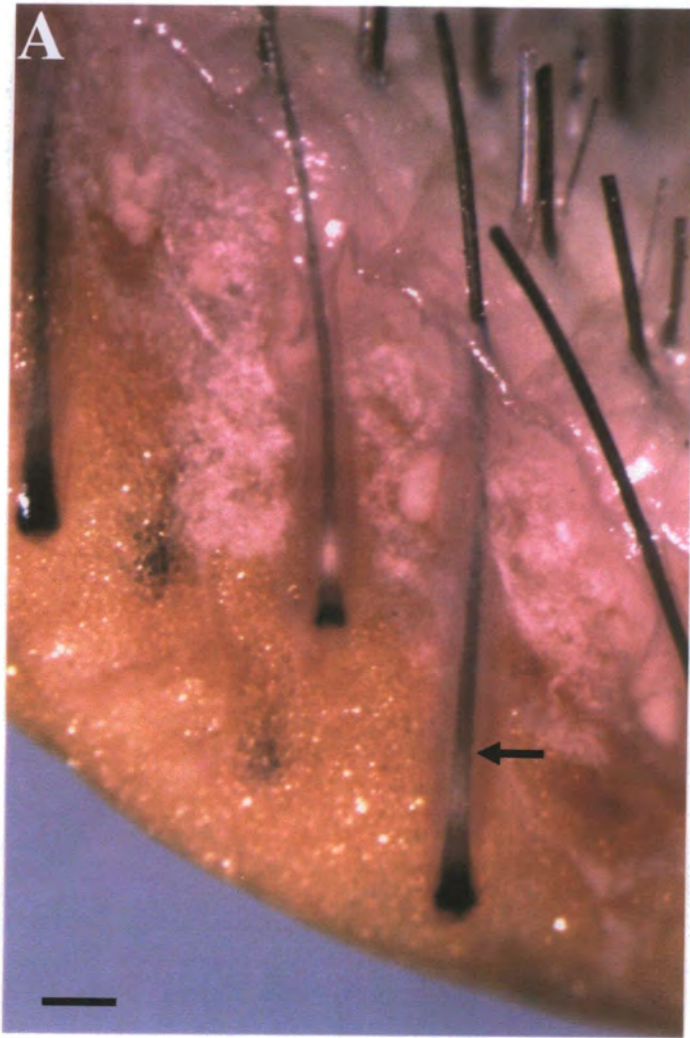
1.2 Structure of skin

Skin is composed of two principal layers, the epidermis and dermis (Fig.1.1).

1.2.1 Epidermis

Epidermis is ectodermal in origin and forms most of the cutaneous appendages including glands (sweat and sebaceous), hair, feather, horn, scale, nail and claw (Kent, 1969). The epidermis consists mainly of a multilayer of epidermal cells, or keratinocytes so named because of the prevalence of intermediate filaments composed of keratin. The epidermis has been regarded as a two-compartment system, the lower "proliferative" compartment encompasses basal and some suprabasal cells, functioning to maintain the germinative cell population and to supply cells to the upper "differentiative" compartment (Lavker & Sun, 1983). Within this compartment epidermal cells produced by the basal layer move upward while undergoing a series of morphological and biochemical changes called terminal differentiation. They finally die and are lost by shedding from the epidermal surface as dead, flattened sheets of keratinized cells. In order to replace the lost cells from the surface and to maintain its steady state, the

Fig. 1.2) schematic representation of different stages in development of feather follicles. After induction occurs between ectoderm and mesenchyme (A), the two components become feather placode and dermal condensation respectively (B). They grow together to form feather buds (C). Elongated feather buds (D) then invaginate into the skin to form feather follicle (E) in which the mesodermal components become dermal papilla while the epidermal components form the papillar ectoderm (reservoir of future feathers), the collar (major sites of cell proliferation) and the feather filament. As a result of cell death in marginal and axial plates (F) the branched structure of the mature feather (G) forms.



epidermis needs constant cell proliferation. It has been postulated that the epidermis continuously supplies the required cells through a population of specific cells called “stem cells” which are almost certainly situated in the basal layer (Jones, 1996). Stem cells are also thought to be responsible for regeneration of the tissue after injury, for example, during wound healing the lost part of the epidermis regenerates cells from its basal layer or from the outer root sheath of the hair follicle, thought to be another source of stem cells (Lavker *et al.*, 1993).

In addition to keratinocytes, the epidermis also contains other cell types including melanocytes, Langerhans cells and Merkel cells. Melanocytes are derived from the neural crest and lie in the basal layer of the epidermis. The outstanding function of melanocytes in the skin is the synthesis melanin via a multistep process that starts with an enzymatic oxidation of the amino acid L-tyrosine which is then catalysed during a biochemical cascade, and finally culminates in the formation of melanin pigment (see Slominski & Paus, 1993). The melanin which is packed in membrane-enclosed vesicles called melanosomes, is then transferred through cytoplasmic processes from melanocytes into the keratinocytes of the basal layer. Langerhans cells are bone marrow derived and during the embryonic stage enter the epidermis (Kim & Holbrook, 1995). These cells are frequently found in epidermis and they are characterised by slender cell processes radiating from the cell body extending into spaces between the surrounding keratinocytes. The function of the Langerhans cells is now known as an element of the body’s immune system where they act like macrophage cells. Merkel cells like keratinocytes are epidermal in nature and are found in the epidermal ridges of developing epidermis as well as the outer root sheath of developing hair follicles (see Kim & Holbrook, 1995). In adult human hair follicles these cells are distributed in the presumptive bulge area (Narisawa *et al.*, 1994a). Merkel cells are neuroendocrine cells and thought to be involved in sensory perception (Kim & Holbrook, 1995).

1.2.2 Dermis

Beneath the epidermis is the dermis, a thick and tough layer of connective tissue, which arises from the embryonic mesoderm (Browder *et al.*, 1991). In comparison with the epidermis it contains relatively loosely packed cells, mainly fibroblasts with some other cell types including lymphocytes, macrophages and mast cells. Fibroblasts are

usually flattened stellate cells with long ovoid nuclei. These cells not only play a central part in the morphogenesis of skin and its appendages during embryonic development but their presence is also vital during postnatal life. Fibroblasts continuously support the skin and repair it after wounding. When skin is injured the fibroblasts nearby migrate into the wound, proliferate and produce large amounts of collagen matrix which help to repair the damaged site (Young, 1975). Moreover, they synthesise and secrete macromolecules, which constitutes majority of the extracellular matrix (ECM). In addition to its role in determining the physical property of the dermis and skin in general, it is now known that ECM provides an appropriate environment through which cells can migrate and a substratum for their adhesion and guidance. Two main classes of ECM molecules are glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and fibrous proteins, which are functionally divided into two groups. One group is structural proteins consisting of collagen and elastin and the other, adhesive proteins, that help cells attach to the matrix. The main adhesive proteins of the ECM are fibronectin, laminin and tenascin (Bard, 1990).

Within the dermis there is also a sparse population of macrophages which have the ability for phagocytosis. Macrophages not only remove dead cells and cellular debris but they also form the first line of body's defence against infection by ingesting and destroying invading bacteria. They also have a role in immunological defence system through processing and presenting antigens to lymphocytes capable of producing protective antibodies. Lymphocytes are represented in small numbers in the dermis and play key roles in the immune system. Mast cells are very large cells distinguished by having numerous basophilic granules in their cytoplasm. They are sparsely distributed in the dermis and are also involved in the immune system (Fawcett, 1994).

1.2.3 Basal lamina

At the dermal-epidermal junction there is a flexible thin (40-120 nm thick) layer which is composed of ECM components (Yamane *et al.*, 1996). This layer normally called the basal lamina, separates the two compartments and supports the epidermis. The epidermal basal cells adhere to this structure via specialised, calcium activated adhesion plaques, termed hemidesmosomes (Jones & Green, 1991). In addition to mechanical support, the basal lamina transmits messages between the dermis and epidermis. This is

believed to play an important role during embryonic development of skin and its appendages (Westgate *et al.*, 1984). Furthermore, the basal lamina determines cell polarity, influences cell migration, organises the proteins in the plasma membranes of adjacent cells, induces cell differentiation and serves as a specific highway for cell migration (Alberts *et al.*, 1994). With transmission electron microscopy it has been shown that the basal lamina consists of two distinct layers: an electron-dense layer (lamina densa) subjacent to the epidermis but separated from it by an electron-lucent layer (lamina lucida). In some tissues, there is also a third layer (lamina fibroreticularis) which connects the basal lamina to the underlying connective tissue. Sometimes the term “basement membrane” is used to describe the composition of all three layers.

The precise composition of the basal lamina can be variable, but according to most references it usually contains type IV collagen, laminin, fibronectin, hyaluronic acid, heparan sulphate and some other proteoglycans (Stanley *et al.*, 1982; Yurchenco & Schittny, 1990; Fine, 1994; Yamane *et al.*, 1996). Collagen type IV is the major and universal structural component of the lamina. It forms the meshwork of the basal lamina, which other components bind to. Laminin is one of the first ECM proteins synthesised in the developing embryo and has adhesion sites for the cell membrane. Proteoglycans have many functions in the maintenance of tissue structure, for example, they have roles in relation to cell adhesion and motility, proliferation and differentiation (Kjellen & Lindahl, 1991). Fibronectin is an adhesive glycoprotein and is connected with many biological processes including cell attachment and migration (see Hynes & Yamada, 1982; Ruoslahti, 1988).

1.3 Morphogenesis of skin

The primitive integument of an embryo is formed by two major embryological elements: the epidermis which originates from a surface area of the early blastula, and mesoderm or mesenchyme which is brought into contact with the inner surface of the epidermis during gastrulation (Rook *et al.*, 1992). Early on in development, the epidermis consists of a single layer of cells. Later it becomes double, having an external flattened periderm and an inner stratum germinatum (basal layer) which is the reproductive layer. In the meantime, the mesenchyme condenses below the germinative layer and the rudiment of the future dermis is formed. Then in the epidermis a third layer

of cells the stratum intermedium (intermediate layer) appears between the periderm and germinative layer. Subsequently the periderm is converted into a cornified layer (stratum corneum) and the stratum intermedium forms three layers, the of stratum spinosum, stratum granulosum and stratum lucidum (Sengel, 1976).

Corresponding to these events, the dermal mesenchyme increases in thickness and various types of connecting fibres appear in the extracellular spaces between mesenchymal cells. At later stages the mesoderm is called the dermis. The mesoderm not only gives rise to the dermis but its presence is important for the development of the epidermis. It is also essential for the induction processes that underpin the morphogenesis of specialised epidermal structures called skin appendages.

1.4 Morphogenesis of skin appendages

During embryonic development, the histogenesis and morphogenesis of many organs and structures including the kidney, gut, lung, thyroid gland depends on the existence of complex interactions between epithelial and mesenchymal tissues. Similarly, in developing skin, the skin appendages (hair, nail, feather, scale and mammary glands) form as products of precisely timed and localised reciprocal interactions between embryonic epidermis and specialised populations of dermal cells. Both of these tissues are essential for normal morphogenesis and pattern formation of skin appendages. Evidence from *in vitro* and *in vivo* experiments (Wessells, 1961 & 1962; Dodson, 1967; Mackenzie & Hill, 1981; Hill & Mackenzie, 1984; Mackenzie *et al.*, 1987) has revealed that after separation from one another neither mesenchyme nor epithelium are able to continue their normal development alone. However, when separated tissues are recombined, they can re-interact and produce normal appendages (Briggaman & Wheeler, 1971 & 1978). The reason for this mutual dependence is the presence of bi-directional molecular signalling between epithelium and mesenchyme (Rawles, 1963; Davidson, 1993), which determines the type, shape, size and pattern of skin and its appendages (Sengel, 1976). Any disruption of these tissue interactions results in the failure of dermal and epidermal cells to perform their normal role which in turn, causes defects in the skin and its appendages (Sawyer, 1983). Furthermore, these interactions and tissue dependence are not restricted to embryonic development. A similar situation exists throughout adult life where continuing tissue interactions are responsible for the

maintenance of dermis and epidermis (Mackenzie, 1994).

Much of our current knowledge about the cascade of events, their timing, and the signalling molecules involved in the morphogenesis of skin appendages is derived from epithelial-mesenchymal recombination techniques *in ovo* or *in vitro* (Billingham & Silvers, 1967; Dhouailly, 1973, 1975 & 1977; Sengel, 1976 & 1986; Chuong *et al.*, 1996). The bulk of evidence that has been provided from these recombinations has revealed that although in vertebrates the epithelial-mesenchymal interactions create various appendages with different functions in different body sites of different species, there are many similarities in these interactions. Nevertheless, it is worth noting that because most investigations in this area have been performed on feather, tooth and hair morphogenesis it is possible that some specific events are not entirely matched in other skin appendages.

Prior to initiation of skin appendage formation, the embryonic epidermis has been organised into basal, intermediate and periderm cell layers. At this time the underlying dermis consists of mesenchymal cells loosely organised in a watery matrix containing blood vessels nerve elements and some ECM molecules (Kaplan & Holbrook, 1994). Formation of an epithelial placode is the first morphological indication that the process of skin appendage morphogenesis has begun, and it coincides with local proliferation of epithelial cells (Wessells, 1965). Several signalling factors have been proposed as being important in the formation of these placodes such as FGF-2 (du Cros *et al.*, 1993, Song *et al.*, 1996) and BMPs (Lyones *et al.*, 1990; Bitgood & McMahon, 1995; Chuong *et al.*, 1996). Later, the epithelial placodes in turn express molecules or factors including Shh (Nohno *et al.*, 1995; Noveen *et al.*, 1995; Bitgood & McMahon, 1995; Ting-Berreth & Chuong, 1996a,b), Wnt-7a (Chuong *et al.*, 1996) and Msx (Noveen *et al.*, 1995), which induce dermal condensations in the underlying mesenchyme. The condensed mesenchyme shows an increased concentration of ECM molecules e.g. fibronectin (Gibson *et al.*, 1983; Mauger *et al.*, 1982 & 1987), cell adhesion molecules e.g. NCAM (Jiang & Chuong, 1992), tenascin (Kaplan & Holbrook, 1994), growth factors e.g. TGF β 2 (Ting-Berreth & Chuong, 1996b), and other molecules which make it distinguishable from the inter-condensation mesenchyme. The condensed mesenchyme signals back through these molecules to the epithelium to induce Msx-1 and Msx-2 in the epidermis which regulate growth and morphogenesis of skin appendages (Ting-

Category	Molecule type	Feather follicle	Hair follicle	References
Growth factors	BMP-2		*	Lyons <i>et al.</i> , 1990
	BMP-4		*	Jones <i>et al.</i> , 1991a
	FGFs	*	*	Moore <i>et al.</i> , 1991 du Cros, 1993 Chuong <i>et al.</i> , 1996 Widelitz <i>et al.</i> , 1996
	EGF		*	Moore <i>et al.</i> , 1991
	TGF- β	*	*	Jones <i>et al.</i> , 1991b Chuong, 1993 Paus <i>et al.</i> , 1994 Ting-Berreth & Chuong, 1996
CAMs	Tenascin	*	*	Jiang & Chuong, 1992 Chuong <i>et al.</i> , 1993 Tucker, 1991 Kaplan & Holbrook, 1994
	N-CAM	*	*	Chuong & Edleman, 1985 Chuong <i>et al.</i> , 1993 Kaplan & Holbrook, 1994
	L-CAM	*		Chuong & Edleman, 1985 Chuong <i>et al.</i> , 1993
	I-CAM		*	Kaplan & Holbrook, 1994
	Cadherin		*	Hirai <i>et al.</i> , 1989
	Integrins	*		Jiang & Chuong, 1992
	Oncogenes	myc		*
myb		*		Desbiens <i>et al.</i> , 1991
ets-1		*		Desbiens <i>et al.</i> , 1991

Continued from previous page

Category	Molecule type	Feather follicle	Hair follicle	References
Differentiation markers	keratins	*	*	Haake <i>et al.</i> , 1984 Lynch <i>et al.</i> , 1986 Presland <i>et al.</i> , 1989 Chuong <i>et al.</i> , 1996
Transcription factors	Hox	*	*	Chuong <i>et al.</i> , 1990 & 1993 Bieberich <i>et al.</i> , 1991 Stenicki <i>et al.</i> , 1998
	Msx	*	*	Noveen <i>et al.</i> , 1995 Stelnicki <i>et al.</i> , 1997
	LEF-1		*	Zhou <i>et al.</i> , 1995 Kratochwil <i>et al.</i> , 1996
	Hedghogs	*	*	Nohno <i>et al.</i> , 1995 Bitgood & McMahon, 1995 Tingberreth & Chuong, 1996 Noveen <i>et al.</i> , 1996
	Wnt-7a	*		Chuong <i>et al.</i> , 1996
ECM molecules	Collagen	*		Mauger <i>et al.</i> , 1982
	Fibronectin	*		Mauger <i>et al.</i> , 1982
	Proteoglycans	*	*	Kitamura, 1987 Geotinck & Carlone, 1988 Kaplan & Holbrook, 1994
Retinoic acids		*	Hardy <i>et al.</i> , 1990 Chuong <i>et al.</i> , 1992	

Berreth & Chuong, 1996a). Hox genes and other molecules possibly determine the phenotype of skin appendages.

The account presented above by no means describes all molecules and factors that are reported to be involved in the process of skin appendage morphogenesis. There are many other molecules that have been shown to play a role in this process. For example lymphoid enhancer factor 1 (LEF-1), has recently been shown to be expressed in epithelial placode during hair follicle morphogenesis in mouse. It has been suggested that this factor plays a mediator role during epithelial-mesenchymal interactions (Zhou *et al.*, 1995). This idea is supported by the fact that transgenic mice with LEF-1 over-expressed in the ectoderm showed abnormalities in the positioning and orientation of hair follicles (Zhou *et al.*, 1995). Moreover, phenotype of LEF-1 null mutants have reduced number of hair follicles (Kratochwil *et al.*, 1996).

In addition to a detailed histological description of skin appendage morphogenesis which can be found in Sengel (1976), the expression and distribution of different signalling molecules and homeobox genes have been reviewed by Widelitz *et al.* (1997). Nevertheless, some of the molecules which have been experimentally identified to be associated with or play a role in morphogenesis of skin appendages can be found in Table 1.1.

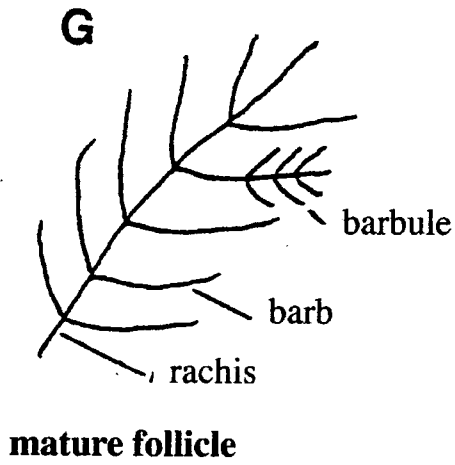
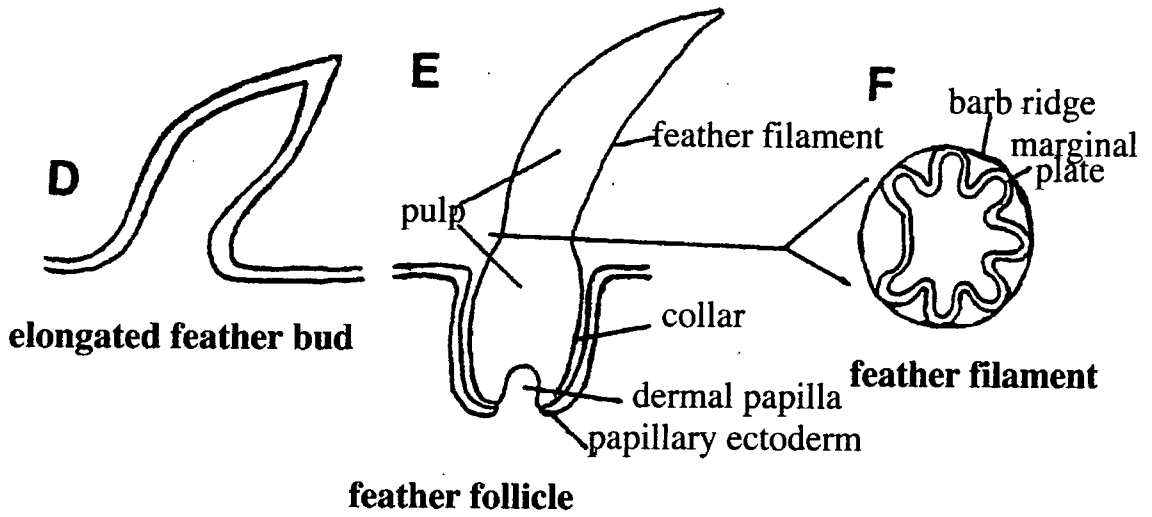
1.4.1 Morphogenesis of the feather follicle

In feather morphogenesis the processes of epithelial placode formation and dermal condensation are similar to that described above. In chick embryos the first histological signs of feather formation consisting of an epithelial placode and underlying dermal condensation appear in the skin about six days after incubation on spinal lumber regions (Wessells, 1965; Wessells & Evans, 1968). The epithelial placode is characterised by specific morphology of its basal cells which increase in height (Wessells, 1965), but no difference is seen between the placode and interplacode epidermis in terms of cell density. However, cell density is considerably higher in the dermal condensations in comparison with neighbouring regions. Dermal cells within the dermal condensation later penetrate the overlying epidermal concavity, thus the feather bud grows upwards on the skin surface. While the bud progressively grows upward, the epidermis at the base of the bud invaginates into the dermis to form a feather follicle. As a result, the

A
ectoderm
mesenchyme

B
placode
dermal condensation

C
feather bud



mesodermal component forms the lower dermal papilla and upper feather pulp, while the epithelial component becomes the papillar ectoderm, collar, and feather filament which lies on the skin surface. The papillar ectoderm situated at the lowest part of follicle is an epithelial cell reservoir for future feathers, while the overlying collar is the proliferative region where new epithelial cells are added to the growing feather. The feather epithelium starts to form barb ridges consisting of alternating marginal plates, and barb plates, the latter being the seats of barb and barbules. Cells in the marginal plate are later committed to programmed cell death creating distinct spaces between barb plates (see Chuong, 1993). The epithelial cells of the barb plate mostly keratinize and form the first generation of branched structures, the barbs. Another cycle of cell death later occurs among the barb cells generating the second branched structures, the barbules. The adult feathers then go through several molting cycles in which feathers are detached at the level between the collar and papillar ectoderm. A new feather grows when the regenerating papillar ectoderm interacts with the dermal papilla and neighbouring mesodermal components (see Chuong, 1993). A detailed description of feather morphogenesis has been provided by Sengel (1976) but schematic diagrams of this process cited from Chuong (1993) can be found in Fig. 1.2.

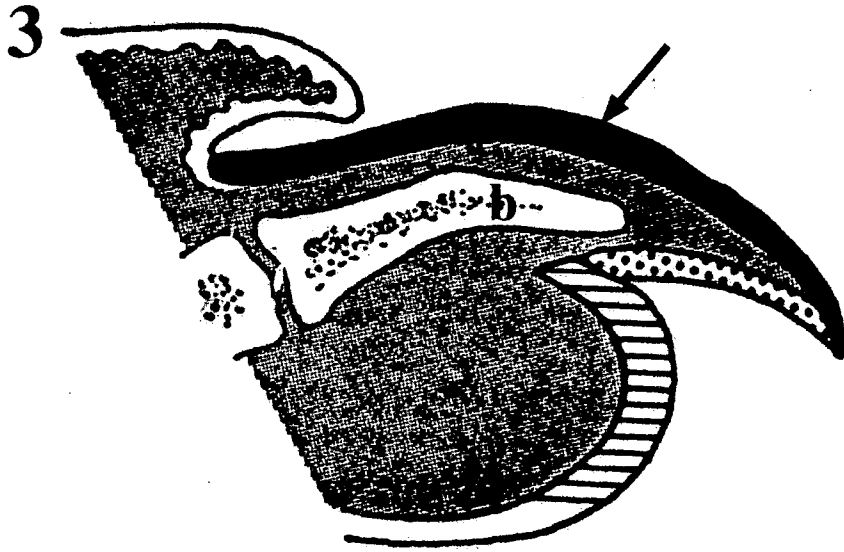
1.4.2 Morphogenesis of the nail and claw unit

Despite some ultrastructural and biochemical differences the basic structure of these two organs is similar. Apparently the nail in human has developed from claws (Fig. 1.3) existing at the end of the distal phalanx in lower mammals (Baden & Kvedar, 1991). The nail is a hard and durable epidermal appendage located on the dorsal tip of the distal phalanx of each finger. It protects the end of the digits and serves as a multi-purpose tool. Nail plate is composed of multiple layers of horny cells firmly cemented together and filled with keratin proteins. Nail is the product of the nail apparatus or nail unit that consists of four anatomically distinct compartments; the nail folds, the nail matrix, the nail bed and hyponchium (Sinclair *et al.*, 1994).

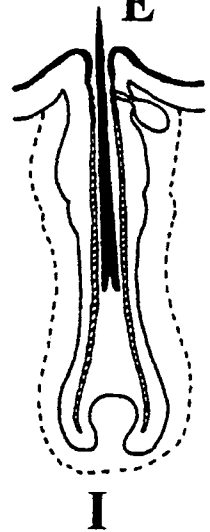
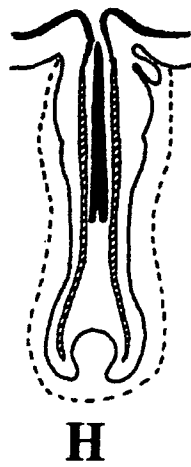
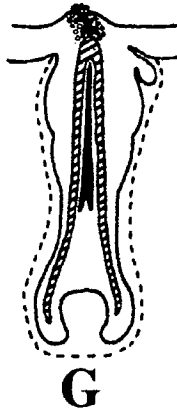
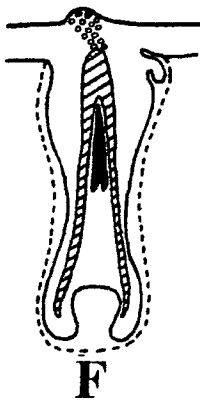
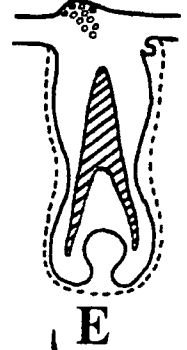
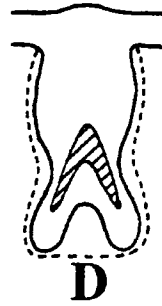
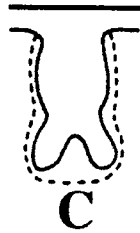
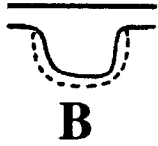
In humans the nail can first be recognised in a 10-week-old embryo (Zaias, 1963). In the embryo, prior to nail development the entire digit is covered by an undifferentiated two-layered epidermis consisting of periderm and stratum germinatum. Later, a distinctive smooth and shiny area, the claw field, begins to show on the tip of

Fig. 1.3) A diagrammatic longitudinal section through the rat finger tip showing the position of the claw plate (arrow) in relation to finger bone (b).

Fig. 1.4) Stages in the development of vibrissa follicles in the mouse. Initial hair bud (A), hair nodule (B), hair bulb and formation of dermal papilla (C-D), hair cone (E), formation of inner root sheath and hair shaft (F-H), hair eruption (I).



4



 Inner Root Sheath

 Keratinized Hair

finger. It is well delineated by two grooves, one distal and one proximal. After that, the epidermis here becomes three layered with a middle stratum intermedium (Baden & Kvedar, 1991). At this time, in the proximal portion of the nail field, a group of cells invaginate in a horizontal direction, passing inwardly into the underlying mesenchyme, towards the base of the distal phalanx. This cells mass forms the nail fold, which lies within the mesenchyme, parallel with the overlying epidermis. The nail fold is subdivided into two regions, a deeper layer (the nail matrix), and a superficial layer. Later, the upper cells of the matrix begin to keratinize, and these keratinized cells gradually fuse into the compact nail plate. As new material is added to the nail plate from the cells of the matrix, the distal portion of the plate is pushed progressively toward the end of the digit (Nelson, 1953).

1.4.3 Morphogenesis of the hair follicle

Hair follicle morphogenesis is a complex process that again occurs as result of the dermal-epidermal interactions. The first sign of hair development is crowding of nuclei in the basal layer of the epidermis, the so-called primitive hair germ or pre-germ stage (Serri & Cerimele, 1990), which in humans become evident around 60 days of gestation (Holbrook & Minami, 1991). The pre-germ passes rapidly into the hair-germ stage; the basal cells become taller, the nuclei become elongated and the structure starts to grow downward into the dermis where it is surrounded by the accumulation of mesenchymal cells. The downward growth and elongation of the hair germ, visible as a cord of epithelial cells, ends at the hair peg stage. The terminus of the hair peg flattens, forming a concave, bulb-like structure, which begins to enclose the associated mesenchymal cells. The epithelial cells located immediately above the mesenchymal cells are now called the matrix cells, and give rise to inner root sheath and the fibre. Later, the matrix cells almost completely encompass the underlying mesenchymal condensation, which is now termed, the dermal papilla. The remaining mesenchymal cells along the sides of the follicle become the follicular dermal sheath. The epidermal matrix cells proliferate and form a cone-shaped collection of elongated cells that grow into the centre of follicle toward the skin surface. The cells in the hair cone take their origin from specific regions of the matrix and thus become organised into the concentric layers of the inner root sheath and hair shaft. Keratinization within the hair shaft begins at the

approximate level of the junction of the bulb and lower follicle, while inner root sheath layers differentiate at a higher level. The hair is formed from cells that stream centrally from the matrix and are pushed upward to eventually emerge from skin surface (Holbrook *et al.*, 1989). In humans, the first lanugo hairs emerge from the surface of the skin around 19 weeks of gestation. A detailed description of hair follicle morphogenesis can be found elsewhere (Sengel, 1976; Serri & Cerimele, 1990; Hardy, 1992). Nevertheless, a schematic illustration of hair embryogenesis taken from Hardy (1983) is shown in Fig. 1.4.

1.5 Hair follicle in adults

The hair follicle is a unique system. Although small, it is highly organised with a great molecular complexity, and it embodies many fundamental biological processes seen in different organs. Consequently, the hair follicle has attracted the attention of many researchers as a model system for investigating epithelial-mesenchymal interactions, pattern formation, organogenesis, differentiation and cycling (see Stenn & Eilertsen, 1996). In comparison with other mammals in which hair plays a vital part in the body's thermal regulation, humans are relatively hairless and hair has no known significance for survival of the species (Messenger, 1993). However, throughout human history, hair has held great practical, agricultural, and even religious importance (Jahoda & Reynolds, 1996). Perhaps it has been because of this importance that research on biology of the hair began very long time ago. It is said that Malpighi (1628-1694) made the first observations on the structure of hair follicles by dissecting and describing the structure of very large vibrissa follicles on the lower lip of horses, mules and asses (see Camacho & Montagna, 1997). However, information on the detailed structure of hair follicle, their distribution, pattern of growth on the body, their embryology, and anatomy was published much later (Dry, 1926; Chase, 1954; Melargno & Montagna, 1953). Since that time organ and tissue culture techniques (e.g. Hardy, 1949 & 1969; Jahoda & Oliver, 1981; Oliver & Jahoda, 1981) as well as immuno-histochemistry (Westgate *et al.*, 1984; Couchman & Gibson, 1985; Commo & Bernard, 1997) and *in situ* hybridisation (Danilenko *et al.*, 1995; Panteleyev *et al.*, 1997) and homologous recombinations methodologies have been applied to the hair follicle. In addition to establishing the morphology, behaviour, proliferative potential and inductive capacity of

follicle derived cells, these techniques have given us molecular insights into the functions of follicle cells in development and cycling (Jones *et al.*, 1991a,b; Moore *et al.*, 1991; Messenger *et al.*, 1991a,b; Seelentag *et al.*, 1996; Akiyama, *et al.*, 1996; Panteleyev *et al.*, 1997; Rogers *et al.*, 1997; Byrne, 1997; see also Moore, 1989; Stenn *et al.*, 1994a & 1996; Stenn & Eilertsen, 1996; Danilenko *et al.*, 1996). In recent years in addition to natural mouse mutants e.g. angora and hairless (see Ahmad *et al.*, 1998), the genetically engineered transgenic animals have also been used to identify genes involved in the morphogenesis and cycling of the hair follicle (Mann *et al.*, 1993; Luetkeke *et al.*, 1993; Guo *et al.*, 1993 & 1996; Hebert *et al.*, 1994).

The hair follicle is probably the only structure that undergoes profound cyclic changes throughout adult mammalian life. Every cycle is typically defined as having three phases: anagen, the stage during which the follicle is regenerated and a new hair produced; catagen, the stage during which hair production ceases and the hair follicle regresses; and telogen, the stage during which the follicle is at rest (Dry, 1926). Due to those multiple stages in the hair follicle cycle, it is supposed that the control is derived by several factors. However, despite a wealth of investigations into the hair follicle cycle, exploring many signalling molecules having an influence on the hair cycle, it is still unclear which molecules control this cycle? It is also well known that during this cycling there are changes in associated systems that might influence the cycle; for example, in the vascular, lymphocytic, basement membrane and the interfollicular epithelial/dermal compartments, but whether these changes are causal, effect or associative (see Paus, 1996) is not clear at this time.

At present, and in the context of hair follicle cycling, of the follicular systems mentioned above the dermal compartment of the follicle particularly the dermal papilla appears to have the most obvious influence on in the cycle. The dermal papilla is a pear-shaped body of specialised fibroblast cells (Randall, 1996). It resides in the follicle where it is surrounded within an inverted cup of epidermal cells, normally termed matrix cells. Much of the present evidence suggests that the dermal papilla both initiates and terminates follicle growth (see Jahoda & Oliver, 1990; Randall, 1996), however the molecular mechanism by which the papilla regulates these events is still unclear.

Recently, several molecules have been characterised in follicular tissues believed to be associated with the hair cycle. Three groups of these molecules are fibroblast

growth factor (FGF5), transforming growth factor- β (TGF β), bone morphogenetic protein 2 and 4 (BMP2 and BMP4). FGF5 has been shown that it functions as an inhibitor of the anagen phase (Hebert *et al.*, 1994), TGF- β is considered to be involved in the anagen-catagen transition (Seiberg *et al.*, 1995; Paus *et al.*, 1997a), and BMPs appear to inhibit the proliferation of epidermal matrix cells (Blessing *et al.*, 1993).

In addition to follicle-derived factors it has been shown that several systemic factors are possibly involved in the control of the hair follicle cycling. In this context, for example, a variety of steroid hormones including estrogens and androgens can influence the hair follicle growth and cycle. It has been suggested that these hormones exert their effect through the dermal papilla (Oh & Smart, 1996; Randall, 1996; Hibberts *et al.*, 1998). However, there is considerable complexity in this area as it remains a mystery why, for example, the same androgens produce opposite effects in different body sites (see Messenger, 1993).

According to the so-called bulge hypothesis one of the crucial signals in the hair cycle is also governed by the epithelial compartment of the follicle. This hypothesis postulates that those epidermal cells that are responsible for production of hair shaft during anagen have a limited proliferative ability. This idea states that the follicle enters catagen when the replicative ability of these cells runs out. The follicle starts the next growth stage after the dermal papilla activates stem cells in the bulge, which supplies new cells capable of dividing (Cotsarelis *et al.*, 1990; Sun *et al.*, 1991; Lavker *et al.*, 1993; Miller *et al.*, 1993). However, this hypothesis leaves unexplained how the dermal papilla knows when to activate stem cells and what controls the papilla reaction?

1.6 Hair follicle stem cells

The hair follicle is in constant self-renewal, which continues throughout the lifetime of an individual. It is believed that in all self-renewal systems such as the hair follicle, cells with a great capacity for proliferation must exist (Potten *et al.*, 1979). These cells normally termed "stem cells" must also be the depository of all the information necessary for suitable differentiation to occur. Therefore, their progeny inherit and then express the specific differentiation that is the functional landmark of the tissue. The hair follicle basically consists of two cell types, epidermal and dermal. Thus, it is logical to suppose that for each cell type a reservoir of stem cells must exist in the

follicle to provide differentiated progeny for the lifetime of the animal.

1.6.1 Epidermal stem cells

It was once believed that follicular stem cells reside in the lower end of the follicle, the bulb, (Kligman, 1959; Montagna, 1983). However, based on labelling of follicular cells with radioactive thymidine *in vivo* Cotsarelis *et al.* (1990) put forward the bulge hypothesis, which suggests that the follicular stem cells are located in the bulge region. As I will discuss later in the next chapter, one property of stem cells is that they are believed to be slow cycling. Cotsarelis *et al.* (1990) showed that the bulge cells retained thymidine label for a longer period than bulb cells, which indicated that the former cells are slow cycling cells. Later, in work culturing different epithelial cell populations from the follicle, and by using clonal analysis evidence was produced in support of this idea (Kobayashi *et al.*, 1993). However this point has not yet been fully resolved because there are credible arguments against this idea. For example, the work of Oliver (1967a), showed that it is possible to form hairs in rat follicles that lack a bulge, and Inaba *et al.* (1979), showed that axillary human follicles are able to regenerate after removing most of the follicle including the bulge area.

1.6.2 Dermal stem cells

The hair follicle contains two populations of dermal cells, the dermal papilla and dermal sheath. The dermal papilla is an important part of the hair follicle and has powerful inductive influences over follicular and afollicular epidermis (see Jahoda & Oliver, 1990; Reynolds & Jahoda, 1991a). Interestingly though, microsurgical manipulations have demonstrated that in the follicle, the papilla can be replaced by the other population of dermal cells, the dermal sheath (Oliver, 1966a,b).

The dermal sheath is continuous with the basal stalk of dermal papilla. It runs along the whole length of the follicle and forms a sleeve surrounding the epidermal compartment. The dermal sheath is separated from the epidermal part by a thick basement membrane, called glassy membrane. Although it has not yet been proved, there is considerable evidence that dermal sheath cells might serve as dermal stem cells within the follicles. For example, as just mentioned after removal of the papilla, the dermal sheath is able to regenerate a new dermal papilla and restore hair production in the

follicle (Oliver, 1966a,b). Secondly, implantation of a piece of the dermal sheath into a deactivated rat vibrissa follicle results in activation of the follicle (Horne & Jahoda, 1992). However, to fully prove this point and also to clarify the exact role of the dermal sheath in hair follicles more investigations are required.

1.7 Dermal-epidermal interactions

The primary inductions that result in the formation of the basic plan of the embryo including determination of embryonic polarisation and generation of the embryonic layers (gastrulation), and the secondary inductions that occur later during organogenesis, depend on interactions that take place between different cell populations (Wessells, 1977). These interactions result in developmental changes in one or both of the interacting tissues and are the main mechanism by which the genetic potential is channelled into the co-ordinated sequence of events necessary to develop structure and function (Mackenzie, 1994). As a result of numerous studies, many genes and gene products have been found to be expressed during these interactions, but the real molecular mechanisms that create and regulate these interactions is still unclear. The pattern of histogenesis and morphogenesis of integument and its appendages including hair follicles is also determined by interactions occurring between the mesenchyme and epithelial tissues during embryonic development (Mackenzie *et al.*, 1987). However, the interesting point about the hair follicle is that this organ is one of the few organs in which dermal epidermal interactions also continue throughout maturity (see Jahoda & Reynolds, 1993). The hair follicle undergoes cyclic periods of growth, regression and regrowth involving interactions between dermal and epithelial cells which recapitulate many events seen during development (Randall, 1994a). There is the likelihood that the intrinsic signalling mechanisms that control the adult hair follicle might be the same as those involve in embryonic morphogenesis (Jahoda *et al.*, 1992). Thus, hair follicle components can be used to investigate the unknown mechanisms involved in dermal-epidermal interactions during adult life.

The bulk of our current understanding concerning the nature of dermal-epidermal interactions in adult hair follicles has originated from studies performed on large rat vibrissa follicles (see Jahoda & Reynolds, 1996). These studies that were pioneered by Cohen (1961) and subsequently followed by Oliver and colleagues (see Jahoda & Oliver,

1990; Jahoda, 1993) have revealed that dermal and epithelial components of hair follicle are able to recapitulate embryonic interactions. More specifically, in these experiments the inductive properties of the follicles principle dermal component (dermal papilla) in reformation of hair follicles was established (Oliver, 1967b, Jahoda *et al.*, 1984, Horne *et al.*, 1986). Transplantation experiments showed that freshly dissected dermal papillae can induce hair growth when implanted into inactivated follicles (Oliver, 1967b). Moreover, the culture of dermal papilla cells and subsequent cell re-implantation experiments demonstrated that even cultured papilla cells are able to elicit hair growth in homeotopic or heterotopic sites (Jahoda *et al.*, 1984; Jahoda, 1992). Advances in isolation and cultivation of follicle cell types including dermal papilla, dermal sheath and epithelial cells has allowed a broader exploration of adult inductive potential and cell interactions to be carried out.

A large body of information about the inductive ability of follicle dermal and epidermal cells has emerged from dermal-epidermal interactions *in vivo*. However, in general, a problem that exists in study of dermal-epidermal *in vivo* is that it is not possible to exactly determine the role and abilities of individual cell types. It is due to the presence of many variables which may influence the interactions, such as different cell types and superimposed influences of systemic factors of the blood circulation, that *in vitro* model systems have been developed to mimic dermal-epidermal interactions, excluding other factors that might govern the interactions. In these models dermal and epidermal cells are cultured and then recombined with each other and are subsequently grown in culture media or in a host animal. Apart from dermal cells, follicular epidermal outer root sheath (ORS) cells have shown that they are suitable for study of dermal-epidermal interactions (see Limat *et al.*, 1991 & 1995). These cells which are known to be involved in epidermal regeneration during wound healing, can be easily isolated from the follicle and cultured *in vitro*. An offshoot of this culture has been the use of ORS cells in dermal-epidermal recombinations for making skin models. Using these models it has been shown that ORS cells are not only able to interact with dermal cells to reconstruct a normal skin-like structure (Noser & Limat, 1987), but they also show the ability to initiate follicle formation in the reconstituted structure (Watson *et al.*, 1994). However, to date the dermal compartment of these skin models has been the skin fibroblasts and the chance has not been given to the follicular dermal cells to show their

interactive abilities with epidermal cells. Given that relatively little is known about follicular dermal sheath it is important to establish whether adult cultured dermal sheath cells can be involved in the creation of a normal skin in association with ORS cells.

Moreover, in the context of dermal epidermal interactions it has been documented that embryonic dermal and epidermal tissues from heterotopic (from different body sites of the same animal) and heterospecific (from animals with different zoological-class origins) regions are also able to initiate reciprocal interactions when recombined together (see Sengel, 1976). In these recombinations, the dermal and epidermal compartments of different types of skin are separated in an anatomically intact and viable form allowing the production of heterotopic and heterospecific recombinants- e. g. mouse epidermis with chick dermis-which are then transplanted to compatible hosts. This approach has been used in various species and the results obtained have demonstrated that during embryonic stages, morphogenetic messages from "foreign" tissues could be understood and correctly interpreted (see Sengel, 1976). In this context it is interesting to investigate whether these inductive messages from foreign tissues can be understood in maturity.

1.8 Aims and objectives

The general objective of my research was to investigate particular properties and interactive capabilities of epithelial and dermal cells derived from skin appendages. Currently there is considerable interest in the epithelial stem cells of the hair follicle. Others have investigated the proliferative characteristics of epithelial cells from different regions of the follicle *in vivo* and *in vitro*, and reached firm conclusions about the location of stem and transient amplifying (TA) cells in the hair follicle and the role of different epidermal cell populations in the hair growth cycle. My first aim was to examine the same question with a direct experimental approach, and for this I developed a plucking procedure using the rat vibrissa follicle because it can be employed for quantitative as well as qualitative experimentation. In the context of this question, previous plucking studies have revealed two interesting points: i) plucking of a hair follicle can result in the initiation of new hair growth (Chase, 1954), and ii) in the vibrissa follicle plucking leaves a small population of germinative epidermal (GE) cells (the main site of cell proliferation during anagen) at the base of the follicle (Jahoda,

1982). However, in these studies the question of which cells participate in the formation of the new hair after plucking has remained unanswered. Thus, I attempted to clarify this question by scrutinising morphological events and the location of cell proliferation and cell death in plucked follicles at different intervals post-plucking. I then investigated the effects of repeated plucking on individual follicles, looking at the cellular source of hair growth renewal each time as described above, and then attempting to determine for how long follicles can produce hair fibre as well as the effects on growth cycle parameters such as rate and duration. I also directly tested whether the residual GE cells left behind after plucking were able to produce a new fibre without contribution from epidermal cells of the upper part of the follicle.

There have been some discrepancies in previous reports describing the proliferative and clonogenic abilities of epidermal cells from different regions of the hair follicle *in vitro*. Therefore I investigated the growth of hair follicle epidermal cells with emphasis on a comparison between bulb epidermal cells and outer root sheath cells from the upper follicle.

Relatively little attention has been paid to the properties of follicular dermal sheath cells, therefore a substantial part of my research was directed at revealing more about the characteristics and interactive abilities of these cells. Previous studies had revealed that dermal sheath cells show similarities with both follicle dermal papilla cells and skin fibroblasts (Reynolds *et al.*, 1993b). Therefore as well as comparing dermal sheath cells with the above cell types, I specifically questioned whether, in combination with epidermal cells, the sheath cells would reveal more of the appendage inducing characteristics of papilla cells, or like fibroblasts, could become part of the interfollicular skin dermis.

The inductive abilities of the adult hair follicle dermal papilla are well documented and here I investigated the ability of intact dermal papillae, dermal papilla cells and different hair follicle cell combinations to induce hair follicles in a novel (dorsal skin) site. Finally, in embryonic recombination experiments interactions between dermal and epidermal cells from skin appendages of widely different origin are possible. For example, embryonic avian dermis from a feather-forming region is capable of initiating hair follicle formation when combined with mammalian epidermis. Here I investigated the extent to which dermal cells from another mammalian skin appendage (the claw) and

from adult bird feather dermal papillae were capable of dermal-epidermal interactions *in vivo*?

All of the above-mentioned investigations are represented in the following chapters;

Chapter 2: Proliferative abilities of hair follicle epidermal cells *in vivo*.

Chapter 3: Hair follicle epidermal cells *in vitro*.

Chapter 4: Dermal-epidermal interactions in various skin appendages.

Chapter 2

Proliferative abilities of hair follicle epidermal cells *in vivo*

2.1. Introduction

The hair follicle provides a fascinating and readily accessible structure for studying various aspects of animal biology. In particular, the cyclic nature of hair growth makes the adult follicle an intriguing paradigm of developmental activities whose importance is becoming increasingly apparent (du Cros, 1993; Hebert *et al.*, 1994; Stenn & Eilertsen, 1996; Stenn, *et al.*, 1996). The precise mechanisms and signals (cellular and molecular) which drive this cycle are still unclear and remain fundamental targets for hair biologists. According to one of the prominent ideas, the follicular dermal papilla plays a central role in the process of hair cycle control (Oliver, 1970). Chase (1954) proposed that an inhibitor of proliferation called a “chalone” produced in the dermal papilla controls the hair cycle. This inhibitor accumulates during anagen causing entry into catagen when present in sufficient concentration. According to this theory, dispersal of the inhibitor during the telogen stage leads to initiation of the next anagen phase. In support of this concept Paus *et al.* (1990) found an inhibitor of hair growth in extracts of mice telogen skin which was absent in anagen skin.

Some growth factors are also among the possible candidates thought to control the hair cycle. Hebert *et al.* (1994) reported that FGF5, localised to the outer root sheath, probably have a role in regulating this cycle through controlling the duration of anagen phase. Mice engineered to be homozygous for null allele of the FGF5 gene showed abnormally long hair, that suggested FGF5 might function as an inhibitor of hair matrix proliferation and hair elongation. However, since homozygous negative mice do eventually enter into catagen, the possibility arises that another factor(s) may be involved in controlling the duration of anagen.

The proliferative capability of progenitor cells in the hair follicle has been recently put forward as one of the main factors that could play a part in timing of hair growth. It is believed that hair follicles, like all self-renewing systems, possess a repertoire of stem cells, with high proliferative capabilities, which play a central role in

hair growth, regeneration and differentiation. According to most definitions (Gilbert & Lajtha, 1965; Lajtha, 1979; Potten *et al.*, 1979; Lavker & Sun, 1982, 1983; Wright & Alison, 1984; Messenger, 1993), stem cells are long-lived, well-protected, and primitive, with very low rates of mitotic activity. In most models, these cells are stimulated by particular tissue demands and respond by entering a proliferative state and producing daughter transient amplifying (TA) cells (Lavker & Sun, 1982). The latter cells are more rapidly dividing and serve to amplify the population but have a limited capacity for division before they undergo terminal differentiation.

Despite the unanimity amongst investigators about the existence of epidermal stem cells in the hair follicle, there are different views as to their location. Many authors have assumed that hair follicle epidermal stem cells are situated in the lowermost end bulb region of the follicle, where dividing cells give rise to the fibre and other differentiated products during the growth phase of the cycle (Kligman, 1959; Van Scott *et al.*, 1963; Pinkus, 1978; Cotsarelis *et al.*, 1989). On the other hand, recent investigators have proposed the “bulge hypothesis” which suggests that the stem cells reside in the upper outer root sheath (ORS) of the follicle, in a region close to the point of insertion of the arrector muscle (Cotsarelis *et al.*, 1990; Sun *et al.*, 1991; Lavker *et al.*, 1991, 1992, 1993; Miller *et al.*, 1993; Kobayashi *et al.*, 1993; Wilson *et al.*, 1994a, 1994b). In human scalp hair follicles, some suggest that the stem cells are located in this bulge area (Yang *et al.*, 1993), while others consider their source to be below the midpoint of the follicle but well above the hair bulb (Rochat *et al.*, 1994).

Amongst evidence put forward to support this hypothesis is the fact that after removing the lower third of a follicle, the remainder of the follicle regenerates a new fibre (Oliver, 1966a,b,c, 1967a,b; Inaba *et al.*, 1979; Ibrahim & Wright, 1982; Matsuzaki *et al.*, 1996). The new epidermal bulb cells must have originated from above the level of transection and it has been proposed that they may come from higher up in the follicle, specifically the bulge region (Cotsarelis *et al.*, 1990, Lavker *et al.*, 1993). Further to this, cells from the mid and upper parts of the follicle have been documented to have greater proliferative potential compared with bulb cells *in vitro* (Kobayashi *et al.*, 1993; Yang *et al.*, 1993; Moll, 1995 & 1996). Even more obviously during the resting (telogen) stage of most follicle types the lower half of the follicle shrinks and retracts to the level of the bulge site (De Weert *et al.*, 1982). In human follicles, the bulge region cells also express

keratin 19 which has been associated with stem cell location (Lane *et al.*, 1991; Narisawa *et al.*, 1994b; Michel *et al.*, 1996).

A part of the bulge hypothesis is that it provides an explanation for one aspect of hair cycle control. This hypothesis states that anagen is initiated by interaction between the dermal papilla and stem cells of the bulge region, which results in activation of these cells. This activation leads to proliferation of some bulge stem cells which produce TA cells. Later the TA cells move downward and end up in the bulb where they undergo constant proliferation during anagen. According to the bulge hypothesis, the germinative epidermal (GE) cells of the bulb are TA cells whose limited proliferative potential runs out at the end of each growing phase resulting in the cessation of hair growth and entry into catagen. If this idea is correct, it provides a solution to one of the most interesting and mysterious questions in hair follicle biology, namely, what controls the duration of the growth phase of the cycle?

Evidence for the relative proliferate potentials of the bulge and bulb epidermal cells has come largely from the *in vitro* cultivation of ORS-type cells from different levels of the follicle (Kobayashi *et al.*, 1993; Yang *et al.*, 1993; Moll, 1995). Up to now, this question has not been investigated directly *in vivo*, therefore this part of the study concentrated on examining the proliferative potential of the epidermal cells that remain in the follicle after the fibre has been plucked.

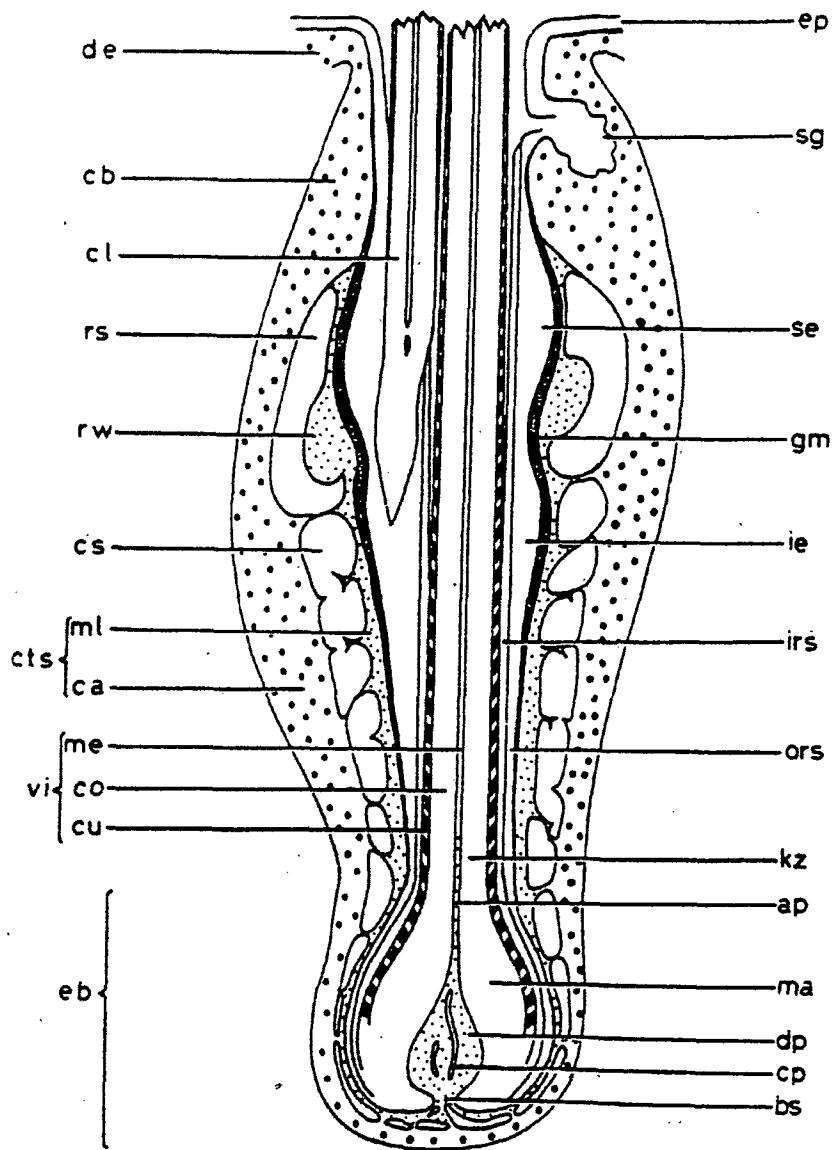
One of the above mentioned characteristics of stem cells is the belief that they occupy a protected environment in relation to possible injury. It has been shown that when a growing hair fibre is plucked, essentially two main sub-populations of epidermal cells are retained in the follicle. One is the small ring of germinative epidermal cells at the lowermost part of the follicle bulb (Oliver, 1965; Young, 1977; Ibrahim & Wright, 1978; Jahoda, 1982; Bassukas & Hornstein, 1989) and another, the population of outer root sheath (ORS) cells in the bulge region (Cotsarelis *et al.*, 1990; Moll, 1995). As Collins (1918) for the first time showed when a hair is plucked from its follicle, it always causes the initiation of a new hair fibre production. This poses the question of where the new fibre might come from? In other words which residual cells (GE or bulge region cells) participate in the formation of the new hair after plucking?

The large tactile sinus follicles on the mystacial pad were first exploited for investigations of hair biology by Cohen (1961, 1964 & 1965). Subsequently, this system

has been widely used as a model for hair growth studies because of several advantages. The large size of these vibrissa follicles provides a good model for dissection and manipulation of individual components. They are arranged in a constant and similar orientation pattern in both sides of the upper lip. This not only assists in distinguishing the location of every single follicle on the upper lip but as each follicle has an identical counterpart on the opposite side which is in the same stage of cycle, one side can be used as a convenient and reliable control for experimental procedures. Moreover, while the growth cycle of many follicle types are modulated by hormonal or environmental influences (see Ebling, 1990; Randall & Ebling, 1991; Rose *et al.*, 1995; Schilli *et al.*, 1997), these follicles have a cycle that is regulated internally with remarkable precision, and whose origin apparently derives from ontogenic follicle development (Oliver, 1965). Thus, the growth of a follicle can be predicted to within a day, and the length of its fibre to within one or two millimetres (Ibrahim & Wright, 1975). Finally, a wealth of background data from experiments conducted during the past 30 years is available for use in interpretation of results.

For the above reasons in the present study to investigate directly some of the questions highlighted above I also adopted these follicles as my model. A schematic representation of the essential compartments of the rat vibrissa follicle, taken from Oliver (1965) is shown in Fig. 2.1. In this study I first used histology, electron microscopy and immunohistochemistry with BrdU labelling to track the origin of the bulb epidermis that regenerates after plucking. I then directly tested the proliferative capacity of germinative cells by repeated plucking and fibre growth measurements. In parallel the presence of apoptotic cells was localised in plucked follicles at different intervals. Finally, the lower end of plucked follicles was transected *in vivo* to explore the regenerative potency of the germinative cells in the absence of the upper outer root sheath epidermis.

Fig. 2.1) Diagram of a large anagen whisker follicle with details of muscle attachments and blood and nervous supply to the follicle omitted. (ap) apex of papilla, (bs) basal stalk of papilla, (ca) capsule, (cb) conic body, (cl) club vibrissa, (cc) cortex, (cp) capillary, (cs) cavernous sinus, (cts) connective tissue sheath or dermal sheath, (cu) cuticle, (de) dermis, (dp) dermal papilla, (eb) end bulb, (ep) epidermis, (gm) glassy membrane, (ie) inferior enlargement, (irs) inner root sheath, (kz) beginning of keratogeneous zone, (ma) matrix, (me) medulla, (ml) mesenchymal layer, (ors) outer root sheath, (re) ring sinus, (rw) ring wulst, (se) superior enlargement, (sg) sebaceous gland, (vi) growing vibrissa. (From Oliver, 1965).



2.2 Materials and Methods

2.2.1. Animals

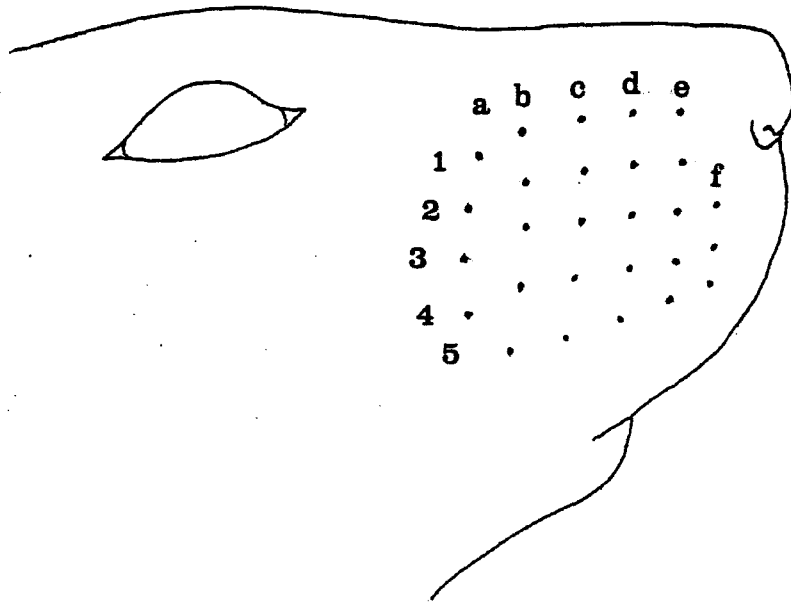
Inbred PVG hooded rats of both sexes, aged between 2-4 months, were supplied by the animal unit (Durham University, Durham, UK). Rats were kept under conventional husbandry conditions. They were fed with commercial irradiated food pellets and received de-ionized water. Rats were housed in polypropylene cages with stainless steel filter covers. The cages were filled semi-weekly with white pine shaving. A photoperiod of 12:12 h light: dark cycle was employed. Experimental animals were housed individually to prevent damage to whiskers by fighting.

2.2.2 Notation of follicles and measurement of hair fibre length

For the purpose of this study it was essential to ascertain the location of each individual vibrissa follicle on the mystacial region. Therefore, prior to an experiment a normal animal was killed by an overdose of CO₂ and its whiskers were observed under a binocular microscope (Nikon SMZ-10, Japan). According to Oliver (1965) there are normally 54 major follicles positioned symmetrically in both sides of animal face in a uniform and well-defined pattern, 27 being on each side. The follicles were identified according to the annotation of Oliver (1965) (Fig. 2.2). On each side, the mystacial region consists of five horizontal rows which are numbered 1 to 5 in a dorso-ventral direction and 6 vertical rows lettered a to f in a posterior-anterior direction. Regarding the nomenclature, each individual follicle used experimentally was recognised and recorded using this combination of numbers and letters, for example **1b** is the position of the follicle which is located on the most posterior horizontal row and second row from the top.

Prior to any manipulation, the length of the fibre (or fibres) was also measured on relevant follicles using a flexible, transparent and graded capillary tube (internal diameter 1mm). To measure the fibre length the tube was placed over the individual hair and while it was held flat against the skin surface the length at the tip of fibre was recorded. In follicles that had two fibres the longer and thicker club fibre, was initially separated from the shorter and thinner growing fibre, and then their lengths were

Fig. 2.2) Diagrams illustrating the distribution pattern of the major vibrissa follicles on the mystacial pad of rats.



	a	b	c	d	e	
1						
2						f
3						
4						
5						

measured individually. All observations and measurements were carried out under a binocular microscope (Nikon SMZ-10, Japan).

2.2.3 Procedures for study of normal hair follicles in various phases of the cycle

2.2.3.1 Follicle isolation and growth phase classification

Using a pair of scissors an incision was made at the posterior side of the mystacial pad parallel to the most posterior row (row a). The anterior edge of the incision was clamped using a pair of artery forceps, and the skin pad was rolled backward to expose the follicles. By means of a pair of fine scissors, the exposed follicles were then cleaned of surrounding connective tissue. Individual follicles were carefully gripped round their neck region at the point at which they joined with the skin and removed from the mystacial pad.

After isolation of the follicles, the growth cycle stage of each isolated follicle was established using external criteria based on the length and arrangement of the fibres as follows:

Mid-anagen, the follicle possessed two fibres and the length of the growing fibre was between one to two thirds of its respective club fibre length; Catagen: the follicle had a single fibre and a swollen end bulb region; and telogen: the follicle again had a single fibre but showed a pointed end bulb. Subsequently, combinations of all three types of follicles were processed for histology, immunohistochemistry or electron microscopy.

2.2.3.2. Histology

The follicles were individually fixed in 4% formalin saline for 24 hours. They were dehydrated through a graded series of ethanol (70% and 95% for 2 hours in each and 100% for three hours), cleared in histoclear (National diagnostics) for three hours with three changes and infiltrated with melting paraffin wax overnight with three changes. The follicles were then oriented with the point of nerve entry to the follicle at the side and embedded in the paraffin wax. Seven microns thick longitudinal sections were cut using a microtome (Leitz1512, Germany) and stained with a combination of Alcian blue, Weigert's Haematoxylin, and Curtis's Ponceau S.

2.2.3.3. Electron microscopy

The follicles were individually fixed, as described by Karnovsky (1965), in 4% paraformaldehyde, 2.4% glutaraldehyde and 0.1 M sodium cacodylate for 1 hour at 4 °C. Specimens were then post fixed in a 1% osmium tetroxide solution buffered with 0.2M sodium cacodylate for 30-60 minutes at room temperature. Afterward, the follicles were dehydrated through a graded series of ethanol (70%, 95% for 15 minutes each and 100% for 30 minutes). Follicles were then infiltrated with intermediate solutions of ethanol/propylene oxide (1:1 for 30 minutes) and pure propylene oxide for another 30 minutes. Infiltration with resin was carried out first for 30 minutes with 1:1 absolute ethanol/araldite, followed by pure araldite for a further 30 minutes. Araldite resin was composed of 10 ml araldite resin CY212, 10 ml DDSA, 1 ml dibutyl phthalate and 0.4 ml BDMA. Follicles were oriented and embedded in pure araldite and then left at 60 °C for polymerisation. Semi thin sections of 1µm in thickness were cut on an Ultracut microtome (Leica AG, Austria) and stained with 0.1% toluidine blue for light microscopy. Ultra thin sections (70 nm) were floated onto copper grids (100 square mesh, Agar aids), and stained with 1% uranyl acetate and lead citrate for 25 and 10 minutes respectively. Stained ultra thin sections were examined using a Philips 400T transmission electron microscope.

2.2.3.4 Immunohistochemistry

i) Detection of cell proliferation in follicles by BrdU

Cell proliferation can be studied by monitoring the incorporation of 5-bromo-2-deoxy-uridine (BrdU) into cellular DNA of cycling cells which binds in place of thymidine. Cells that have incorporated BrdU into DNA are usually detected using a monoclonal antibody against BrdU and a conjugated secondary antibody.

a) BrdU labelling

The procedure employed here was essentially that described in the BrdU detection kit (Boehringer Mannheim) as applied to tissues. Immediately after isolation, follicles were immersed in minimum essential medium (MEM) with glutamax supplemented with conventional antibiotics (100 U/ml penicilin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, all supplied by Gibco, Life Technologies, UK) and

containing 10 µmol/l BrdU label. In some cases prior to labelling, follicles were transected in two halves to test the penetration of BrdU into the specimens. Specimens were incubated at 37 °C in the presence of 95% O₂ and 5% CO₂ for 1 hour. To remove any unincorporated BrdU, specimens were washed three times in washing buffer and then incubated in the same buffer at 37 °C for 20 minutes. Labelled follicles were immersed in Tissue-Tek embedding fluid (Miles Inc, USA) within aluminium foil boats, and oriented for cutting. The blocks were then snap frozen by floatation in liquid nitrogen.

b) BrdU detection (using fluorescein conjugated antibody)

Follicles were cut longitudinally on a Bright cryostat at -20 °C. Five micron thick frozen sections were heat adhered onto poly-L-lysine coated glass slides- slides that had been incubated in 0.1% poly-l-lysine solution (Sigma, UK) for 30 minutes removed from solution and then allowed to air dry overnight at room temperature. Sections were then fixed in 70% ethanol (in glycine buffer, 50 mmol/l, pH 2) at -20 °C for 30 min. Specimens were washed three times with PBS and then incubated with anti-BrdU solution (Boehringer Mannheim), for 1 hour at 37 °C. Sometimes, in order to facilitate the penetration of antibody into cells, the sections were first treated with pepsin (1 mg/ml in 0.01-N HCL). Sections were washed three times with PBS (phosphate buffered saline) and then immersed for 1 hour in rabbit anti-mouse Ig-fluorescein (Dako A/S, Denmark) diluted 1:40 in PBS containing 70 µm/ml of Evan's blue to provide a background counter stain. The slides were washed in PBS as described above, and mounted in an anti fading agent (Citifluor, Agar Aids).

Labelled sections were examined with a Zeiss Axiovert 135 fluorescence microscope (Carl Zeiss, Germany) equipped with epi-illumination and an I₂ filter combination (excitation band 450-490-nm, stop 510-nm). Photographs were taken on Fuji 400 professional (Provia, Japan) colour transparency film, imported into Adobe PhotoShop, assembled and printed on a Kodak XLS 8600 PS printer.

ii) Localisation of cell death in follicles

Follicles were embedded in tissue-Tek and frozen in liquid nitrogen immediately after dissection. Six-micron longitudinal frozen sections were cut as described above and applied onto poly-l-lysine coated slides. Sections were left to air-dry at room

temperature before being fixed in acetone for 10 min at -20°C . Specimens were washed 3x5 min with PBS and incubated in C-Jun/AP-1 rabbit polyclonal antibody (diluted 1:30, Santa Cruz Biotechnology) which detects cell death (Horton *et al.*, 1998) overnight at room temperature. After washing 3x5 min with PBS, the sections were incubated in the second antibody (anti-rabbit Ig, diluted 1:30 with PBS, containing 70- $\mu\text{m}/\text{ml}$ Evan's blue) for 1 hour. Specimens were washed as above and then mounted with Citifluor. Labelled sections were examined either under a fluorescence microscope as described above for BrdU labelling sections or with laser scanning confocal microscope (Biorad MRC 600 confocal).

Moreover, to provide positive control specimens, rat embryos at day 15 were dissected from a pregnant animal and their limbs bud was snap frozen in liquid nitrogen. Six-micron longitudinal sections were stained with the cell-death antibody in the same manner as described above. Negative control procedures included a first incubation of sections with PBS in place of the primary antibody, followed by incubation in the secondary antibody as normal.

2.2.4 Plucking experiments

2.2.4.1 Follicle selection

In plucking studies the large follicles on the three most posterior rows were employed. These follicles are large which makes it easier to study their histology and do fibre measurements. Moreover, to provide accuracy and uniformity throughout the experiment, only mid-anagen follicles were used.

2.2.4.2 Plucking of fibres

In order to pluck the follicle fibres, rats were anaesthetised by fluothane (Mallinckrodt Veterinary Ltd, UK) inhalation. Under a binocular microscope the position of mid-anagen follicles on the mystacial pad was recorded. To facilitate the plucking of the growing fibres and to maximise the amount of material removed, for each selected follicle the club hairs were first plucked, using a pair of watchmaker forceps. The growing fibre was then gripped low down close to the skin surface and then carefully and smoothly pulled up. The proximal end of each plucked fibre was immediately examined under the binocular microscope to ensure uniform results. When

the procedure had been carried out correctly the plucked fibre had an even inverted cup-shaped epidermal matrix attached to its base (Fig. 2.3). Non-uniformly plucked follicles were noted and discounted from experimental use although their histology was examined at the end for any possible interesting behaviour. Normally 5-7 follicles were plucked in each rat. After the plucking procedure was completed most animals were allowed to recover and then kept in isolated cages. The animals with plucked follicles were later employed for one of the following experiments; single plucking, multiple pluckings, or follicle amputation.

2.2.4.3 Single plucking

To examine the events following a single plucking the experimental animals were killed immediately (time 0) after plucking or at 6, 12, 24 and 48 hours, and subsequently at 4, 7 and 9 days post-plucking. The plucked follicles were isolated from the mystacial pad as described above but to achieve consistency and to avoid inaccuracy, special handling and further specific checks were carried out at this stage. Because the plucked follicles had a soft and delicate internal structure it was necessary to handle the follicles gently. Moreover, before processing the follicles for examination, the follicles were carefully observed under the dissecting microscope to detect any abnormalities. Where the follicles had not been plucked properly they displayed a greater amount of matrix material left in their bulbs. Furthermore, in a few cases a small piece of club fibre was found to be still present in the upper part of follicle. In all such cases, these abnormal plucked follicles were discarded from the study.

The plucked follicles displaying standard criteria were alternatively processed for histology, electron microscopy and immunohistochemistry for detection of cell proliferation and cell death using similar procedures to those described above for normal follicles. Table 2.1 shows the number of plucked follicles processed for each experiment.

2.2.4.4 Multiple consecutive plucking and length measurements

In order to further determine the sequence of morphological and replicative events following multiple plucking, follicles were initially plucked as above. Moreover, for this part of the study, prior to plucking, for each follicle the lengths of the growing fibre and club hair were measured and recorded respectively. Subsequently the

Fig. 2.3) Procedures involving in plucking (A-C) and amputation (D-E) of vibrissa follicles. **A)** A schematic diagram of a follicle prior to plucking. **B1)** A plucked fibre corresponding to **B2** shows a vibrissa that has been plucked from a mid-anagen follicle. Note the even inverted cup shape of the epidermal matrix at the base and that the epidermal root sheath material has been stripped from the follicle along with the hair. **C)** A follicle after plucking showing two main subpopulations of epidermal cells left behind after plucking, germinative epidermal (GE) cells at the base and outer root sheath (ORS) cells in the upper part of the follicle. In between these two, scattered ORS cells are also left behind at the sides of the hair shaft cavity. For plucking and amputation experiments the plucked follicle (**C**) was cut just above the follicle bulb (**D**). The two sections: the bulb and the upper follicle (**E**) were left separated in vivo.

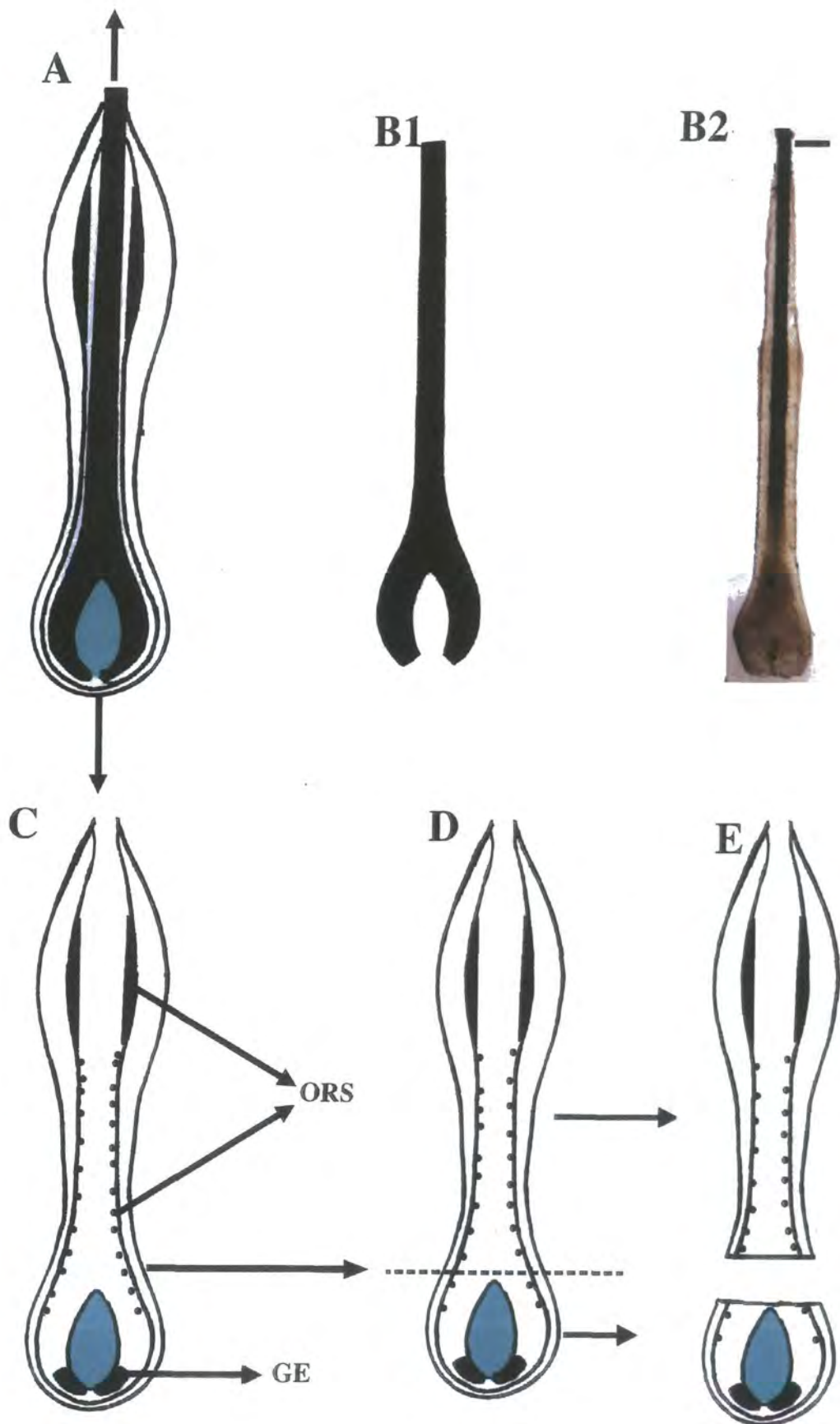


Table 2.1) Tabulation of the numbers of rat vibrissa follicles employed for plucking experiments.

Type of experim.	0 time	6 hours	12 hours	24 hours	48 hours	4 days	7 days	9 days
Normal histology	5	8	8	9	6	5	4	3
Electron micros.	-	4	3	4	3	4	2	1
BrdU labelling	4	8	8	7	6	7	6	4
Cell death	3	4	3	4	4	4	-	-

anatomical position of each individual experimental follicle on the mystacial pad was observed regularly until a new fibre emerged from the skin surface. After this the length of this new fibre was regularly measured at intervals of between one and three days. To measure the fibre length, the animal was anaesthetised as described above and the positions of the experimental follicles were identified. Then, using the graded capillary tube the length of the regenerated fibre was measured. The procedure of measuring hair length continued at regular intervals until the length of the fibre reached to between a third to a half of its respective club length (normally this was achieved 20-25 days after previous plucking). For the period just prior to the next plucking, fibres were measured even more frequently, often daily. At this stage following a final measurement of fibre length the follicles were plucked again in exactly the same manner as described above. On 4 rats, follicles were plucked twice. The whole process of plucking and measuring was repeated for a third occasion on three more rats, while two animals underwent four, five and six successive plucking always with continuous measurement of regenerated fibre lengths. Irregular plucked follicles were again discarded from the study at any point. Regular measurement of fibre lengths enabled the growth rate of each hair to be monitored for each cycle. The total length of fibre produced by each follicle was calculated as the sum of the lengths of the original growing, and the regenerated, fibres. This was then compared with original club fibre length, which had been measured just before the first plucking. It is noteworthy that on animals whose follicles were plucked for one, two, three, or four times a combination of vibrissa follicles on the two most posterior vertical and the uppermost horizontal rows were employed. However on animals in which five successive plucks were performed on their follicles, only the follicles on the most posterior row comprising the largest follicles, were utilised.

Animals were normally biopsied 6 or 24 hours following the last pluck of each sequence, and the isolated experimental follicles either fixed in formal saline for histology, or processed for BrdU immunolabelling as described in section 2.2.3.4. In one rat, after the third plucking, the follicles were allowed to grow to their terminal length while still undergoing regular measurement. The rat was then killed and the follicles fixed for histology.

2.2.4.5 Plucking and transection of follicles *in vivo*

In order to investigate the regenerative ability of the plucked end bulbs *in vivo*, rats were anaesthetised by intramuscular injection with 0.08 ml hypnorm (Janssen Pharmaceuticals Ltd) and then intraperitoneally with 0.06 ml of Diazepam (Roche Products Ltd) or with an approximate dosage depending on body weight. When anaesthesia was induced, the whisker follicles on the most posterior row (row a) were exposed as described previously in section 2.2.3.1. Using fine scissors the upper three-quarters of the follicles were cleaned free of connective tissue. The club and growing fibres of the exposed follicles were plucked respectively and the follicles were then transected just above the bulb region (Fig. 2.3). This left the plucked, amputated bulb surrounded by connective tissue and separated and completely away from the upper section, which was free from connective tissue and still attached to the skin by its uppermost portion. After each operation, the mystacial pad was closed by stitching with three or four, 5-0 coated vicryl sutures (Ethicon) and the animal was allowed to recover in an isolated cage. The operation was never observed to cause any deleterious effects on the animal's feeding or drinking behaviour. A total of 16 follicles from 4 rats, were used in this section of the study.

Furthermore, in order to compare the regenerative ability of the isolated end bulb at different stages of post-plucking, on another four rats the vibrissa follicles were first plucked as described above. However, after the plucking the animals were kept in solitary cages for 1, 2, and 4 days at which points the plucked follicles were exposed and their bulbs were transected in the same manner as outlined above.

In all cases, twenty days after the operations the animals were killed by an overdose of carbon dioxide. The two separated segments of each of the experimental follicles were removed and placed in 4% formal saline. The bulbs were cleaned of connective tissue and then photographed along with their upper follicle portions. Both elements were then processed for histology as described in section 2.2.3.2. In one case the specimens were stained with Haematoxylin and Eosin.

2.3 Observations and Results

2.3.1 Characteristics of follicles at different stages of the hair cycle

2.3.1.1 Anagen follicles

Throughout most of anagen the lower end of follicles appears as a bulbous shape (Fig. 2.4A) comprising a central dermal papilla enclosed, apart from its basal stalk, by the epidermal component, usually termed the hair matrix (Fig. 2.4B). The dermal papilla appears large, distally extending into the medulla of the growing hair shaft, and its extracellular matrix (ECM) displays a high affinity to Alcian blue (Fig. 2.4C). Ultrastructurally, as previously reported (Young, 1980), papilla cells showed various shapes with long cytoplasmic processes oriented in all directions (Fig. 2.4D). The cells were distinguished by a large round or oval nucleus (Fig. 2.4E) and were well spaced and evenly distributed inside a loose ECM. Their cytoplasm was granular and contained normal cytoplasmic organelles (Fig. 2.4F). The cells demonstrated extensive endoplasmic reticulum (ER) associated with a mass of ribosomes. Mitochondria and Golgi apparatus were located in the more peripheral regions of the cytoplasm while numerous free ribosomes were distributed in all parts of the cytoplasm (Fig. 2.4G). The bulb epidermal cells were separated from the papilla by a basement membrane (Fig. 2.4H). The epidermal cells were attached to this membrane by hemidesmosomes. The epidermis consisted of multi-layered and densely packed cells (Fig. 2.4I) which exhibited specific characteristics in the lower and upper regions of the bulb. In the lowest region (germinative), these cells were round, small in size with a large nucleus (Fig. 2.4J). Their cytoplasm possessed several mitochondria and a mass of free ribosome but was not rich in other organelles (Fig. 2.4H). Desmosomes were visible at low density at the junction of these cells. As cells moved upwards they gradually altered either in morphology or in cytoplasmic constituents. Thus, in the upper part of the matrix, epidermal cells tended to be large and elongated (Fig. 2.4K). Epidermal cells in this region were connected to neighbouring cells with many more desmosomes than cells in the lower area (Fig. 2.4L). In pigmented follicles a large number of melanin granules were present in the cell cytoplasm and distributed intracellularly through most of the epidermal matrix width (Fig. 2.4M). Above the bulb, epidermal cells were undergoing terminal differentiation

and keratinization products appeared in the cell cytoplasm (Fig. 2.4N).

The epidermal compartment, in turn, all through the length of the follicle (except its neck), was enveloped by a sheath of dermal cells called the dermal sheath (Fig. 2.4B), which at the lowest part of the follicle was joined to the basal stalk of the dermal papilla. At the interface of these two components, the epidermal and dermal sheath, there was a distinct basal lamina, normally termed the glassy membrane (Fig. 2.4O).

When follicles were labelled with BrdU for localisation of cell proliferation, the distribution of BrdU labelling in anagen follicles was found to be in agreement with previous studies which used other proliferative markers (Tseng & Green, 1994; Commo & Bernard, 1997) (Fig. 2.5A). Briefly, extensive cell proliferation was observed within the epidermal cells of the matrix region, particularly at the lowest level (Fig. 2.5B). Scattered labelling was seen in the middle and upper regions of the follicle along the hair shaft, basically in the outer root sheath cells attached to the glassy membrane (Figs. 2.5C-E). In my observations no cell division was seen in the dermal component of the follicle, neither in the dermal papilla nor in the dermal sheath. To make sure that the label penetrates to all regions of the specimens in some cases follicles were first transected in two halves and then labelled with BrdU. The pattern of BrdU-labelled cells that was observed in these follicle halves was similar to that seen in the whole follicles.

Follicles were also examined for detection of cell death using a C-Jun/AP-1 polyclonal antibody (see section 2.2.3.4). In this case, to demonstrate that the cell death antibody was specific and would only stain apoptotic cell death, the immunohistochemical reaction was first performed on a positive control tissue. In development of limbs in vertebrates it has been well documented that the cell death process removes specific cells during remodelling of the structure when undergoing morphogenesis. Hence, limb buds of a 15 days rat embryo were initially labelled with the antibody. The antibody was found to be specifically expressed in the distal tip and interdigital regions of the limb buds (Figs. 2.6A-B), corresponding to where cell death has been shown to occur previously (see Browder *et al.*, 1991). This observation suggested that the antibody reacts with an antigen that is expressed in specific apoptotic cells. Based on this evidence I utilised the antibody for detection of cell death in the plucked follicles. In anagen follicles examined for cell death no marking was found in follicles neither in the bulb area nor in the upper part of the follicle (Figs. 2.5F-H),

although occasionally in the upper part of the follicle one or two single epidermal cells were labelled with cell-death antibody. Moreover, in two sections one or two marked cells were found in the epidermal cells of the bulb region. These cells were situated in the upper half of the bulb in an area close to the dermal papilla. Apart from this, no marked cells were localised in the bulb, neither within the papilla, nor in the epidermal component.

2.3.1.2 Catagen follicles

In longitudinal histology sections, the catagen phase was distinguished by the involution of the dermal papilla's apex, which collapses to one side of the dermal papilla (Figs. 2.7A-B). At this stage the dermal papilla still is large in size, but the number of cell layers in the epidermal matrix decreases as its organisation altered (Fig. 2.7A). In the mid-bulb, cell organisation in the epidermal matrix adjacent to the papilla was found to have collapsed and cells degenerated possibly as a result of apoptosis (Fig. 2.7A). BrdU labelling of follicles (Fig. 2.7C) showed that at the onset of catagen the labelling began to reduce in the bulb GE cells. However, compared to anagen follicles more labelled cells were observed in the upper half of the follicle bulb. Above the bulb, as in anagen follicles, scattered marked cells were found within the ORS cells along the length of the fibre (Fig. 2.7C).

2.3.1.3 Telogen follicles

During telogen the lower end of the follicle is narrow and pointed (Fig. 2.8A). In histology the bulb is characterised as a small knob at the end of the follicle (Fig. 2.8B). The dermal papilla is small and surrounded by a strand of epithelial cells (Fig. 2.8C). The papilla cells were found to be densely packed, round in shape, and relatively small with far fewer cytoplasmic projections visible (Fig. 2.8D). Their cytoplasm was confined to a narrow space between nuclei with a noticeable reduction in cytoplasmic organelles. In the epidermal component of the bulb changes had happened as well. In addition to a reduction in the number of cell layers, the matrix cells were found to be large and elongated. The cells were still separated from the dermal sheath cells by a glassy membrane, which was now much thicker than that observed in the bulb region of anagen follicles (Fig. 2.8E). Epidermal cells at the tip of the matrix, which during anagen is the

site of GE cells, also displayed a similar morphology to those in upper regions. These cells were also large with extended nuclei (Fig. 2.8F). Although, there are reports that during telogen in rat vibrissa follicles a subpopulation of epidermal cells is left behind at the lowest extremity of the bulb (Reynolds & Jahoda, 1993), in this study I could not track these cells. During telogen, the hair fibre (no longer growing) had detached from the epidermal cells of the matrix and moved upward to form the club (Fig. 2.8B).

In telogen follicles which were labelled with BrdU, marking was found in cells both in and above the bulb. In the bulb area cell division was only observed in epidermal cells located in the upper part of the bulb (Fig. 2.9A). No marking was found in the lower part of the matrix where massive cell proliferation was observed in anagen follicles. In areas above the bulb the marked cells were mainly concentrated within the ORS cells of the mid-region of the follicle and a few cell divisions were also detected in the upper half of telogen follicles (Figs. 2.9B-D). No staining was found in the dermal component of the follicle (the dermal papilla and dermal sheath) similar to what was described for anagen follicles.

Examination of the telogen follicles with a cell death antibody (Fig. 2.9E-G) revealed a large number of labelled cells in the lower third of the follicle between the dermal papilla and the lifting club fibre. The antibody was mainly found in the upper half of the bulbs in cells surrounding the dermal papilla but no labelling was observed within the papilla cells (Fig. 2.9E). In contrast to BrdU labelling, in which marked cells in this region was found within the basal epidermal cells attached to the glassy membrane, cell-death labelling was observed within cells close to the dermal papilla. Above the bulb, the marked cells were located within the narrow tube which formed as a result of the upward migration of the club fibre (Fig. 2.9F). Once again, the labelled cells were not seen in the ORS laterally attached to the glassy membrane but they were found in the centre. Some stained cells were also found in cells around the base of the club fibre. In the upper part of the follicle, the density of labelling diminished so that only 1 or 2 fluorescent cells were seen in the upper half of each follicle section (Fig. 2.9G).

2.3.2 Changes to follicles after fibre plucking

When a growing vibrissa hair was plucked, not only the fibre but also most of the ORS and the epidermal matrix of the bulb were removed (Fig. 2.3B2). A small

population of germinative matrix cells was consistently left behind at the lowest extremity of the follicle (Fig. 2.10). Above the follicle end bulb, an incomplete layer of basal outer root sheath cells remained attached to the glassy membrane at the sides of the hair shaft cavity, which was filled with blood and debris. Towards the top of the appendage, in the presumptive bulge region, a substantial outer root sheath was retained (Fig. 2.10).

Normally plucking did not damage the hair follicle dermal papilla, which initially retained anagen typical characteristics at the base of the follicle. It remained pear-shaped with a distinct apex, while proximally the basal stalk was surrounded by a ring of darkly stained germinative cells as described above. Dermal papilla cells were well spaced within a glycosaminoglycan rich ECM and the whole structure was ramified by capillaries (Fig. 2.10). Nevertheless, in few cases the plucking led to damaging (Fig. 11A) or displacement of the dermal papilla solely, or along with, germinative epidermal cells (Figs. 2.11B-C). In these cases the tissues were lifted from the follicle base and left in the upper part of the follicle, inside the hair shaft cavity.

2.3.2.1 Single plucking

i) Histology

Histological examination of plucked anagen vibrissa hair follicles at increasing intervals post-plucking revealed the main processes of regeneration in the plucked follicles. Six hours after plucking the follicles demonstrated an anagen histology in their dermal components, with a pear-shaped papilla which occupied a large area in the bulb, similar to anagen follicles. The papilla apex extended distally into the hair shaft cavity inside the interrupted outer root sheath left after plucking. Proximally the papilla was continuous with the follicle dermal sheath via a well-defined basal stalk (Fig. 2.12A). Papilla cells were distributed evenly in the tissue and their ECM showed a high affinity to Alcian blue stain. Due to removal of epidermal matrix the papilla cells in the mid-region of the papilla were in direct contact with the glassy membrane that was originally located at the matrix-dermal sheath junction. However, at the base, the papilla was surrounded by the residual GE cells which were small, round and retained a strong reaction to basophilic stain- an indication of their mitotic activity (Young & Oliver, 1976). No major changes were observed over the subsequent 12 hours in the follicle

bulb (Fig. 2.12B). In 70-80% of the plucked follicles a cyst-like structure now started to appear inside the hair shaft cavity higher up in the mid-half of the follicle. The cyst was composed of a blood-filled hole and was surrounded by two or three layers of tightly connected epidermal cells (Fig. 2.12H). In 50-60% of the follicles only a single cyst was observed inside the hair shaft cavity, but in 10-20% of specimens, another one or two similar structures became visible higher up in the hair shaft cavity in subsequent days (Fig. 2.12I). These cysts were visible in the follicles up to 7 days post plucking.

After 24 hours a clear expansion was seen in the epidermal component of the bulb which had now surrounded most of the lower part of the papilla (Fig. 2.12C). Nevertheless, the apex and upper region of the papilla was still denuded and no epidermal matrix was visible at the sides of the papilla. The residual germinative cells were still present at the lowest region of the new growing matrix as darker stained cells, while a subpopulation of regenerated cells with lighter staining was discernible just above them. The dermal papilla still retained an anagen morphology with rich ECM, but in the upper papilla some abnormal spaces appeared between cells corresponding to the level at which the plucked epidermal component had been removed and was absent from the papilla basement membrane. Higher up the follicles, the hair shaft cavity was now filled with cells which morphologically resembled outer root sheath cells.

At 48 hours post-plucking, the new regenerated region of the growing matrix had broadened and expanded upwards with evidence of internal differentiation distally. At this stage, the matrix had expanded to nearly enclose the papilla whose tip alone was free from epidermal cells (Fig. 2.12D). In the majority of follicles (75-90%), a new type of cyst-like structure was apparent in the outer region of the growing epidermal matrix. Cells in the cyst showed an abnormal density and different arrangement to their neighbouring epidermal cells. Inside, the dermal papilla demonstrated a slight reduction in size but cells were still well spaced within a rich ECM, which still well stained by Alcian blue.

At 4 days post-plucking, the papilla had condensed down with a distinct loss of its ECM bulk and specifically in glycosaminoglycan labelling. It was completely surrounded by the new regenerated matrix, which by this time was continuous with the epithelial cells filling the hair shaft cavity. The cyst, originally inside the matrix, had moved upwards and was now seen above it (Fig. 2.12E). At 7 days, fibre growth had

resumed and a new fibre was now visible growing in the follicle. At this stage the papilla was still relatively condensed with a reduction of ECM compared with the mid-anagen specimens, however, the epidermal matrix was clearly producing a new fibre (Fig. 2.12F). By 9 days, the follicle had returned to an anagen-typical morphology with a large and pear-shaped papilla accompanied by a broadened and proliferating epidermal matrix producing all the expected differentiated epidermal cell components (Fig. 2.12G). By this time, a regenerated fibre had emerged from the skin surface of some follicles. When the diameters of the regenerated fibres were compared with fibres of the same length from normal follicles it was found that the regenerated fibres had similar diameter to those observed in the normal follicles.

ii) Cell division

In the plucked mid-anagen follicles mitotic activity was investigated at the same post-plucking intervals as described above for histology. BrdU localisation of follicles immediately after plucking (Fig. 2.13A) revealed that the pattern of cell proliferation was relatively similar to that described for anagen follicles. In the bulb region, in addition to the strong expression of the label within the residual germinative cells some staining was also found in the upper bulb region (Fig. 2.13B). Dividing cells were found in the middle part of the follicle corresponding to the position of ORS cells left attached to the glassy membrane (Fig. 2.13C). BrdU labelling in the upper follicle was patchy and very sparse (Fig. 2.13D). Labelling was only observed in the epidermal component of the follicle.

Six hours post-plucking (Fig. 2.14A), a significant reduction was found in the total amount of labelling. In these follicles intense BrdU labelling was again observed in the GE cells that remained in the follicle base. However, at this stage, staining was strictly confined to these cells (Fig. 2.14B), with little or no indication that division was occurring in either the basal ORS cells of the hair shaft (Fig. 2.14C), or the more substantial ORS cell reservoir higher up (Fig. 2.14D). At 12 hours (Fig. 2.15A) staining was still strong in the germinative region (Fig. 2.15B) but was now also visible among the ORS cells above the bulb (Fig. 2.15C), while remaining virtually non-existent in the upper half of the follicle (Figs. 2.15D-E). At 24 hours (Fig. 2.16A) the matrix cells remained strongly labelled (Fig. 2.16B) and there was now extensive staining present higher up in the hair shaft (Fig. 2.16C). The mid-follicle hair shaft cavity had by this

stage become filled with a solid core of ORS-like cells (Fig. 2.16A), but there was only limited cell division apparent in the basal layers of the upper half of the follicle epidermis (Fig. 2.16D). At 2 days post plucking (Fig. 2.17A), cell division was observed in epidermal cells of all regions of follicles (Fig. 2.17B-E). In the bulb (Fig. 2.17B), cell division was still persistent in the epidermal germinative region. The middle region of follicles also stained with the antibody (Fig. 2.17C) but after two days of relatively negative response, cell division was again seen in the upper part of the follicle where the hair shaft cavity was wider (Fig. 2.17D). The pattern of BrdU labelling in 4-day post plucking follicles (Fig. 2.18A) was generally similar to the follicles examined 2 days after plucking. While the upper part of follicles showed dispersed labelling, the intensity of the marking in the GE population was reduced (Figs 2.18B-E) when compared to that observed in the early hours after plucking.

At 7 days post plucking (Fig. 2.19A), the follicles returned to an anagen state in terms of the number of marked cells at the bulb region. In this area the epidermal cells were intensively labelled with BrdU (Fig. 2.19B). Scattered labelling was also observed in the middle and upper parts of follicles within the ORS cells along the length of the new growing fibre (Figs. 2.19C-D). The pattern of cell division within the follicles 9 days after plucking was typical of anagen, with intense labelling in the germinative cells and dispersed marking in the middle section and 1 or 2 labelled cells in the upper part of the follicle (Fig. 2.20).

Overall, these observations indicated that cell division was initially suppressed in the upper part of plucked follicles (Figs. 2.14-15) but took place continuously and predominantly in the germinative region, and that this group of cells also remained proliferative subsequently (Figs. 2.13-20). The distribution of the replicating cells spread with time from the base upwards, but it was around 24 hours post plucking before bulge region cells were labelled (Fig. 2.16). During the period between 2 to 4 days post plucking the BrdU labelling decreased in the bulb (Figs. 2.17-18) region however positively marked cells were persistently observed in germinative cells. Labelled cells had extensively increased at 7 days (Fig. 2.19) in the matrix region and by 9 days post plucking (Fig. 2.20) follicles displayed anagen characteristics (Fig. 2.21).

iii) Cell death

Microscopic examination of the follicles just after plucking (time 0) revealed that in general no cell death was occurring in the follicles. In the bulb and lower half of the follicle not a single marked cell was observed (Fig. 2.22). In the upper half of the follicle only 1-2 cells were stained per section, usually in the region of the club fibre. In follicles 6 and 12 hours post-plucking (Fig. 2.23) no major changes were observed in respect of the number of labelled cells. However, in a few cases 1-2 marked cells were observed in the upper half of the follicle inside the hair shaft cavity, and in the bulb within the dermal papilla apparently corresponding to the position of blood vessels. The number of cells stained with the antibody increased in follicles 24 hours post-plucking. In the bulb area, labelling was observed within the epidermal cells around the papilla apex but a few cells at the tip of the papilla apex also appeared to be marked. No sign of labelling was observed within the cells of the lower half of the bulb, neither within the papilla cells nor in the epidermal matrix cells. In the follicle's mid-region in each cut section between several cells were labelled with the antibody. The labelled cells were situated inside the residual hair shaft cavity. The upper half of the follicles also displayed 2-5 stained cells per section, in the ORS cells of the presumptive bulge region (Fig. 2.24). Moreover, at the top of the follicle where it joins to the skin surface, 2-3 positively labelled cells were observed in the centre of the hair shaft cavity in each section.

Follicles 2 days post plucking displayed a relatively similar marking distribution to that seen in 24-hours follicles although the number of marked cell had reduced (Fig. 2.25). In the bulb region 3-4 stained cells were observed in each section. The marked cells were surprisingly found in the papilla (Fig. 2.25B). In two sections one single labelled cell was also observed within the epidermal matrix but it was located in a region corresponding to the cyst-like structures observed by histology. In no sections was marking found within the GE cells. At this time, the mid and upper regions of the follicles continued to demonstrate some cell death staining, essentially among cells in the centre-line of the hair shaft (Figs. 2.25C-D).

iv) Ultrastructure

In the ultrastructural study of plucked follicles essentially only changes to the

lower third of the follicles were followed. At 6 hours after plucking, the residual germinative epidermal (GE) cells at the lowest region of follicle (Fig. 2.26A) were found to be small and round as seen in anagen follicles (Fig. 2.26B). Epidermal cells were joined together by a few desmosomes similar to that seen within GE cells in anagen follicles (Fig. 2.26C), and their cytoplasm appeared to contain a low number of mitochondria and endoplasmic reticulum but many free ribosomes. The epidermal cells were separated from the external dermal sheath and internal dermal papilla by a distinct glassy membrane and basement membrane respectively. Above the residual GE cells, a fracture plane was clearly visible as a consequence of fibre plucking (Fig. 2.26D). Above this, the basement membrane at the epidermal-dermal papilla interface had collapsed and as a result no distinct border between cells in this region could be identified. In two sections, a few single unusual cell-like structures (Fig. 2.26D-E) which appeared to be apoptotic figures were observed at the fracture level. This characteristic was never found within the underlying GE cells. Dermal papilla cells were irregularly shaped and distributed evenly in the papilla similar to their morphology in anagen follicles (Fig. 2.26F). The papilla ECM was rich in thin fibrils and there was no obvious feature to distinguish it from that of the anagen papilla (Fig. 2.26F).

Similar ultrastructure was found in the follicle bulb 12 hours post plucking. However, above the residual epidermal matrix the first regenerated cells were visible, characterised by their lighter cytoplasm (Fig. 2.27A). This feature was indistinguishable by light microscopy. Above the bulb the hair shaft cavity created by removal of the fibre was a relatively cell-free area (Fig. 2.27B). The cavity was lined with an incomplete layer of ORS cells separated from the outer dermal sheath cells by a thick glassy membrane (Fig. 2.27C). An abnormal observation seen in these follicles was that in some sections single papilla cells were located in such close connection with the epidermal matrix cells so that in lower magnification it appeared that they joined together (Fig. 2.27D). However, at higher magnification a narrow space was identified between these cells (Fig. 2.27E). Interestingly, the epidermal cell at the junction site appeared abnormal in its morphology compared with neighbouring cells and looked as an apoptotic cell (Fig. 2.27D). At 24 hours post plucking (Fig. 2.28A), the epidermal matrix and dermal papilla displayed anagen features similar to those described above (Fig. 2.28B). As demonstrated by histology, the matrix component had by now clearly expanded (Fig.

2.28A). Above the bulb the hair shaft cavity had been invaded by epidermal cells probably originating from ORS cells (Fig. 2.28C).

At 2 days after plucking, unusual characteristics were observed in some epidermal cells situated in the upper and outer region of the epidermal matrix (Fig. 2.29A). This region was equivalent to the area where a cyst-like structure had been observed histologically. Cells within the cyst had unusual polygonal nuclei with dense nucleoli and their cytoplasm showed many granules (possibly trichohyalin) of differing sizes. At higher magnification it was found that in contrast to their normal neighbouring cells, the cyst cells did not display the normal cytoplasmic organelles, but large numbers of desmosomes were found at cell junctions (Fig. 2.29B). A thick glassy membrane was observed at the junction between the epidermal matrix and dermal sheath around which in addition to dermal cells, scattered mast cells were also evident (Fig. 2.29C). An observation that was noted in several other follicles was that dermal sheath cells changed shape from the basal stalk toward the upper region of the bulb (Fig. 2.29C). At the lower region of the bulb, particularly in areas close to the papilla these cells displayed a similar morphology to the papilla cells, but toward the upper bulb they were lengthened and revealed less cytoplasmic projections. At this stage the dermal papilla showed a normal anagen-like ECM and the papilla cells possessed a cytoplasm which was found to be active with numerous mitochondria and a rich endoplasmic reticulum (Fig. 2.29D). Above the bulb, in the middle region of the follicle, the hair shaft had broadened and inside it, an epidermal cyst had formed. The cyst was composed of 2 to 4 layers of ORS-like cells, which tightly surrounded a central cavity (Fig. 2.29E). Despite the presence of some scattered cells in a few sections, no organised structure was visible inside the cavity.

By 4 days post plucking the dermal papilla had condensed down in size and had significantly less ECM volume, while most cells had assumed a round shape and their cytoplasm had decreased (Fig. 2.30A). The epidermal compartment in the lower part of bulb comprised a narrow strip, two or three cell layers wide (Fig. 2.30B). In the upper part of the new bulb, the epithelial component was broader and was already composed of multiple cell layers, aligned in different directions, indicating that channelling of the cells into different derivatives had begun (Fig. 2.30C).

At 7 days post-plucking, the dermal papilla cells still appeared relatively densely

packed with fewer cytoplasmic projections than was seen in anagen follicles (Fig. 2.31A). The epidermal matrix however, had expanded in the germinative region, where cells were small and rounded and showed mitotic figures (Fig. 2.31B). At 9 days, plucked follicle papilla cells and epidermal matrix had reverted to a typical mid-anagen morphology (Fig. 2.32).

Overall, these observations demonstrated that after plucking the follicle maintained its anagen features for up to 4 days by which time a new matrix was restored around the papilla. During this period dermal papilla cells displayed typical anagen morphology with many cytoplasmic projections within a loose and open extracellular environment. After that, the plucked follicles entered a transitional stage during which they displayed catagen or telogen-like characteristics. In this period, that lasted for about 3 days the dermal papilla cells population condensed down and individual cells withdrew most of their projections. During this time in the lowest region of bulb epidermis there was a reduction in the number of cell-layers. By about 9 days post plucking the follicles had resumed normal growth and had assumed an anagen-like ultrastructure.

2.3.2.2 Multiple consecutive plucking

Depilated follicles were normally plucked again 20 to 25 days after the previous depilation. Overall, the percentage of irregular plucked specimens (see section 2.2.4.3) found in these follicles was higher compared with follicles plucked a single time- 15-20% in multiple plucked follicles against 8-10% in single plucking. Nevertheless, in all cases these irregularly plucked follicles were discounted from the experiments.

i) Histology and cell proliferation

To compare the morphology and BrdU labelling of follicles after single and multiple depilations I examined the multiple plucked follicles at intervals of 6 and 24 hours post plucking. Apart from one change relating to the epidermal component of the follicles the histology of follicles 6 and 24 hours after they had been plucked multiple times was essentially identical to that observed after a single pluck (Figs. 2.33-34) (see also above). With repeated plucking, the amount of ORS material removed along with the hair shaft was less than the first time. As a result, after plucking, more ORS cells remained attached and were left behind at the sides of the hair shaft cavity.

The pattern of cell division in multiple plucked follicles as seen by BrdU immunolabelling was correspondingly very similar to that observed after single plucking. Six hours after the third, fourth, fifth and sixth depilations, the GE cells were strongly stained with BrdU (Fig. 2.33A). In some follicles, BrdU labelling was also observed sparsely among the cells of the upper bulb region and lower ORS (Fig. 2.33B). In most of the follicles, no trace of label was visible in the mid or upper ORS, or in the bulge area, however, in less than 10% of the specimens one or two single cells were labelled in these regions (Figs. 2.33C-E). At 24 hours post-plucking, the follicles revealed the same distribution of BrdU as their equivalents had done after a single pluck. The epidermal component of the bulb was still intensely stained with BrdU but some labelling had spread to the upper regions (Figs. 2.34B-D). In general, after both single and multiple plucks, the BrdU-label was confined to the bulb and lower half of follicle in the first twenty four hours at which point it had spread upwards to the bulge region. Interestingly, cell division was not observed in the papilla or dermal sheath during this study, apart from a minority of follicles where BrdU staining was observed within the dermal papilla but apparently coincident with the position of capillary cells (Fig. 2.33B).

ii) Growth measurements of regenerated fibres

A new fibre appeared on the skin surface between 8 and 13 days post plucking. The time taken for hairs to emerge after the first or second plucks was between 8 and 10 days. In the case of fibres that were regenerating for a third, fourth or fifth time, the average time before emergence of fibre at the skin surface slightly increased to between 9 and 13 days. As a result of taking frequent measurements, it was established that emergent fibres were growing at normal rates (between 0.8-1.5 mm/day) which remained consistent for successive plucks (Fig. 2.35, see also Fig. 1 in appendix). Measurement of hair length at short intervals, or daily, shortly before the next plucking revealed that during this period, hairs grew at the normal growth rates. In two cases, follicles showed a marginal decline in growth rate after the fourth and fifth plucking (Fig. 2.36) however, none ceased growing. The fibres were always plucked in a mid-growing phase, nevertheless when follicles were allowed to finish their cycles and, even after multiple plucking, they continued to produce fibres at normal rates and with expected lengths (Fig. 2.37). Experimental follicles were all found to have produced longer fibres

than their normal club lengths when their successive post-plucking growths were added together (Tab. 2.2, see also Tab. and Fig. 1 in appendix). As a percentage of club length, these cumulative length increases generally went up with the number of depilations performed. In one case the cumulative length of the fibre produced by a follicle (1a) after 5 consecutive plucking reached 108 mm which was about three times higher than the club length (38 mm) in its equivalent follicle. The regenerated fibres always revealed similar specifications to normal vibrissa in terms of shape and diameter. However, in contrast to the latter they were not always smooth and straight and sometimes displayed a wavy appearance. A difference was also observed between the growth rates of fibre from large and small follicles in the sense that in smaller follicles the regenerated fibres grew slower than large follicles (Fig. 2.38). Therefore, the average length of fibre growth in small follicles over a certain period of time was normally less than for large follicles (Fig. 2.39). For example, at the same time that the 4a follicles had produced fibres with average lengths of 18 mm the 1c follicles had grown fibres with a mean length of 15 mm.

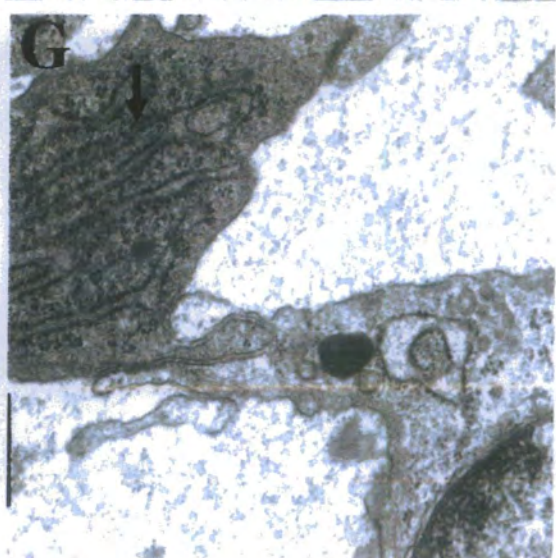
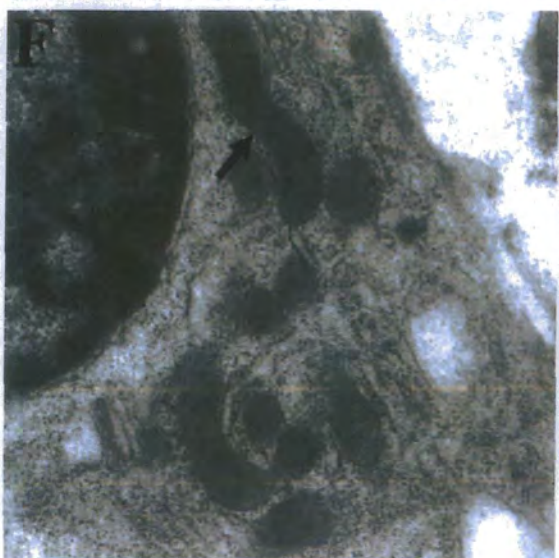
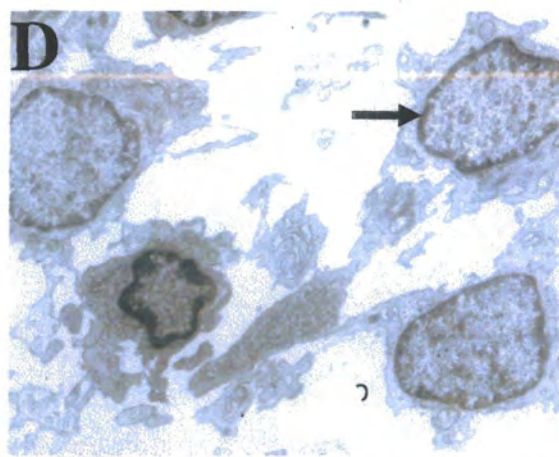
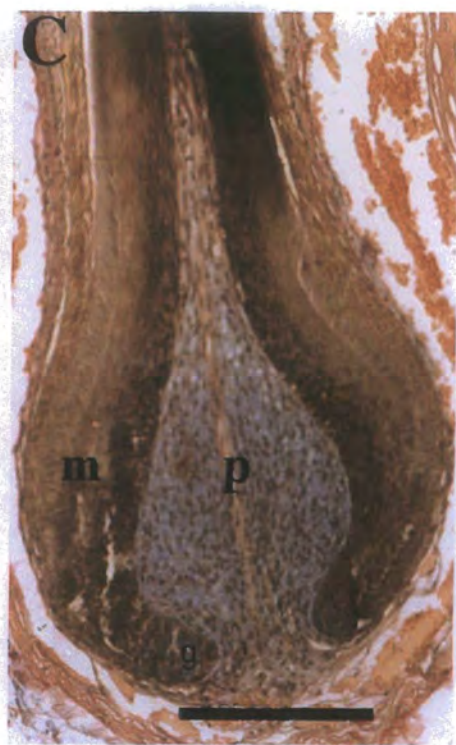
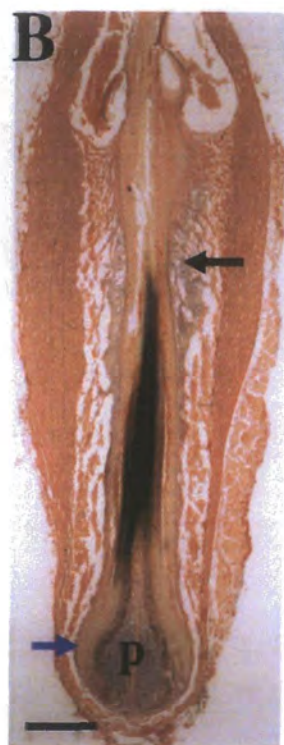
2.3.2.3 Plucking and amputation of follicles *in vivo*

In the follicles that were transected immediately after plucking, out of 16 isolated follicle bulbs, external hair fibre production resumed in eleven (Fig. 2.40A), and one produced a double fibre at biopsy (20 days after amputation). The range of hair length seen was between 0.1 and 3 mm. The fibres were curly, and tightly surrounded by connective tissue. Histologically, these bulbs and their components appeared small. Nevertheless, the epidermal matrix had clearly been regenerated and was still actively proliferating in an anagen state (Fig. 2.40B). In the case of the upper follicle portions, 12 out of 16 produced hair fibres with lengths between 3 and 6 mm. (Fig. 2.40C). Histology revealed that normal-looking end bulbs containing epidermal matrices and pear-shaped dermal papillae had regenerated at the bottom of these still active follicles (Fig. 2.40D).

In order to examine the regenerative ability of these two sections of the follicle, the follicles were transected at different intervals post plucking. In the 4 follicles that were cut in two 24 hours after they had been plucked, external fibres emerged in 2 bulbs and 3 upper sections. In the follicles where the period between plucking and sectioning was 2 days, out of 3 pairs of isolated parts, fibres were produced in 2 bulbs and 2 upper

sections. Finally, when the plucked follicles were segmented 4 days post-plucking, from 4 pairs of isolated segments 2 bulbs and 2 upper sections produced external fibres (Fig. 2.41A). Examination of these follicle sections revealed that in all cases the regenerated bulbs and upper sections had similar characteristics (Fig. 2.41B) to those described above for follicles that were transected immediately after plucking.

Fig. 2.4) Morphology and ultrastructure of the rat anagen vibrissa follicle. **A)** An external view of a follicle displaying a bulbous shape at the base (black arrow). The follicle nerve supply (blue arrow) is also visible. **B)** A longitudinal section through a follicle showing the large dermal papilla (p) and broad epidermal matrix (blue arrow). The follicular epidermis is surrounded by a dermal sheath which is thicker in the upper part of the follicle (black arrow) than the lower regions. **C)** Higher magnification of the follicle bulb showing a large papilla (p) with a distinct apex and extensive extracellular matrix. Except for its base the papilla is covered by a broad epithelial matrix (m). Germinative epidermal cells which by proliferation give rise to the hair fibre are located in the lowest region of the matrix (g). **B** and **C** wax embedded and stained with a combination of Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars **A** and **C** 200µm and **B** 250µm. **D)** Electron micrograph of dermal papilla cells in a follicle. Papilla cells (arrow) are well spaced in an loose extracellular matrix. **E)** Cells show a fibroblastic-like morphology with an oval nucleus and extended cytoplasm containing various organelles including mitochondria (arrow in **F**). **G)** The papilla cells displaying a well-developed endoplasmic reticulum (arrow) with a mass of ribosomes. Dx4600, Ex10000, Fx18000, Gx28000.



Figs. 2.4H-M) Anatomy of anagen follicles

H) Electron micrograph showing an electron dense basement membrane (black arrows) at the interface of the papilla (p) and epidermal matrix (e). The epidermal cells contains many mitochondria and are rich in endoplasmic reticulum and ribosomes. (red arrows) hemidesmosomes.

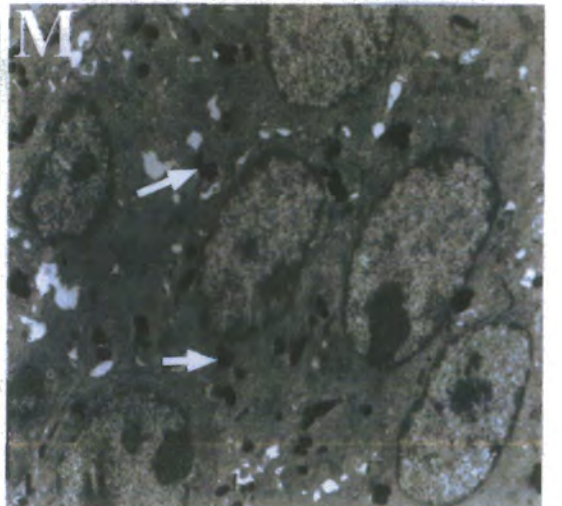
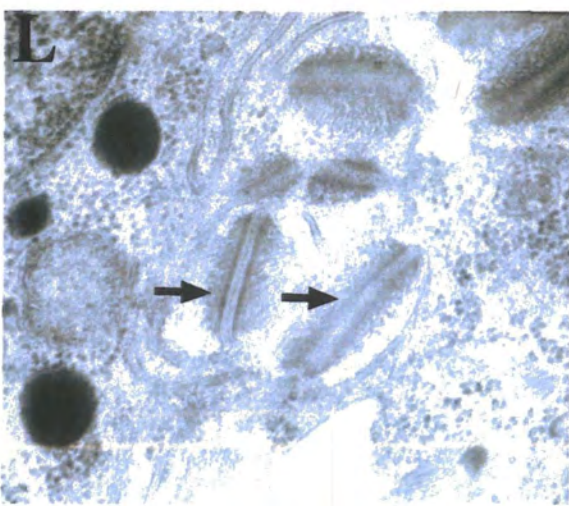
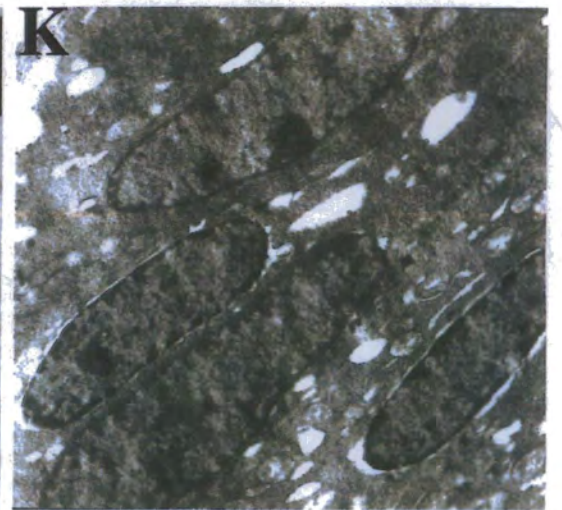
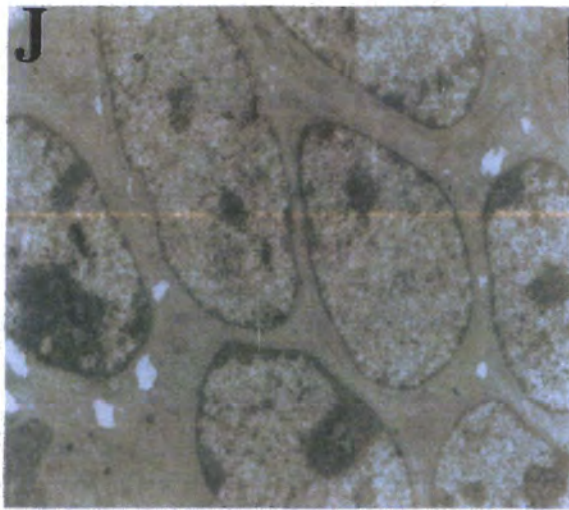
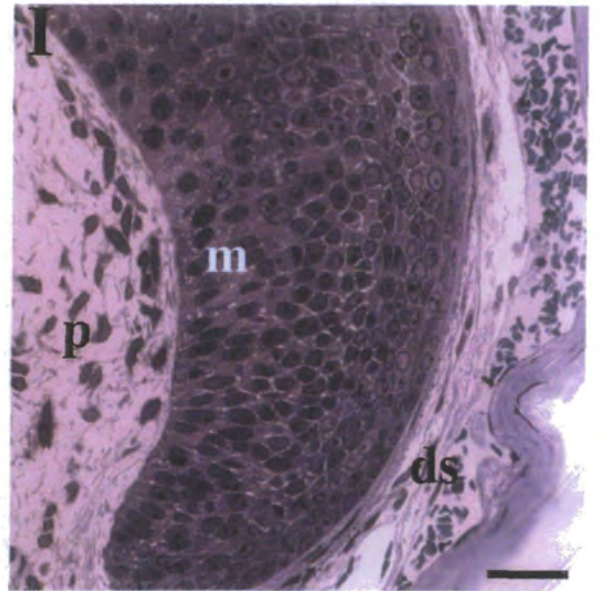
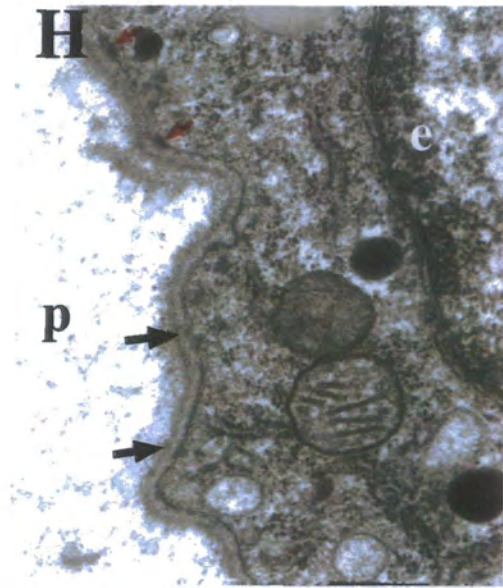
I) Light micrograph of resin embedded section of an anagen follicle. The micrograph displays only the lowest end of the follicle bulb. The papilla (p) is surrounded on all sides by a broad epidermal matrix (m). (ds) dermal sheath. Toluidine blue staining. Scale bar 30 μ m.

J) Electron micrograph of the lower part of the epidermal matrix showing the morphology of GE cells. The cells are small in size and are closely packed together.

K) Cells in the upper part of the matrix have an elongated morphology which is reflected in both the nucleus and cytoplasm.

L) Compared to the lower regions, many desmosomes (arrows) are found at the interface of cells in the upper part of the matrix.

M) In pigmented follicles in the upper matrix melanin pigments (arrows) are present among the epidermal cells. Hx36000, Jx6000, Kx8000, Lx60000, Mx4600.



Figs. 2.4N-O) Ultrastructure of anagen follicles

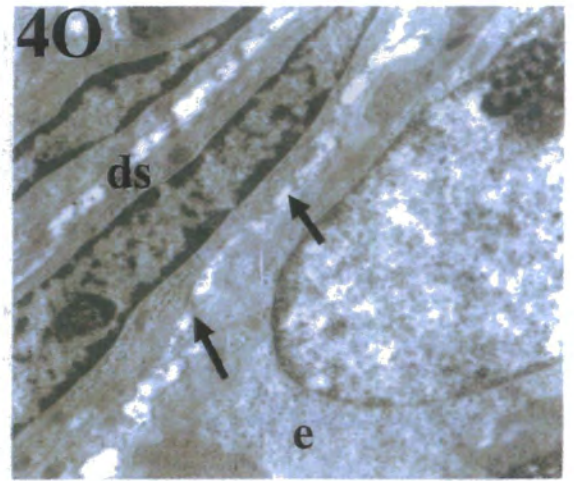
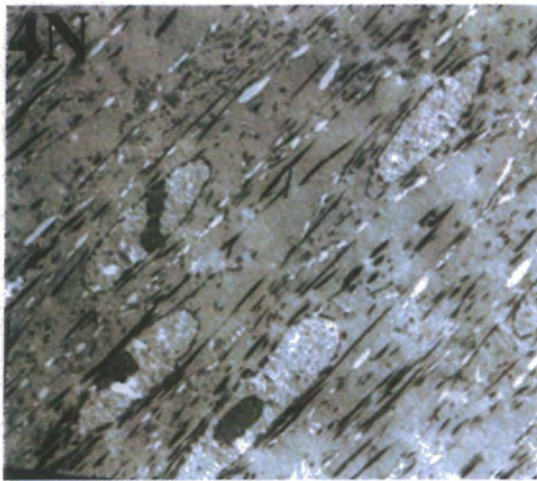
N) Electron micrograph from an area just above the bulb of a follicle showing epidermal cells that are keratinizing. **O)** Epidermal matrix cells (e) are separated from the dermal sheath cells (ds) by a basal lamina called glassy membrane (arrows). The micrograph covers the follicle bulb. Nx2800, Ox6000

Fig. 2.5) Histology (A) and immunohistochemistry (B-H) of anagen follicles. **A)** A longitudinal section through an anagen follicle. Alcian blue, Weigert's Haematoxylin and Curtis's Ponceau.

B-E) BrdU labelling of anagen follicles. **B)** In the bulb region germinative epidermal cells are intensively labelled with BrdU. **C)** Scattered dividing cells are observed in the mid-region of the follicle apparently within the basal ORS cells. Although in this section staining is not seen in the top part of the follicle (**D)** this region was also BrdU-positive (**E)** in the majority of follicle sections.

F-H) Immunolabelling of an anagen follicle with cell death antibody. Virtually no staining was observed in the follicle. **F)** In the bulb region neither the papilla nor epidermal matrix cells are marked, but occasionally in the upper part of follicle one or two single epidermal cells are labelled. No staining is seen within cells of the middle (**G)** and upper (**H)** regions of this follicle. Scale bars A 400µm, B-H 50µm.

Fig. 2.6) Immunolabelling of a developing foot-pad from a 15-day-old rat embryo with an anti-apoptotic antibody displaying labelled cells both in areas close to the skin surface (A) and in interdigital regions (B). Scale bars 50µm.



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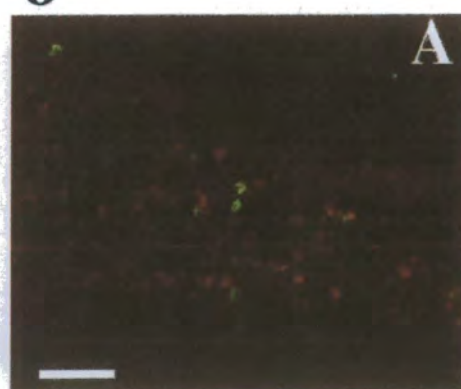
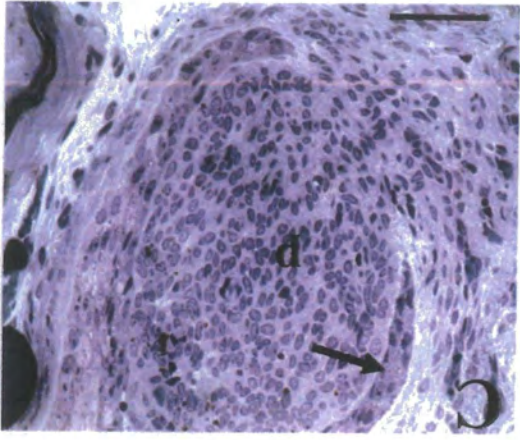
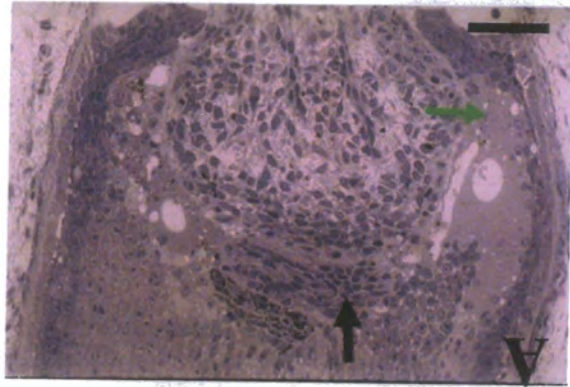


Fig. 2.7) Morphology (A-B) and BrdU labelling (C) of rat catagen vibrissa follicles. **A)** Light micrograph of resin embedded section through the follicle bulb. The papilla apex (black arrow) has collapsed on top of the papilla. Compared to the anagen follicle, the volume of the epidermal matrix is considerably decreased and a population of epidermal cells adjacent to the papilla appears to be undergoing apoptosis (green arrow). **B)** At this stage the hair fibre (f) appears to be separating from the bulb to make a club. **C)** Immunofluorescence micrograph showing the distribution of BrdU labelling in the lower half of a catagen follicle. GE cells in one side of the papilla (p) have ceased dividing while in other side these cells are still dividing. Staining is also seen in the upper part of the follicle bulb. As with anagen follicles, scattered labelling was found in mid-region and the upper half of the follicle. A and B resin embedded and stained with toluidine blue. Scale bars A 50µm, B 150µm and C 50µm.

Fig. 2.8) Morphology (A-C) and ultrastructure (D-F) of rat telogen vibrissa follicles. **A)** Gross morphology of a telogen follicle showing a narrowing and pointed end bulb. **B)** Low power micrograph of a resin embedded section of a telogen follicle. At telogen the bulb is very small and narrow at the base of the follicle. The hair fibre which now is termed, the club, (f) has moved up from the bulb and its base is seen in the lower third of the follicle. **C)** Higher magnification of the bulb region of the previous specimen showing condensed papilla cells (p) surrounded by a thin strand of epithelial cells (arrow). Sections B and C resin embedded and stained with toluidine blue. Scale bars A and B 200µm, C 60µm.



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Figs. 2.8D-F) Ultrastructure of telogen follicles

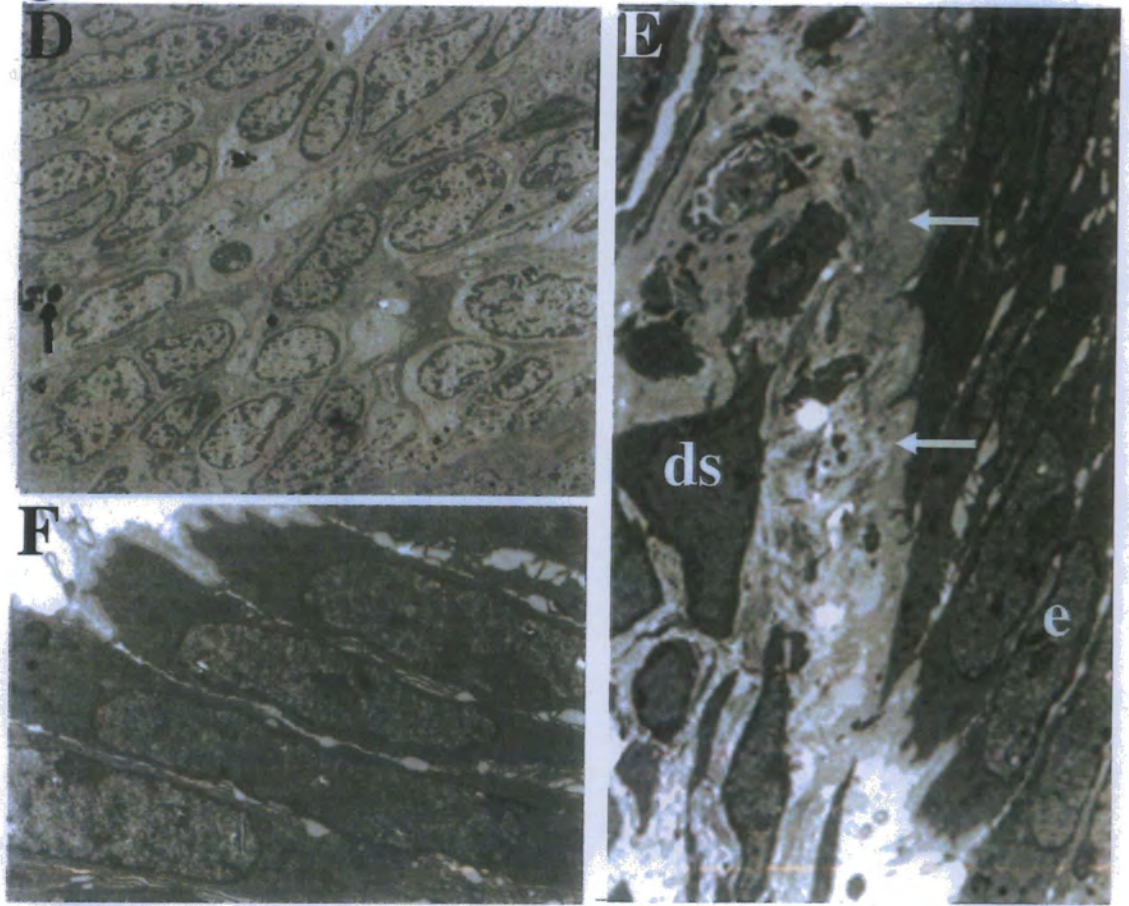
D) Electron micrograph of the dermal papilla in telogen. Papillary cells are tightly packed and residual dense tracts of extracellular matrix remain. Lysosomes (arrow) are common black spots in cell cytoplasm. **E)** The dermal sheath (ds)- epidermal matrix (e) junction in the lower quarter of the bulb region. The epidermal cells are extended with long nuclei. Compared to anagen follicles the glassy membrane (arrows) is very thick. **F)** This micrograph shows the cells in the lowest extremity of the epidermal matrix. Cells are elongated with large nuclei similar to those seen in upper regions. Dx3600, Ex 4000, Fx6000.

Fig. 2.9) Histology (A) and immunohistochemistry (B-G) of rat telogen vibrissa follicles.

B-D) BrdU labelling of a telogen follicle. **B)** The bulb region the BrdU labelling is continuous with that of epidermal cells located in the upper half of the matrix. Scattered dividing cells are also observed higher up of the follicle, mainly in the mid-half (**B-C**). **D)** In the upper half of the follicle cell division is confined to a few ORS cells adjacent to the glassy membrane.

E-G) Immunolabelling of a telogen follicle with the cell death antibody. **E)** In the bulb a few marked epidermal cells are normally seen within epidermal cells surrounding the upper part of the papilla but the labelling is not detected among the papilla cells. **F)** A large number of labelled cells is present in the area between the bulb and the anchorage of the rising club. The stained cells are concentrated in the centre of hair shaft cavity, which at this stage due to upward migration of the fibre, is only filled with ORS cells. Some marked cells are also seen around the base of the club. **G)** Only a few labelled cells are visible in the upper half of the follicle. Scale bars A 160µm, B and C 70µm, D and F 60µm, E and G 130µm.

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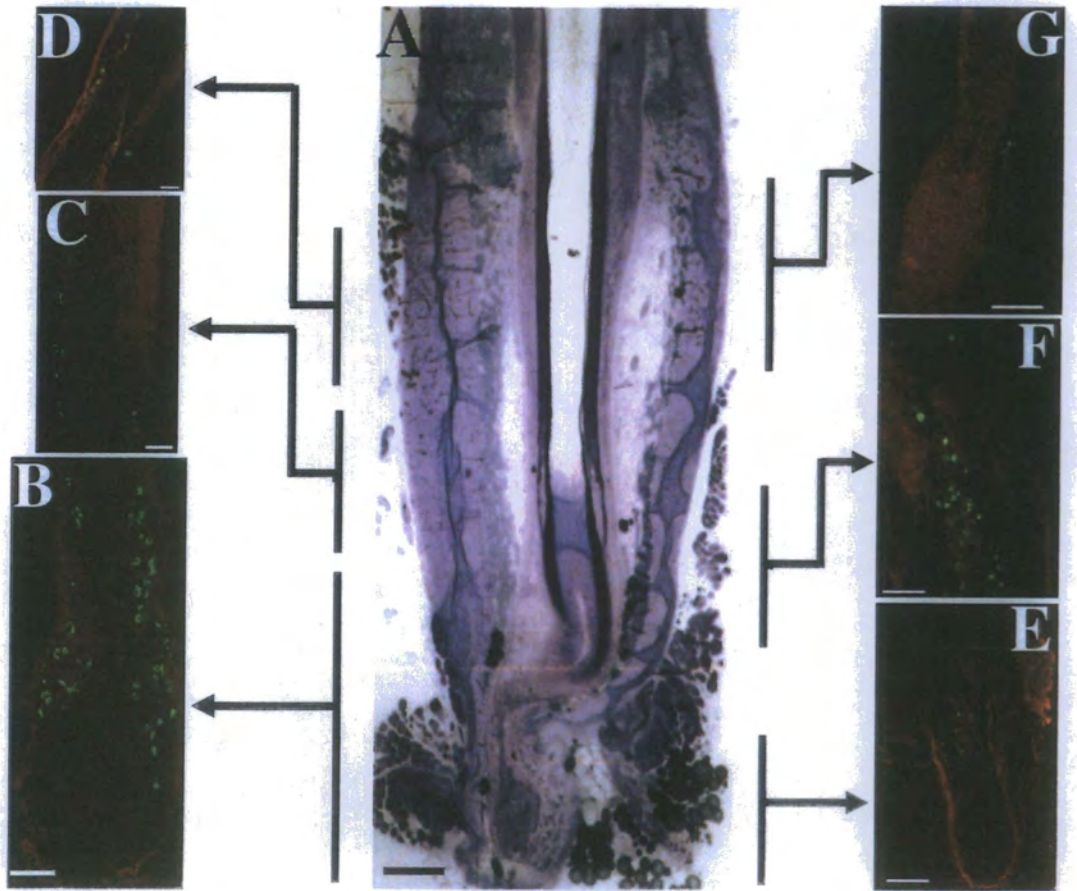


Fig 2.10) longitudinal sections through a mid-anagen vibrissa follicle immediately after plucking of its fibre. Although the main part of the matrix has been removed, a small population of germinative epidermal cells (e) has been left behind in the lowermost portion of the follicle. The hollow tube left through the centre of the follicle was sometimes filled with blood cells and debris. In the upper (bulge) region, a high proportion of outer root sheath cells (ors) has been retained. (p) dermal papilla.

Sections wax embedded and stained with a combination of Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bar 100 μ m.

Fig. 2.11) light micrographs of wax embedded sections through abnormally plucked follicles. **A)** A follicle that was plucked for the third time showing a dermal papilla (white arrow) which has been stretched so that it is seen through all the lower half of the follicle. Moreover, a piece of epidermal matrix (black arrow) has remained at the top of the follicle. **B)** A follicle plucked for the first time. In this case plucking has resulted in displacement of the papilla and GE cells (arrow). They are now seen in the upper section of the follicle. **C)** Higher magnification of the same specimen showing the papilla cells (p) along with GE cells (arrows) on both sides. Scale bars A 150 μ m, B 300 μ m and C 50 μ m.

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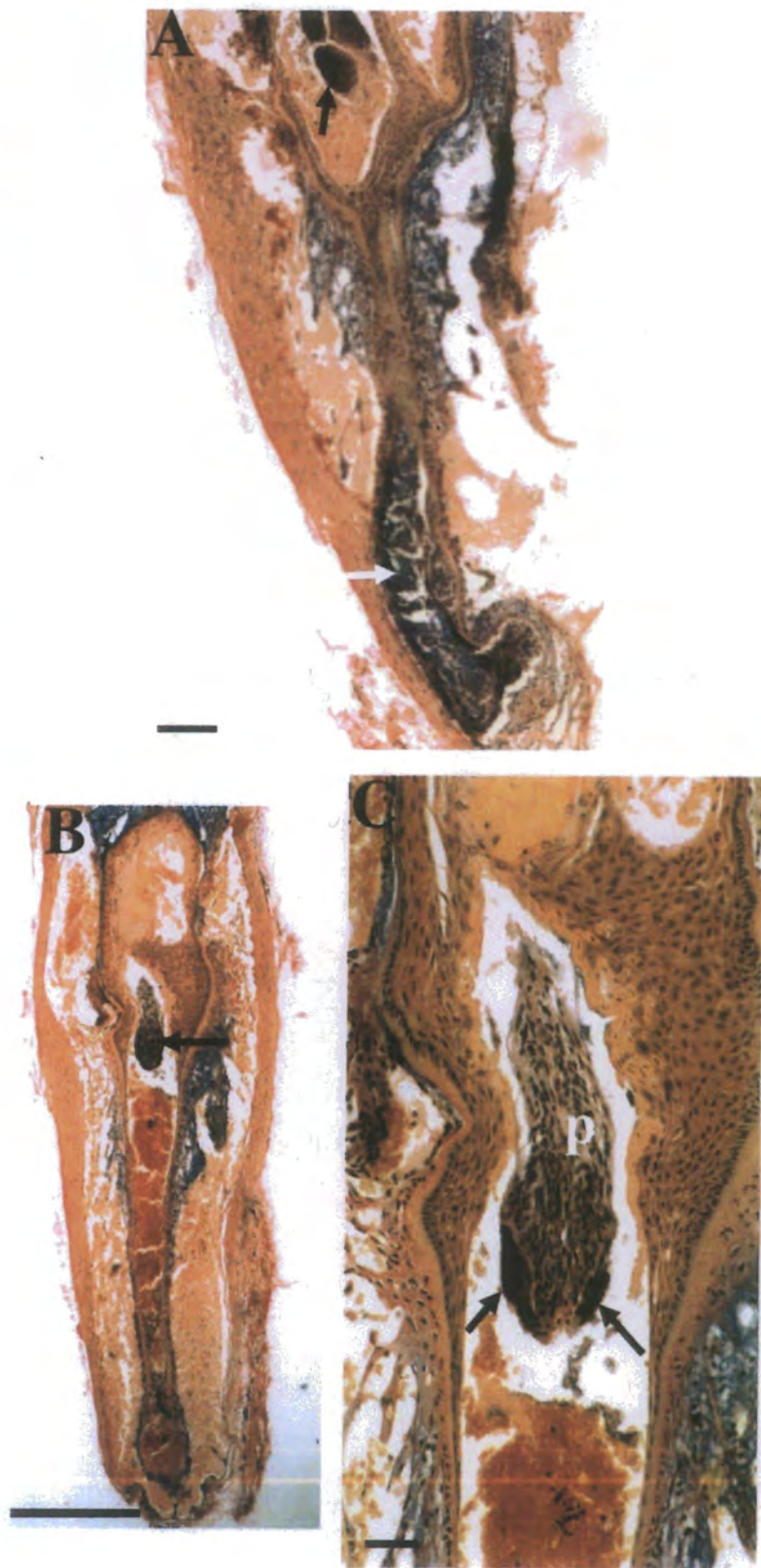


Fig. 2.12) Histology of follicle end bulbs at various stages of regeneration after fibre removal. **A)** 6 hours: GE cells demonstrate a strong basophilic reaction. The dermal papilla (p) is large and rich in ECM. **B)** 12 hours: GE cells appears to be actively dividing as witnessed by mitotic figures, while above the bulb ORS cells are filling in the hair shaft space (arrows). **C)** 24 hours: An expansion of epidermal cells from the GE component is distinguishable. The original germinative cells are still visible at the base as more darkly stained cells, while a new sub population of lighter coloured cells can be seen just above (arrow). The dermal papilla extra cellular matrix is still well stained, but some abnormal spaces can be seen within its upper half where there is no adjacent epidermis. **D)** 2 days: The new epidermal matrix has expanded upwards and nearly surrounds the dermal papilla. At the same time, a group of cells in the expanded outer part of the new matrix differentiated and produced a cyst-like structure (arrow). **E)** 4 days: Around the dermal papilla the epidermal matrix has been restored, and is continuous with the epidermis above. The dermal papilla has condensed down and lost much of its extracellular matrix. The differentiated cyst has now moved upwards and is above the bulb (arrow). **F)** 7 days: Hair fibre production has resumed and the hair shaft can be seen above the bulb region. However, the dermal papilla still looks condensed and the epidermal matrix has not yet reached a normal state. **G)** 9 days: The bulb has returned to a normal anagen appearance, with a multi-layered epidermal matrix, growing fibre and a pear-shaped dermal papilla containing well spaced cells within a rich extracellular environment. **H)** A follicle 24 hours after plucking that has produced a cyst-like structure (arrow) within the hair shaft cavity. **I)** A follicle 4 days after plucking displaying two cyst-like structures (arrows) within the hair shaft region. One of the cysts is seen in the bulge area. Sections wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A-C, E and F 100µm, D, G-I 50µm.

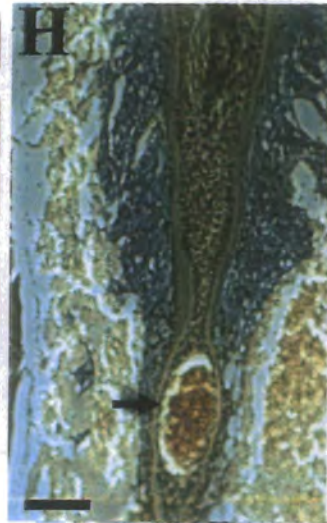
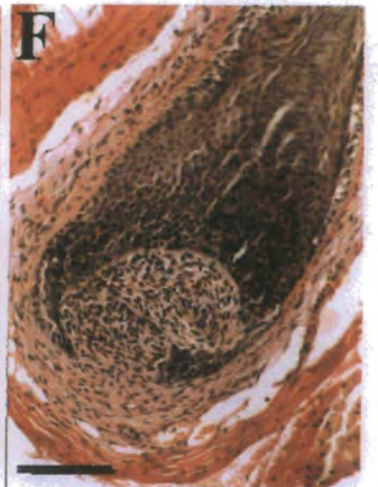
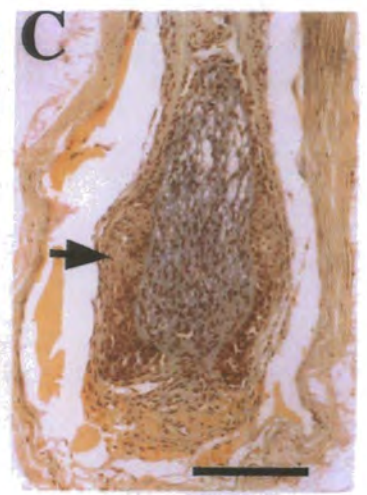
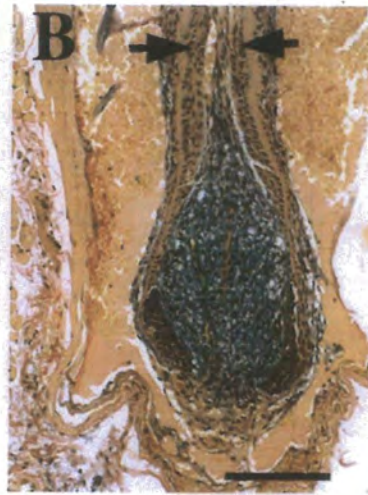
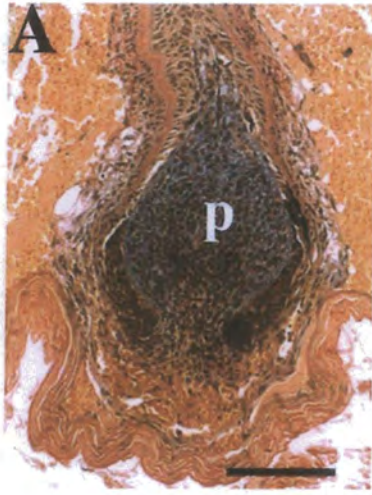


Fig. 2.13 Histology (A) and BrdU-label distribution (B-D) of rat vibrissa follicles immediately after plucking. A) Plucking has removed all the upper part of the matrix and only a small population of germinative epidermal (arrow) cells was seen attached to the lowest region of the papilla (p). B) In the bulb region, a great number of dividing cells are observed within the GE cells. The label was never seen within the papilla cells. C) Labelling is also present as scattered spots within the ORS cells along the middle region of the follicle. In this area labelling is seen within the cells lining the hair shaft cavity which remains after plucking. D) In the upper part of the follicle the number of marking cells decreases and the labelling is confined to a few cells in the presumptive bulge region. (s) hair shaft space. (ors) outer root sheath cells. Section A wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A 140µm and B-D 50µm.

Fig. 2.14 Histology (A) and BrdU-label distribution (B-D) of follicles, six hours post-plucking. A) Along most of the length of the follicle, the hollow tube which, resulted from the plucking of the fibre is largely filled by blood and cell debris. B) Germinative epidermal cells around the base of the papilla (p) are intensely labelled with anti-BrdU antibody. C-D) Above the GE cells, the upper part of the bulb display virtually no label, and similarly, there is no sign of cell division in either mid or upper follicle regions (C and D), (s) hair shaft space, (o) ORS. Section A wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A 160µm, B 150, and C-D 80µm.

Fig. 2.15 Histology (A) and BrdU-label distribution (B-E) of follicles, 12 hours post-plucking. A) In longitudinal section, follicle morphology appears very similar to that observed 6 hours after plucking (Fig. 2.14A), with a gap from removal of the hair shaft still visible through to the upper portion of the follicle. B) GE cells throughout the end bulb are now labelled by the anti-BrdU, with positively stained cells visible right around the dermal papilla. The staining of single cell in the papilla is located at the site of capillaries (arrow). C) Dividing cells are present in the lower quarter of the follicle just above the bulb, although their numbers gradually diminish more distally. D-E) Once again, there is little or no cell division in the middle and uppermost of the follicle. Section A wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A 120µm and B-E 50µm.

Fig. 2.16 Histology (A) and BrdU-label distribution (B-D) of follicles, 24 hours post plucking. A) In addition to the regeneration of a new region of matrix, above the bulb epidermal cells have by now nearly filled-in the space left by plucking (arrows). Unlike the epidermal cells of the matrix, those above are less dense and rather lighter in colour. B) Cell division remains strong in all of the epidermal cells comprising the end bulb. (p) dermal papilla. C) Extremely intense BrdU-label is seen in this lower-mid follicle region, and in particular around a cyst like structure. This concurs with the observed influx of outer root sheath cells to fill the old hair shaft. D) For the first time after plucking (except for time zero) BrdU-label is now observed in this upper follicle area although stained cells are of low density and their distribution limited to the sides. Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S staining for A. Scale bars A 400µm, B-D 50µm.

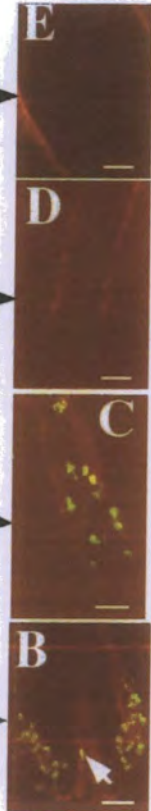
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Fig. 2.17) Histology (A) and BrdU-label distribution (B-E) of follicles, 48 hours post-plucking. A) Epidermal cells comprising the new end bulb matrix have extended around the dermal papilla and signs of differentiation are visible in the epidermal cells situated above the dermal papilla. Higher up the follicle, the old hair shaft space has been completely invaded and expanded by outer root sheath cells. B) The anti-BrdU label still shows dividing cells within the lowest region bulb matrix among GE cells. C) The actively dividing cells that are marked in this section of the follicle still appear to be predominantly in the basal layers of the ORS. D-E) BrdU-label is now seen along most of the length of the follicle, although it diminishes at the highest level. Scale bars A 400µm, B 50 and D-E 100µm.

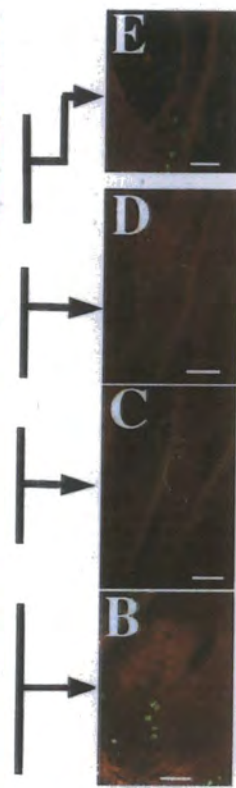
Fig. 2.18) Histology (A) and BrdU-label distribution (B- E) of follicles, 4 days after plucking. A) Epidermal matrix has regenerated around the matrix and cells in the upper region of the bulb appears to be involved in process of differentiation. The matrix shows a reduction in its volume and has fewer cell layers than seen in follicles at previous stages (Fig. 2.21A). The dermal papilla is condensed down and the papillary cells appear packed. B) As with follicles in 48 hours after plucking, relatively few cell divisions are present in the epidermal cells located in the lowest extremity of the follicle bulb. C-D) The upper parts of follicle show a similar distribution of BrdU labelling to that seen in follicles 2 days post plucking (Fig. 2.17). Scale bars A 150µm, B-E 50µm.

Fig. 2.19) Histology (A) and BrdU-label distribution (B- E) of follicles, 7 days after plucking. A) The follicle has regenerated a new fibre which is seen above the bulb. However, the lower epidermal matrix is still narrow and does not display completely normal morphology. Likewise, the papilla is still relatively dense and does not have an apex. B) In terms of BrdU labelling at 7 days post-plucking the follicle shows a typical anagen characteristics. Epidermal cells in the bulb region intensively labelled with BrdU. C-D) Scattered BrdU labelling is also found in the lower third and upper part of follicle similar to those observed in anagen follicles. Scale bars A 150µm, B-D 50µm.

Fig. 2.20) Histology (A) and BrdU-label distribution (B- E) of follicles, 9 days after plucking. Both histologically and immunohistochemically the follicle shows similar characteristics to those seen in anagen follicles. Scale bars A 80µm, B-D 50µm.

Section A in Figs. 2.17-20 wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S

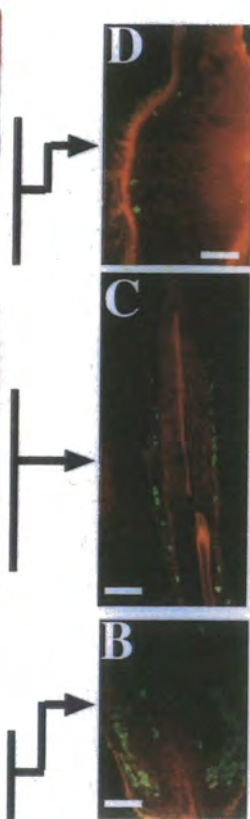
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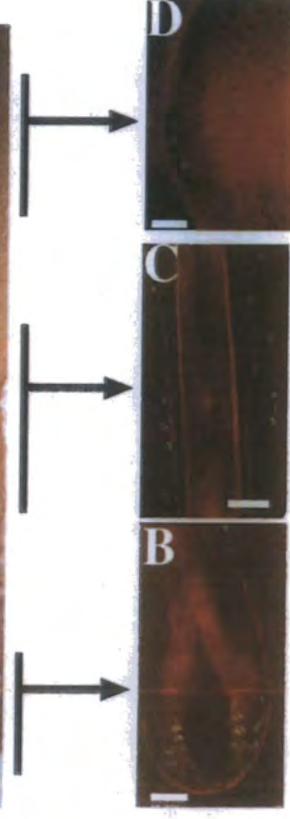
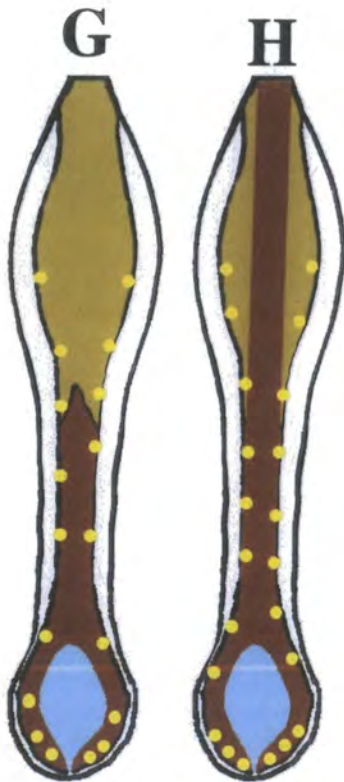
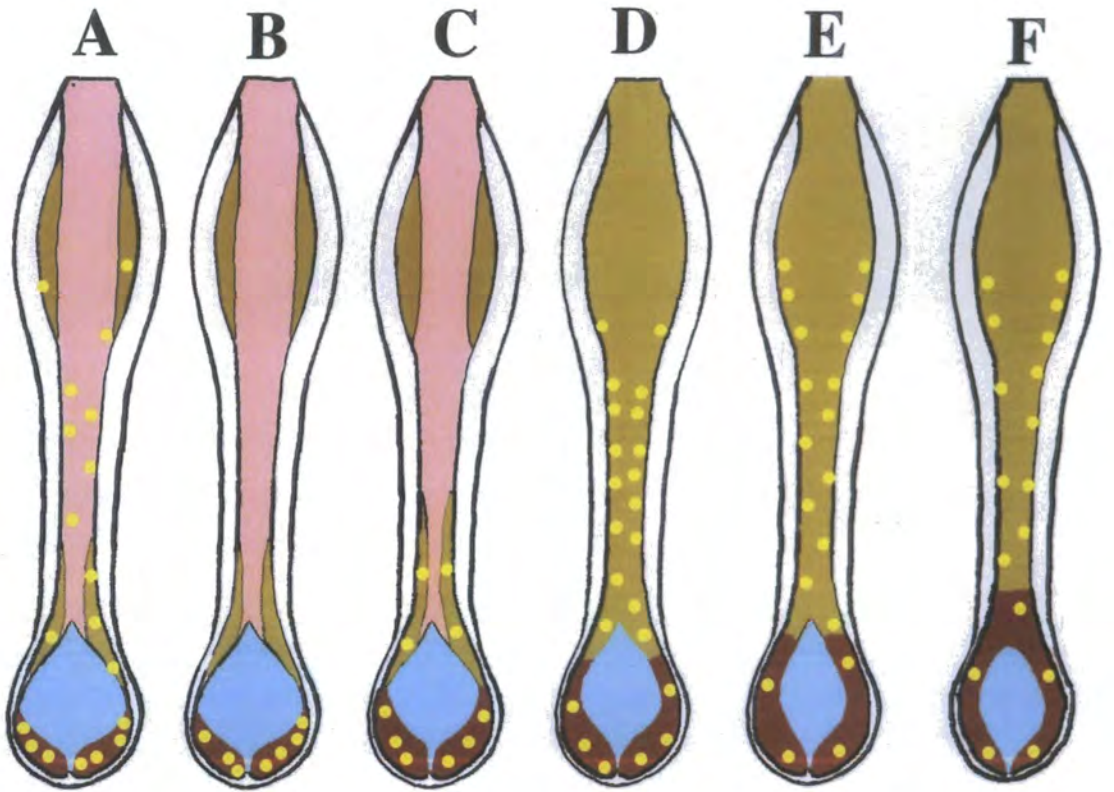


Fig. 2.21) Schematic diagram summarising the overall pattern of cell division seen within follicles after plucking. Initially (at time zero), BrdU labelling is similar to that seen in anagen follicle with intense cell division in the bulb and scattered dividing cells present within the ORS cells of the upper part of the follicle (Fig. 2.13). At six hours post-plucking little or no marking is seen in the cells of the upper half of the follicle and dividing cells are confined to residual germinative cells (Fig. 1.14). Marking then spreads upward through the lower third of the follicle, and by 24 hours post-plucking cell division can be seen throughout all the lower half of the structure (Fig. 1.16). At 48 hours after plucking, BrdU is found over three-quarters of the length of the follicle while the number of cell divisions in the bulb area diminishes (Fig. 2.17). This pattern of staining remains at 4-days post plucking (Fig. 2.18) until day 7, when the intensity of cell division in epidermal matrix again increases (Fig. 2.19). At 9 days post-plucking the follicles display a pattern of BrdU distribution, which is similar to anagen follicles (Fig. 2.20). Marking is constantly observed in the lower (GE) region of the bulb, throughout the period of study.



- Dermal papilla
- Germinative epidermal matrix & Hair fibre
- Outer root sheath
- Hair shaft cavity
- BrdU labelling

Fig. 2.22) Schematic diagram (A) and cell-death distribution (B-D) of follicles immediately after plucking.

A) A schematic illustration of a follicle just after plucking. B-D) No cell death marking is observed in the follicle, either in the lower, or in the upper sections. Scale bars 30 μ m.

Fig. 2.23) Schematic diagram (A) and cell-death distribution (B-D) of follicles 12-hours post plucking.

A) A schematic illustration of a follicle 12 hours after plucking. B-C) No cell death labelling is observed in the lower portion but in the upper region (D) a few cells are marked with the antibody. Scale bars 30 μ m.

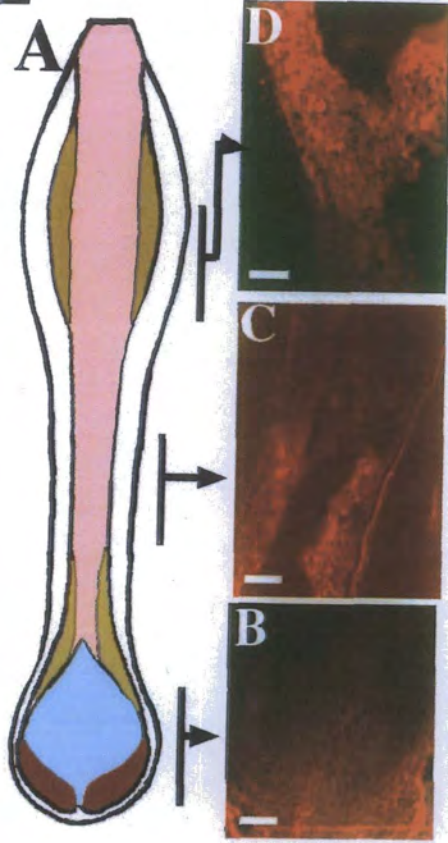
Fig. 2.24) Schematic diagram (A) and cell-death distribution (B-D) of follicles 24-hours after fibre removal.

A) A schematic illustration of a follicle 24 hours after plucking. B) Cell-death labelling is observed in the upper bulb in cells surrounding the papilla. No sign of marking is seen in the lower half of the bulb, either in the papilla cells or in the GE cells. C) Labelling is also observed in cells in the centre of the hair shaft canal. D) Some labelled cells are present inside the hair shaft canal. Again most marked cells are found in the centre of the canal. E) A laser confocal micrograph showing expression of the cell death antibody in the same follicle in more detail. Scale bars B 80 μ m, C and D 50 μ m and E 15 μ m.

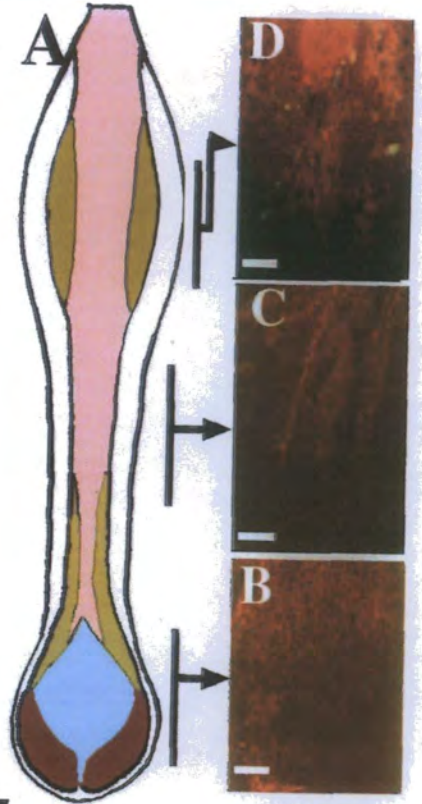
Fig. 2.25) Schematic diagram (A) and cell-death distribution (B-D) of follicles 48-hours after plucking.

A) A schematic illustration of a follicle 48- hours after plucking. B) In the bulb region one or two marked cells are seen around the upper half of the dermal papilla. A single labelled cell (arrow) is also seen within the papilla. Epidermal cells in this area are not labelled by cell-death antibody. C-D) As with the previous stage (Fig. 2.25) some labelling is seen inside the hair shaft canal, but the number of marked cells has diminished. Scale bars 50 μ m.

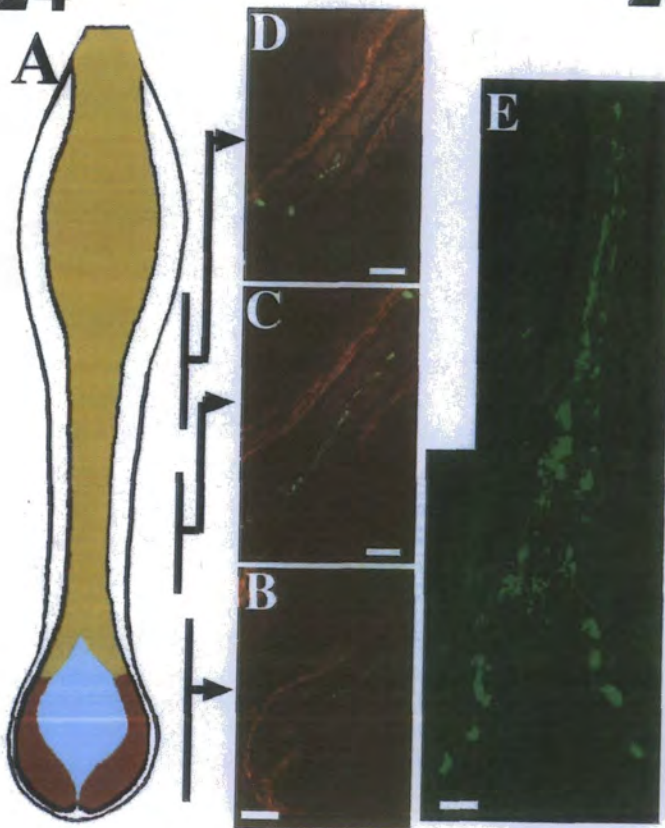
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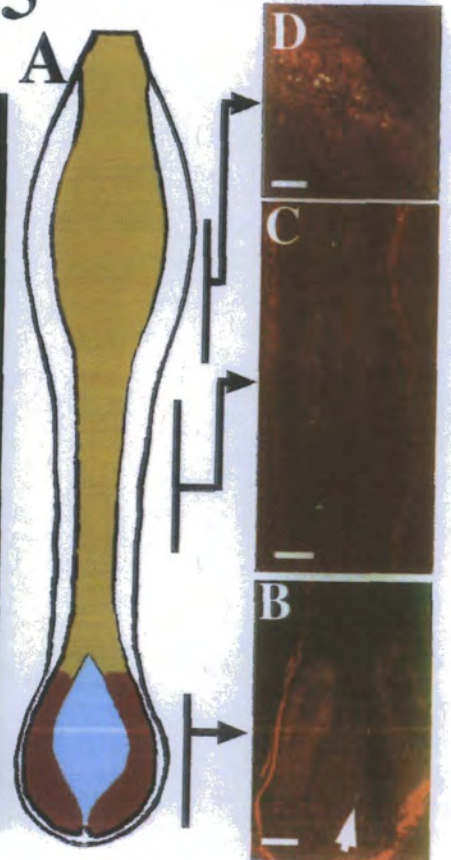


Fig. 2.26) Morphological and ultrastructural characteristics of a follicle 6-hours post plucking. **A)** Light micrograph of a resin embedded section of the lowest region of the follicle bulb. Most of the epidermal matrix cells have been removed with the fibre. However, a small population of germinative epidermal (ge) cells have remained in the lowest region of the bulb attached to the dermal papilla (p). Section stained with toluidine blue. Scale bar 25µm. **B)** Electron micrograph of the lowest part of the bulb showing the germinative epidermal (ge) cells and a part of the dermal papilla (p). The epidermal cells are small in size, similar to those seen in anagen follicles (Fig.2.4). **C)** Junction between two GE cells. The cells have a cytoplasm rich in ribosomes and their membranes attach to neighbouring cell membranes by a few desmosomes (arrow). **D)** Electron micrograph of the bulb region showing the fracture level where the upper matrix cells have been plucked from the lower germinative (ge) cells. In this region the outer dermal sheath (ds) has moved inward and is now seen very close to the papilla (p). In the centre of the micrograph abnormal structures are present which appear to be apoptotic figures. **E)** Electron micrograph of another plucked follicle showing an unusual epidermal cell at the fracture level close to the dermal papilla (p). The cell shows an abnormal morphology and is possibly undergoing apoptosis. **F)** The papilla cells display a fibroblastic-morphology and contain many cytoplasmic organelles. They are well-spaced within a ECM rich in fibrils. Bx2800, Cx100000, Dx2800, Ex22000, Fx4600.

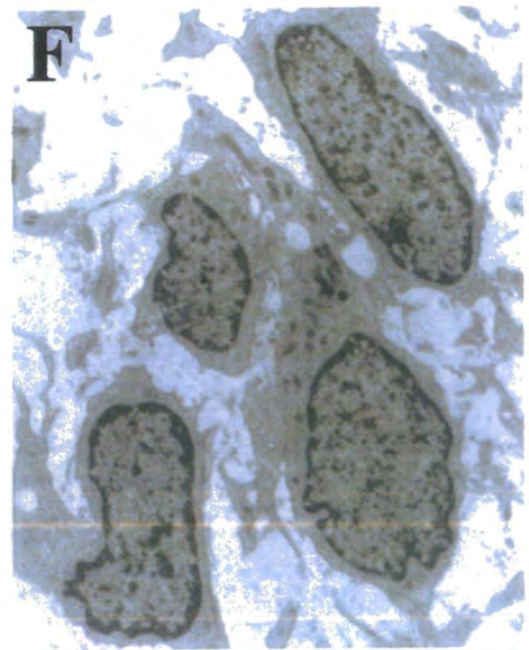
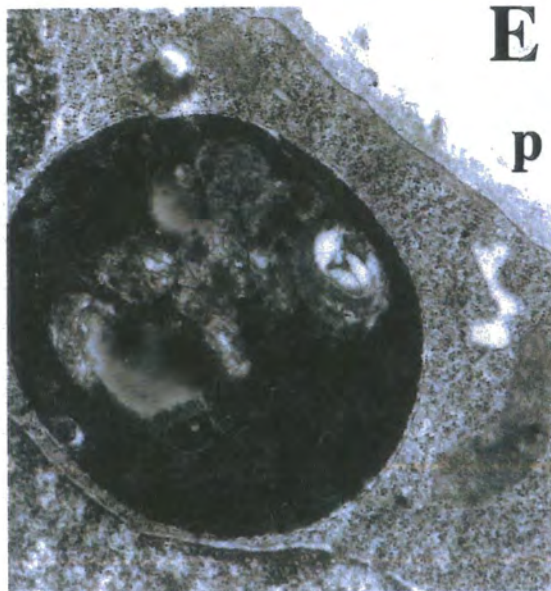
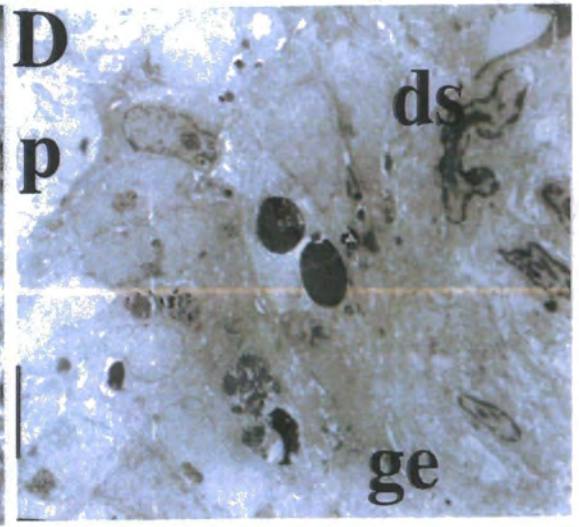
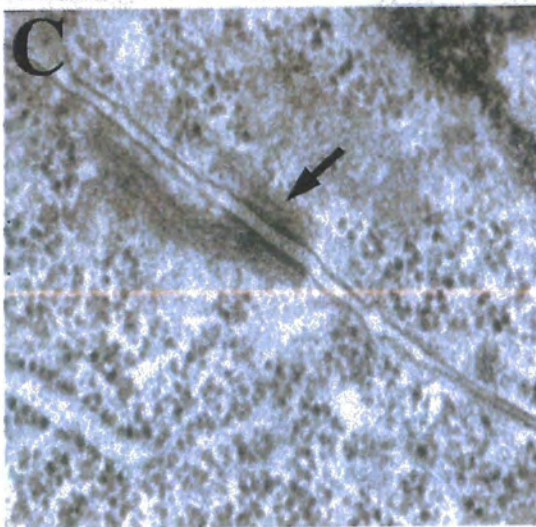
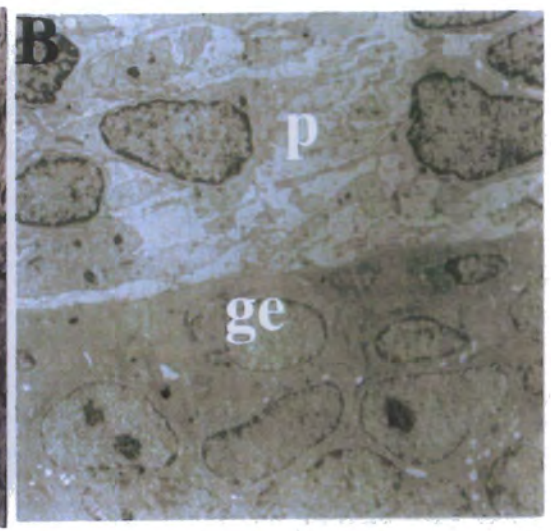
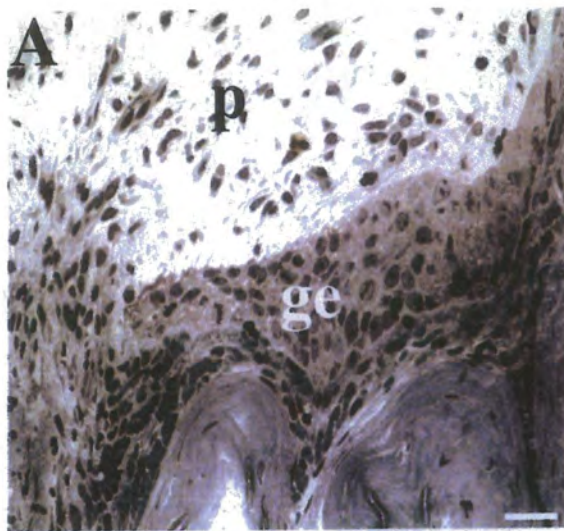


Fig. 2.27) Ultrastructure of a follicle 12 hours post-plucking. **A)** Micrograph shows the beginning of cell regeneration in the epidermal matrix. The epidermal cells (e) which remained in the follicle after fibre removal have produced new epidermal cells (arrow). The dermal papilla (p) contains numerous fibrils. **B)** Micrograph showing the hair shaft cavity (just above the bulb region) created after fibre removal. No specific cells are seen in this area but many fibrils are present. **C)** The hair shaft cavity is lined by an incomplete layer of epidermal outer root sheath cells (ors) loosely attached to a thick glassy membrane (gm). On other side of the membrane, dermal sheath cells (ds) are observed within a ground substance rich in collagenic fibrils. **D)** Micrograph showing a region of the dermal papilla (p) and epidermal matrix (e) junction. A papilla cell (possibly part of the vasculature)) looks to be attached to or closely associated with an abnormal epidermal cell (arrow) of the matrix. The epidermal cell may be undergoing apoptosis. **E)** Higher magnification of the previous specimen showing the junction between the two cells in greater detail. (e) epidermal cell.

A and Cx2800, BX3600, Dx8000, Ex17000.

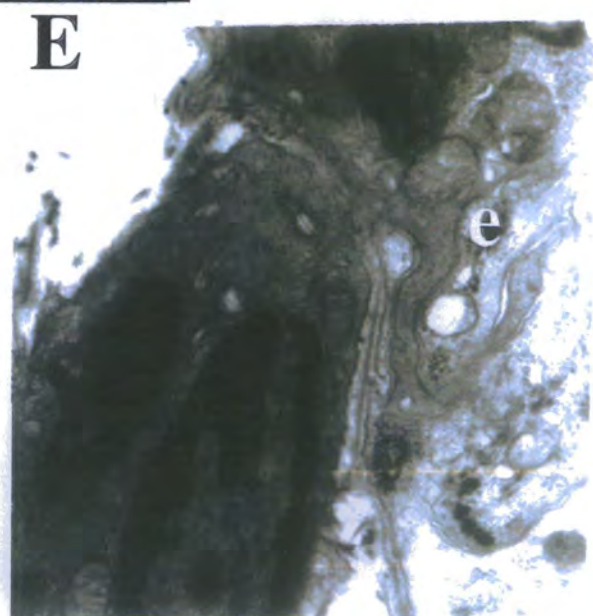
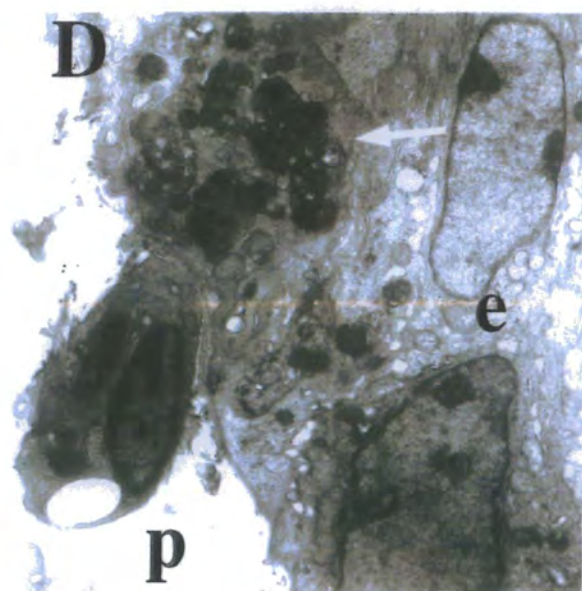
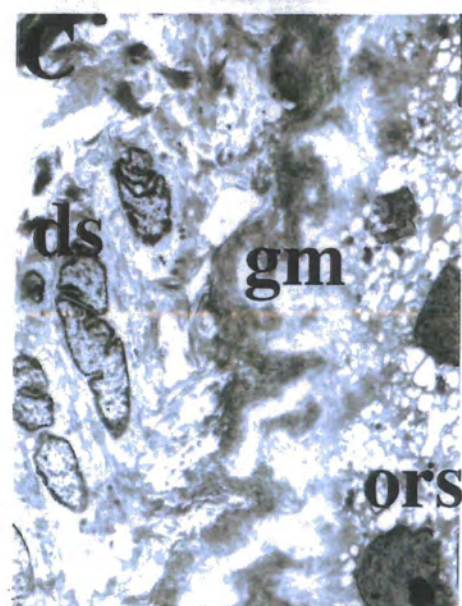
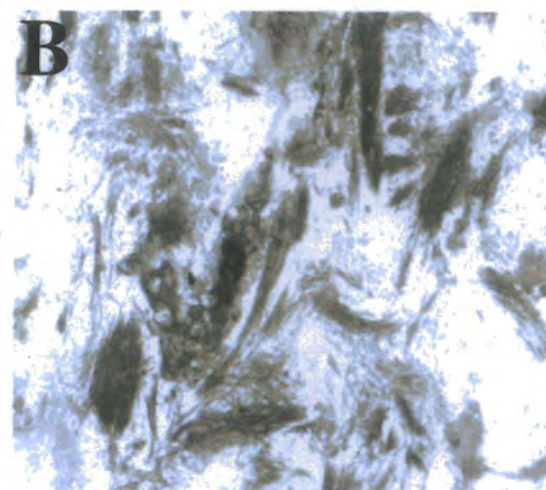
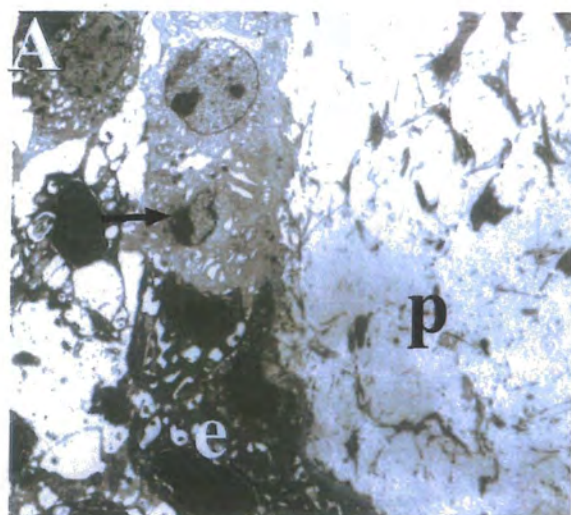
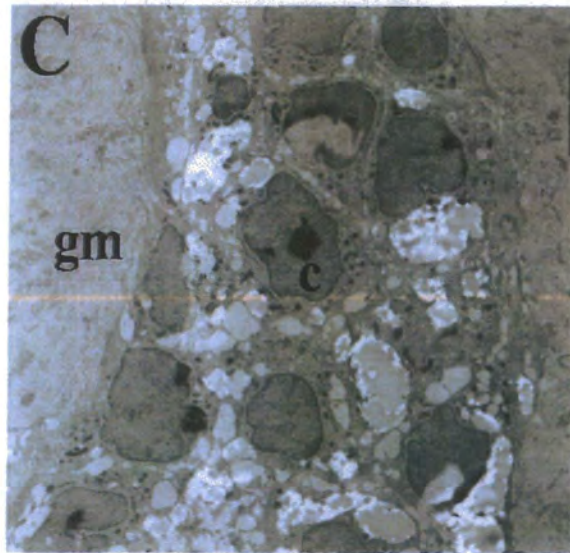
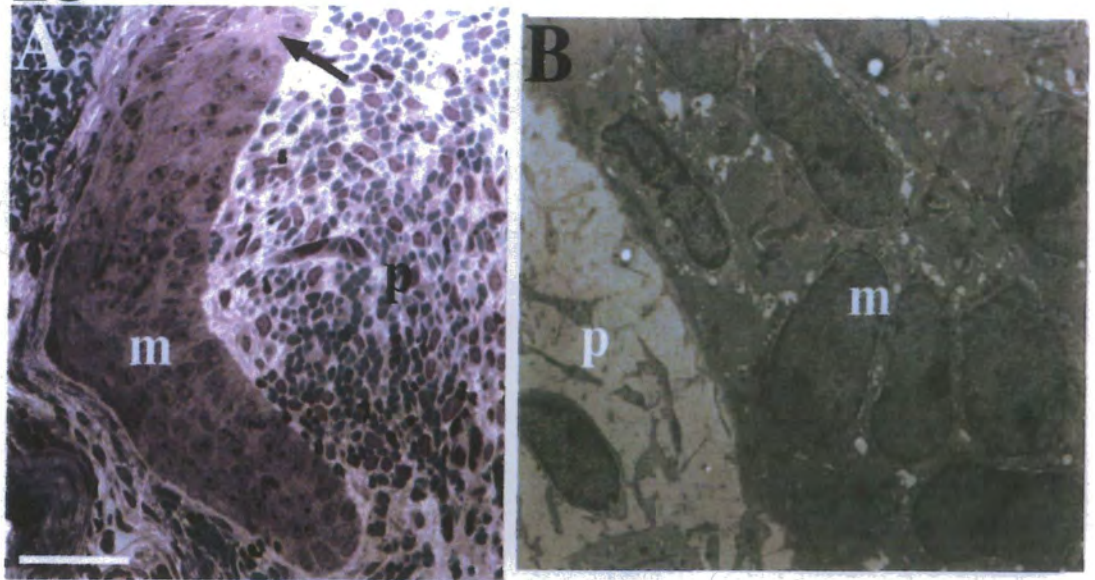


Fig. 2.28) Morphological and ultrastructural characteristics of a follicle 24-hours post-plucking. **A)** Light micrograph of the bulb region of a follicle. The epidermal matrix (m) has partly regenerated, distally, seen as a lighter group of cells (arrow). Blood vessels constituents are also seen within the papilla (p). Resin embedded stained with toluidine blue. Scale bar 50 μm .

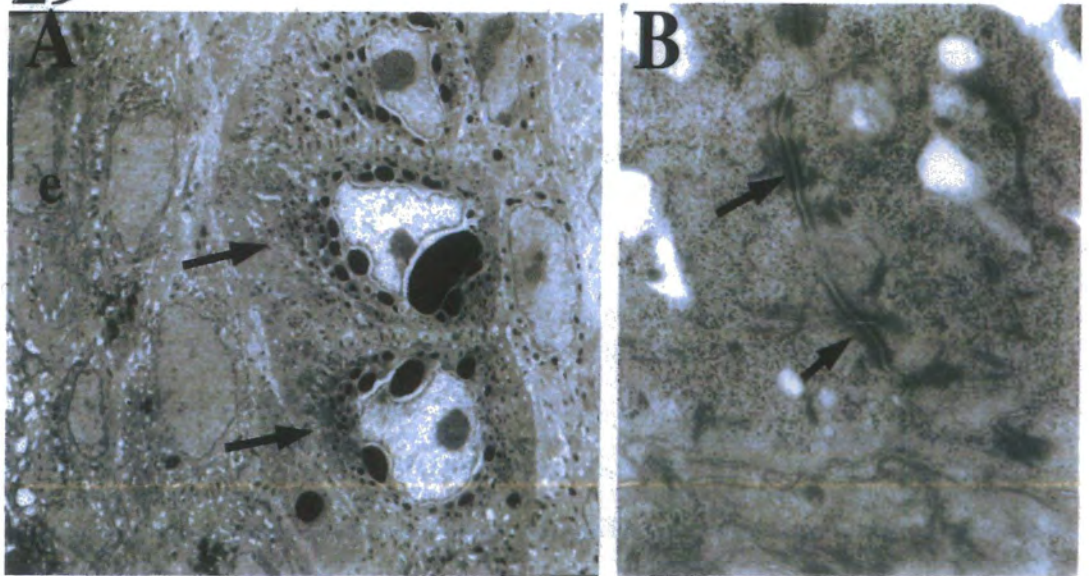
B) Ultrastructurally both the epidermal matrix (m) and papilla (p) display characteristics of their anagen counterparts in terms of cell morphology and density. **C)** Micrograph shows the hair shaft cavity (c), above the bulb, which is now partly filled with epidermal cells but with some extracellular spaces and some abnormal structures are also visible. The cavity is surrounded by a very thick glassy membrane (gm). B and Cx2800

Fig. 2.29) Electron micrographs of follicles 48-hours post plucking. **A)** Micrograph shows cells within a cyst-like structure (arrows) which was within the epidermal matrix (e). In contrast to the neighbouring cells, the cells within the cyst demonstrate abnormal nuclei and cytoplasm. The nuclei have a polygonal shape with a dense nucleolus and cytoplasm contains numerous granules (black spots). **B)** Epidermal cells within the cyst contain few cytoplasmic organelles and they are connected together by a high frequency of desmosomes (arrows). Ax2800, Bx36000.

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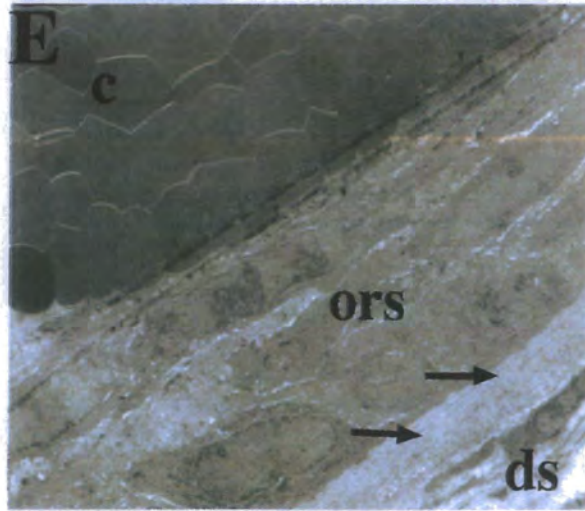
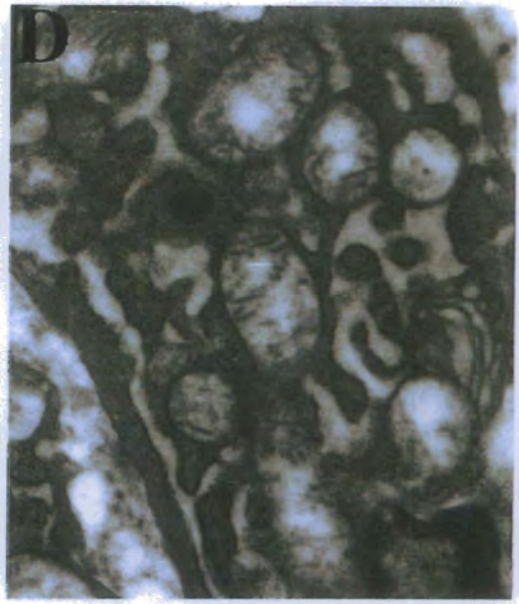
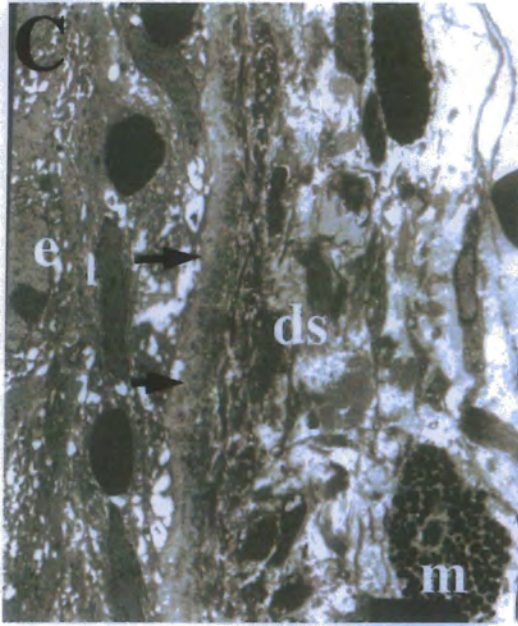


Figs. 2.29C-E) Ultrastructure of follicles 48 hours after plucking.

C) The epidermal matrix (e) is separated from the dermal sheath cells (ds) by a thick glassy membrane (arrows). The dermal sheath cells in the lower area are round but in the upper region they display an elongated morphology. Two large mast cells (m) are also present within the dermal sheath tissue, one at the base and another at the top of the micrograph. **D)** Micrograph showing the cytoplasm of a papilla cell having normal organelles including mitochondria and ribosomes. **E)** A cyst-like structure which has formed inside the hair shaft space. No living cells are seen inside the cyst (c). The cyst is surrounded by 2-3 layers of the epidermal outer root sheath cells (ors). A thick glassy membrane (arrows) separates the epidermal cells (ors) from the dermal sheath (ds). C and Ex2800, Dx28000.

Fig. 2.30) Electron micrographs of follicles 4 days post-plucking. **A)** The papilla cells are tightly packed within an extracellular matrix which contains numbers of fibrils. **B)** Lower region of epidermal matrix (e) containing only 1-2 layers of epidermal cells. Parts of the dermal sheath (ds) and dermal papilla (p) are also seen in the micrograph. At the epidermal-papilla junction a thick basement membrane (arrow) extends finger-like projections into the papilla. A and Bx8000

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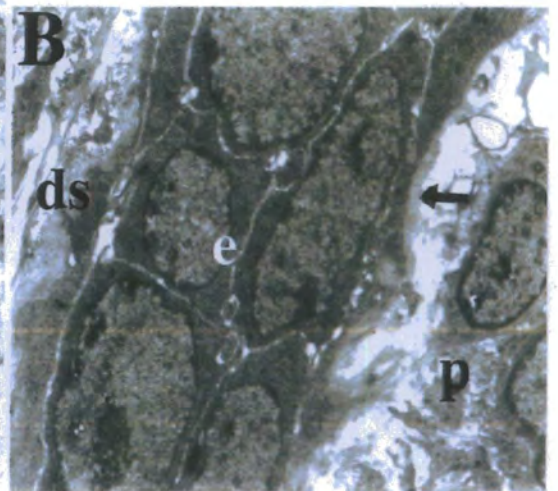
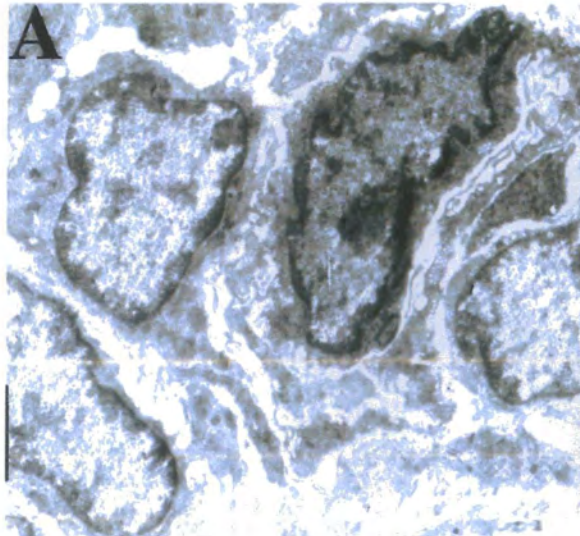


Fig. 2.30C)

Micrograph displaying the upper region of the epidermal matrix (m) in a follicle 4 days pos-plucking. At this point the epidermal component is composed of two cell types which are aligned in different directions. The cells close to dermal sheath (ds) are aligned horizontally whereas the inner cells (b) nearer the papilla show a more vertical arrangement. X2800.

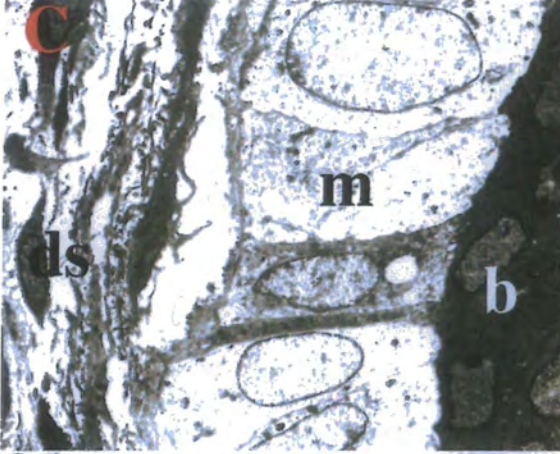
Fig. 2.31) Electron micrographs of follicles 7 days after fibre removal. **A)** The dermal papilla cells (p) are quite close together. The tip of the epidermal matrix (arrow) is also present. **B)** The epidermal matrix (m) has broadened and now contains several cell layers. A dividing cell (arrows) is also present. (ds) dermal sheath. x2800.

Fig. 2.32) Electron micrograph of a papilla cell in a follicle 9-days after depilation. The cell shows a typical fibroblastic morphology with many cytoplasmic projections. At this stage the papilla cells have a large cytoplasm which is well-developed in terms of cytoplasmic organelles. The cells are well-spaced within an extracellular matrix. x6000.

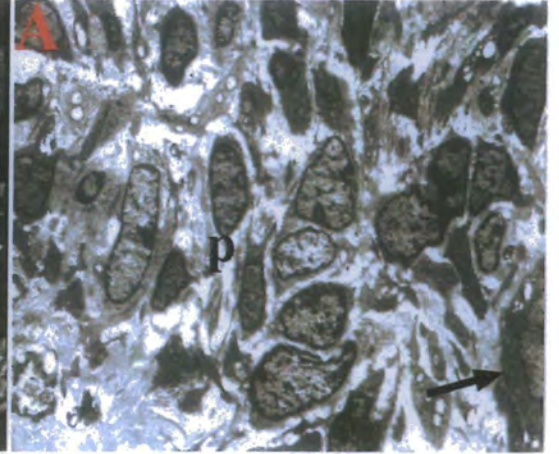
Fig. 2.33) Histology (A) and BrdU-label distribution (B- E) of follicles, six hours after the 6th plucking. Typically, the histology and cell division pattern is similar to that of follicles plucked only once (Fig. 2.14). However, sometimes in these follicles the BrdU labelling was also observed within the ORS cells in areas just above the bulb (C). Within the papilla a few cells were also found to be marked (arrow). Section A wax embedded and stained with Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A 400 µm, B-E 50µm.

Figs. 2.34) Histology (A) and BrdU-label dispersal (B-D) of follicles, 24 hours after a second plucking revealing the same morphology and pattern of cell division in the follicles as that described for 24 hours after the first plucking (Fig. 2.16). The arrow shows an epidermal cyst, which typically appeared in 70-80% of follicles within the hair shaft space. Stain (A) Alcian blue, Weigert's Haematoxylin and Curtis's Ponceau S. Scale bars A 400µm. and B-D 100µm.

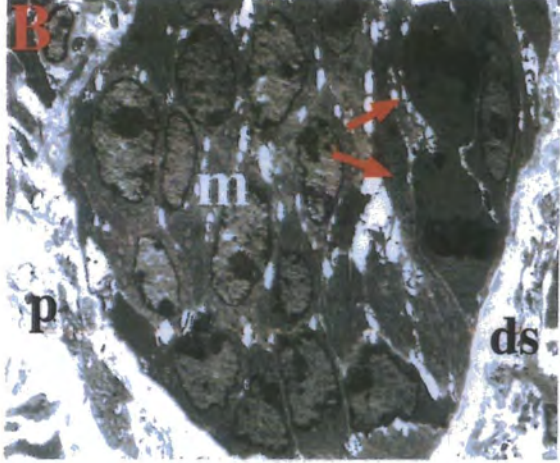
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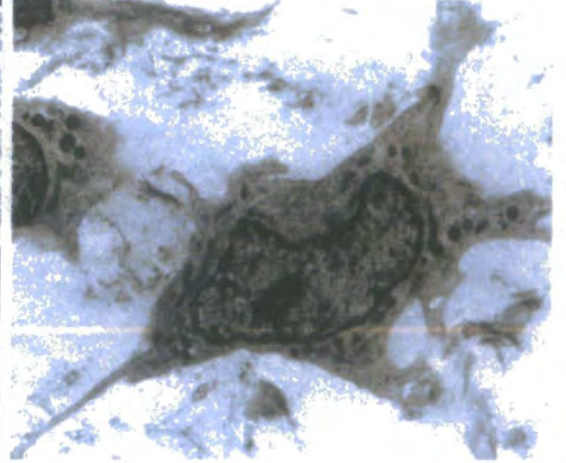
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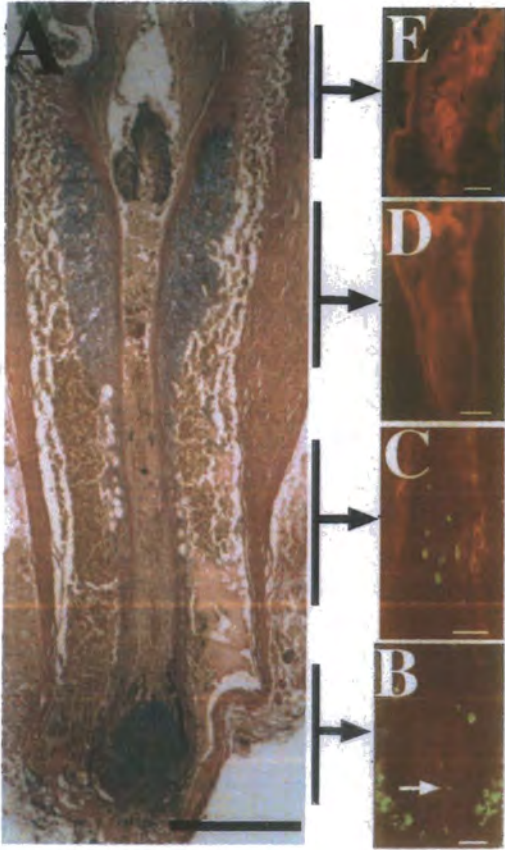
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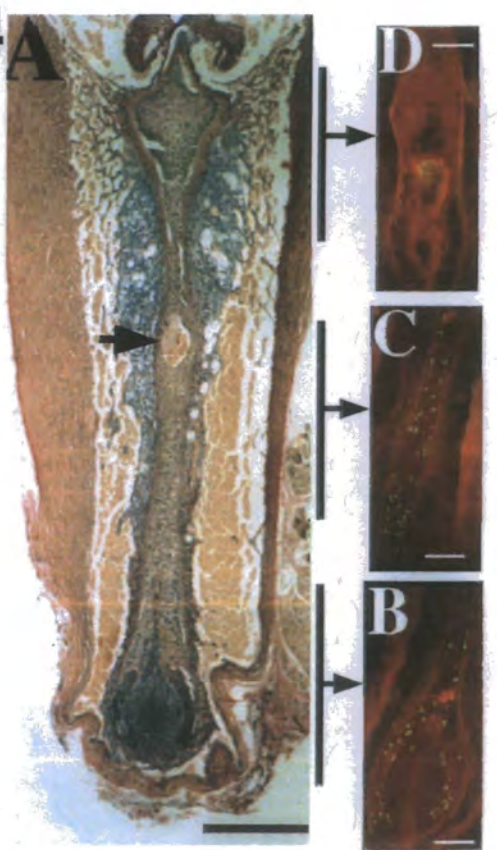


Fig. 2.35) Hair growth curves from a single follicle following five successive pluckings. The fibres were always plucked at the mid-growing stage and the new fibre emerged from the skin surface 8-12 days post-plucking. The average rate of fibre growth was consistently 1.2-1.4 mm/day after each plucking. On average 80% of follicles showed no decline in growth rates after multiple depilations. (Pn) number of plucking.

Fig. 2.36) Hair growth curves following five successive pluckings in another follicle. As seen by the slope of the curves, fibres grew consistently at an average rate of 1.2-1.3 mm/day after the first plucks, but growth rate was reduced (0.8-0.9 mm/day) following the fifth plucking. The total length of hair produced (109 mm) is still much greater than the maximum expected length of the club (51 mm). (Pn) number of plucking.

Fig. 2.37) Hair growth following 3 consecutive pluckings. A hair fibre of normal terminal length and growth rate is produced when the follicle is finally allowed to continue to the end of its growing phase. (Pn) number of plucking.

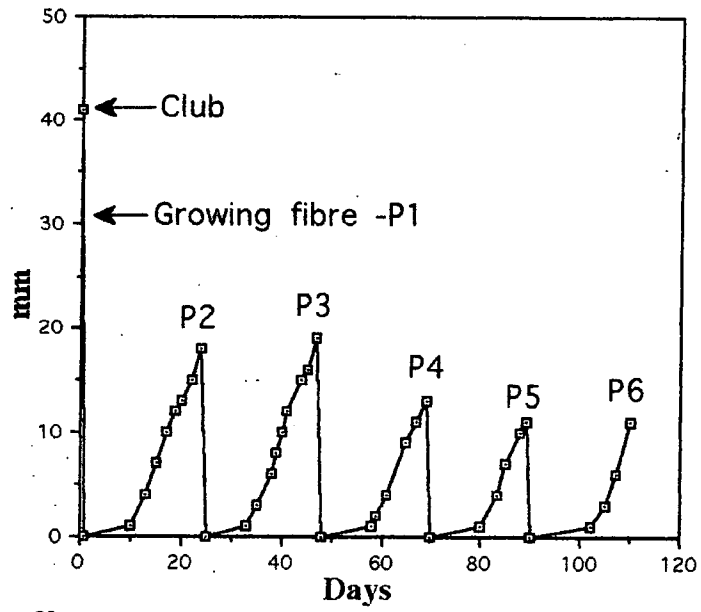
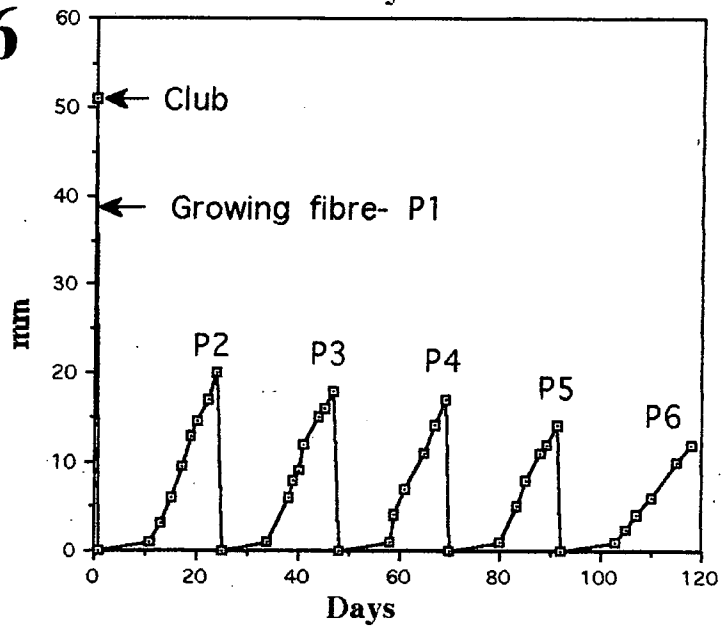
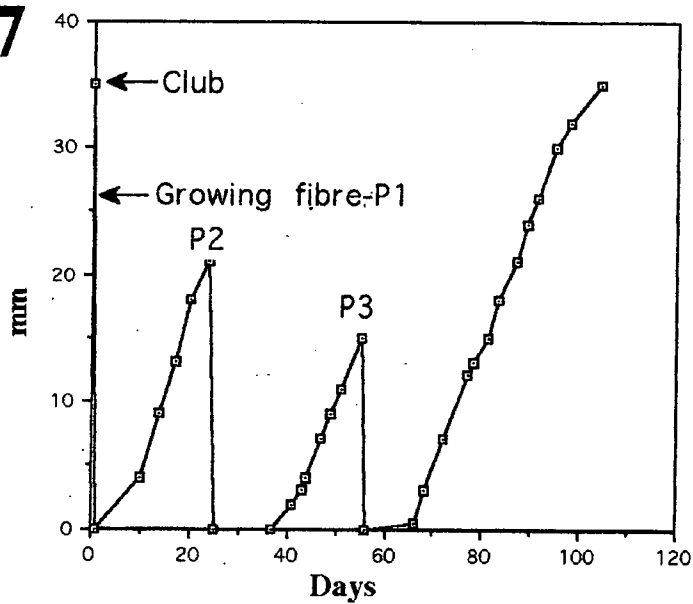
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Table 2.2) Hair lengths produced after single and repeated plucking. The cumulative length of fibres grown by plucked follicles increased consistently after every plucking (column 3). Due to the fact that different sized follicles were utilised, the range of length increases is considerable, and mean values do not go up uniformly with the number of plucks. However, every single follicle cumulatively grew more fibre than its fixed terminal club length.

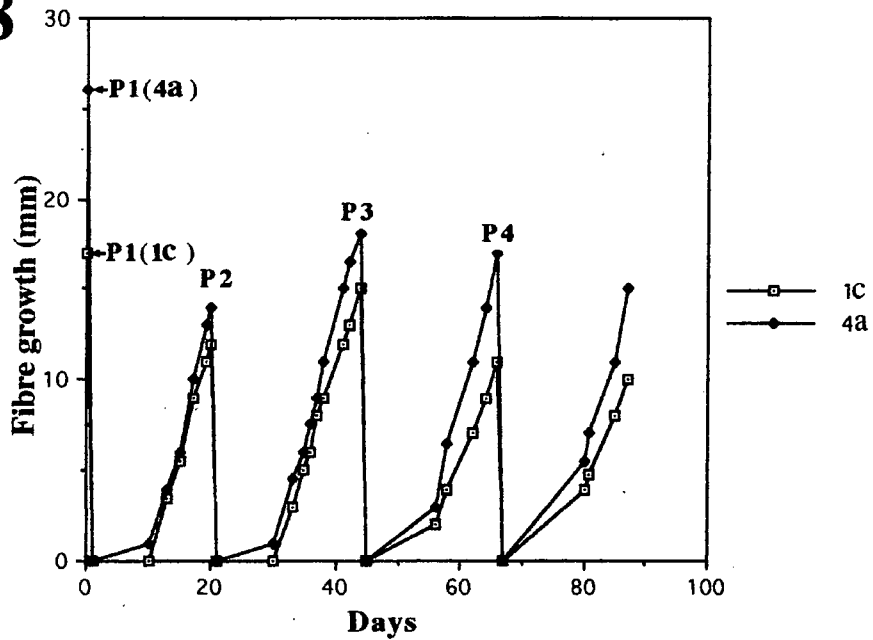
Fig. 2.38) Comparison of hair growth curves of two follicles from different vertical rows of the mystacial pad following 4 successive pluckings. While the growth rate for the larger follicle (row a) was on average 1.2 mm/day, the rate for the smaller follicle (row c) was 1.05 mm/day. (Pn) number of plucking.

Fig. 2.39) Histogram showing a comparison of hair growth in two follicles (Fig. 2.38) from different vertical rows of the mystacial pad at certain times after repeated pluckings. The length of regenerated fibre for the larger follicle (row a), 20 days after each plucking, is longer than that is seen in the smaller follicle (row c). (Pn) number of plucking.

Tab.2

Number of repeat pluckings	Number of follicles measured	Mean of total fibre length (mm)	Mean of club fibre length (mm)	Range of % increase over club	Mean of % increase over club
1	5	58	41	30-66	44
2	6	60.3	31.5	60-120	90.8
3	4	77.5	46.75	42-148	70
4	6	81	26.5	80-265	170.8
5	8	102	38.7	95-184	141.6

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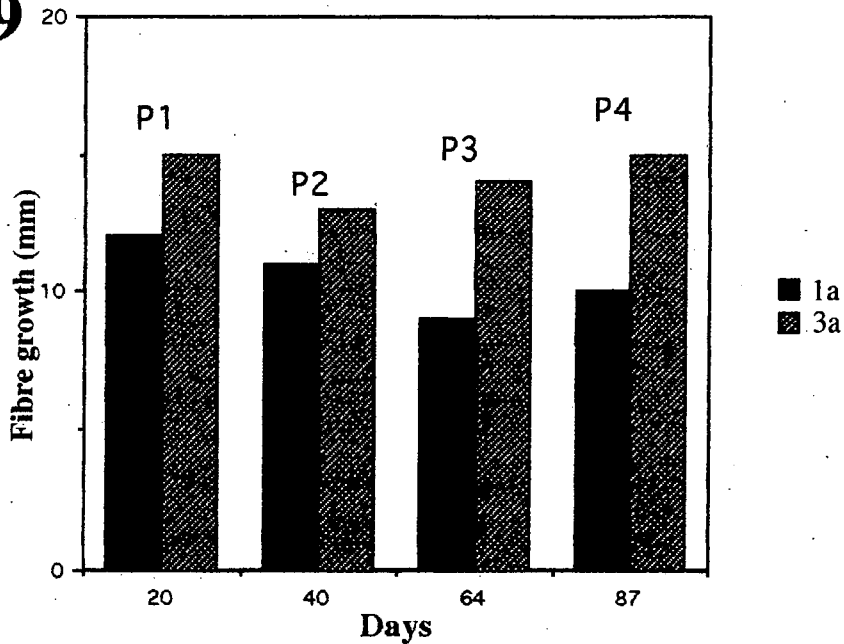
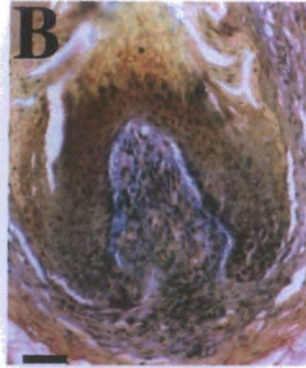


Fig. 2.40) Micrographs of follicles that were plucked and immediately transected, twenty days post-operation. Hair fibres are visible growing from both the lower (A) and upper (C) sections of one follicle. **B)** A longitudinal section through the lower section of the follicle shows that the residual GE cells have produced a new epidermal matrix. **D)** A longitudinal section through the upper part of the follicle showing regeneration of a new dermal papilla, matrix and hair fibre. Stain B and D Alcian blue, Weigert's Haematoxylin and Curtis' Ponceau S. Scale bars A 75µm, B 25µm, C 150µm, and D 40µm.

Fig. 2.41) Micrograph of a follicles that was plucked and transected 4-days later. The two elements were biopsied 20 days after follicle transection. Both the upper and lower (arrow) sections produced fibres. **B)** A longitudinal section through the lower region of a follicle demonstrating the formation of new matrix and hair fibre by germinative epidermal cells. B stained with Haematoxylin and Eosin. Scale bar A 200µm, B 100µm.

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2.4. Discussion

The adult hair follicle occupies an unusual niche in mammalian development because of its cyclical cellular and physiological regeneration. Probably the most intriguing question in hair follicle biology is what controls the timing of this phenomenon (Paus, 1996)? In this study I followed cell activity subsequent to single and repeated fibre plucking and found strong evidence that the germinative cells at the base of the follicle were able to proliferate well beyond the level required to produce fibres of expected length. I have localised cell division by BrdU labelling and demonstrated that cell division occurs only in the epidermal component of the follicle and no cell proliferation was found in the dermal component (dermal papilla and dermal sheath). I have also demonstrated that after plucking GE cells left at the base continued their proliferative activity throughout the process of regeneration of a new hair while ORS cells ceased their division just after plucking, and then restarted division 24 hours later. Following examination of plucked follicle anatomy using light and electron microscopy I have shown that a complete new matrix was established around the papilla 2-3 days post-plucking and this elicited the new hair which emerged from the skin surface 7-9 days later. Moreover, by applying multiple consecutive pluckings to each individual follicle I have demonstrated that the follicle displays the same pattern of cell division after a second, third, fourth, fifth and sixth plucking as those plucked for a single time. This along with histological examination of follicles after multiple plucking confirmed that after each plucking cells of the new epidermal matrix are only derived from the GE cells left behind at the base of the bulb. Furthermore, regular measurement of fibre growth rates at post single and multiple plucking demonstrated that the new fibres grow at the same rate as seen in their 'normal' equivalents. These findings together with the results of amputation experiments militate against the idea that the growing phase of the hair cycle is controlled or restricted by limited division potential in the GE cells (Cotsarelis *et al.*, 1990).

2.4.1 Single plucking

Depilation of follicles has been used to investigate various aspects of follicle

behaviour (Chase, 1955; Johnson & Ebling, 1964; Potten *et al.*, 1971; Ibrahim & Wright, 1975 & 1978; Hale & Ebling, 1975 & 1979; Wilson *et al.*, 1994a), but despite this variety there is little information in the literature concerning subsequent cellular events. Standardisation of the depilation procedure was necessary because; i) it has been shown that plucking of vibrissae at different phases of the growth cycle can affect the timing of subsequent hair growth (Ibrahim & Wright, 1978); ii) it has been documented that plucking can result in the establishment of different break patterns in the follicles (Bassukas & Hornstein, 1989). Regular and detailed observation of the histological events performed here revealed that the residual epidermal compartment shortly after plucking expanded upwardly and restored a new matrix around the denuded papilla. This matrix subsequently produced the regenerated fibre. Later it was established that cells of this new matrix originated from continuing division of the germinative cells left behind in the base of the follicle. This was clear from the constant BrdU staining in the residual GE cells immediately following hair removal which coincided with the continuous anagen-like ultrastructural morphology of cells in the regenerating bulb. Moreover, localisation of cell death in follicles indicated an absence of any degenerative effect on GE cells, indirectly supporting the idea that these cells are actively involved in regeneration process. It had been suggested previously that, after plucking, there was a break in mitotic activity in the residual germinative cells (Ibrahim & Wright, 1978). The new epidermal matrix could not have originated from the upper outer root sheath (or bulge area) of the follicle since matrix regeneration was well under way before the appearance of any cell division in the upper follicle. Plucking of vibrissa and human hair leaves a line of mainly basal outer root sheath cells along the length of the follicle (Reynolds & Jahoda, 1994; Moll, 1995), and cells in the intermediate population located between the bulb and the bulge region have been shown to have considerable proliferative capabilities (Rochat *et al.*, 1994). BrdU labelling of follicles just prior and after plucking revealed that in both cases there are some dividing cells scattered within the ORS between the bulb and isthmus area in addition, of course, to the intense cell division in the bulb region. However, at 6 and 12 hours post plucking, the staining was only restricted to the bulb, and the follicles displayed virtually no staining of the ORS in the area between the bulb and the follicle isthmus. Interestingly, this suggested that the plucking initially imposed an inhibitory effect on cell division activity in ORS cells in

these areas. Although, the mechanism of this inhibition is to date unknown, pro-inflammatory cytokines known to be released from damaged cells after plucking might have a role in this event (Mahe *et al.*, 1996). Irrespective of the mechanism of mitotic inhibition this observation reinforced the point that during the crucial early period of matrix regeneration the ORS cells could not have been involved in new matrix formation. Moreover, reappearance of BrdU labelling in the ORS cells, initially corresponded with the filling in of space created by fibre removal, rather than new matrix formation.

About 4 days after plucking the follicles displayed some catagen or telogen-like characteristics which lasted for 2 to 3 days before the follicles re-appeared as normal anagen. This catagen-like state was partly confirmed by a reduction in number of BrdU stained cells and thickness of the bulb epidermal compartment. However, a number of pieces of evidence suggest that this event did not represent a real catagen. First, during this stage new fibre was continuously growing inside the follicle. This was confirmed by the fact that at 4 days post-plucking when the catagen-like appearance was first seen, the new keratinized fibre had not yet been produced, whereas at 7 days when the follicle began to return to a normal anagen state, the tip of a growing fibre was observed in the lower third or half of the follicles (see Figs. 2.18 and 19). Moreover, BrdU labelling was always seen within GE cells and this coincided with the observation of small and rounded cells in ultrastructural studies. Finally, according to recent investigations, apoptosis is involved in the normal hair cycle and has been detected at the catagen stage in the lower part of the follicle as well as the bulge region (Lindner *et al.*, 1997). However, in this study cell death was found mainly in the upper part of follicle and despite a discrepancy observed in EM and immunolabelling observations no cell death was seen in the GE cells of the matrix. This discrepancy could be due to low number of cell death or the fact that the antibody did not label all the apoptotic cells.

Following bulb matrix regeneration hair was not immediately produced, and the evidence of unusual epidermal differentiation (cyst) in the new upper matrix cells two or three days post plucking might reflect a wound-type response, or relate to localised papilla influences. Morphologically, the upper dermal papilla was specifically affected after being denuded of epidermal cells by the plucking procedure (cell death antibody detected some positive cells in this region) and this may have altered its capacity to

interact locally with the new upper matrix epidermis. However, my *in vivo* amputation experiments proved beyond doubt that the post-plucking residual GE cells were capable of both restoring a new matrix and producing fibre. The ability of intact vibrissa bulbs to grow independently *in vivo* was demonstrated some time ago by Cohen (1961). My work has demonstrated, for the first time, that follicle bulbs which are plucked and then immediately amputated restored a hair-growing matrix. Thus, both matrix regeneration and subsequent fibre growth must have originated from the same residual group of GE cells left after depilation, independently of epidermal cells (or indeed any other influence) from higher in the follicle. Although I clearly showed (by BrdU labelling) that ORS cells after plucking have no contribution in matrix regeneration it still could have been argued that cells were migrating down from higher up in the follicle after initial 24 hours period of dormant mitotic activity following plucking. If cells were contributing to a new germinative cell population this could have been reflected by better (more prolonged) growth in follicles that underwent bulb amputation some time after plucking. This was not found to be the case.

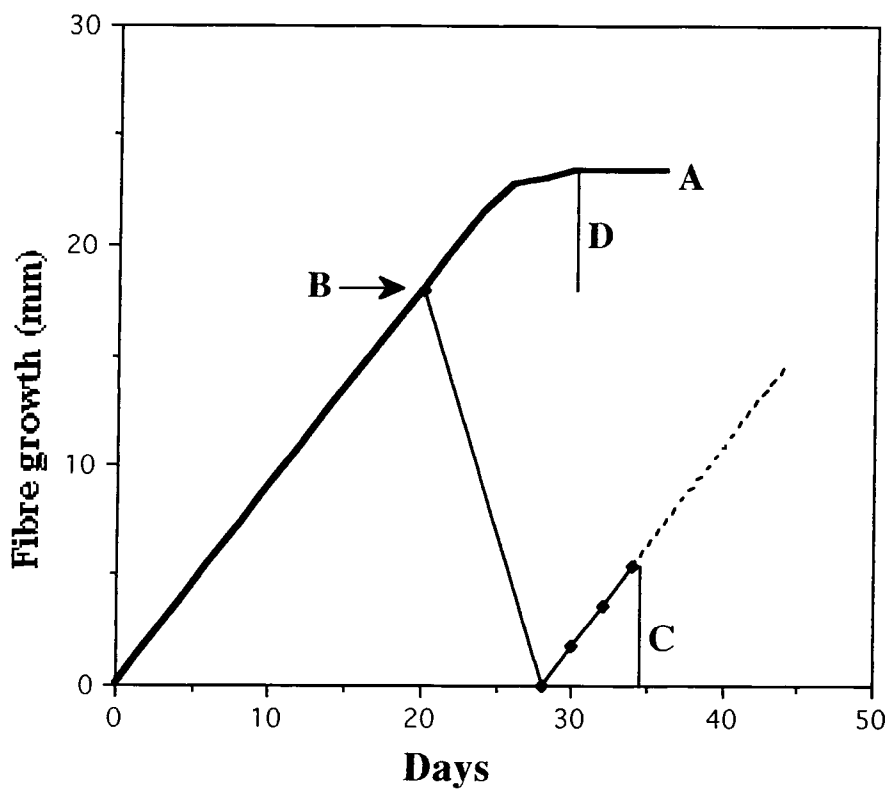
Some may argue that production of short fibres by the plucked end bulbs seen *in vivo* implies that residual GE cells had not been able to proliferate more because their capacity for division had already been reduced. However, because the bulbs were left very deep in the tissue I believe this point is related to the physical obstacles which prevented further fibre growth. This suggestion was confirmed by two observations: i) morphological examination of the bulbs revealed that in contrast to the normal vibrissa these regenerated fibres were abnormally curly and trapped within the connective tissues, ii) histologically bulbs displayed an anatomy similar to that seen in normal anagen follicles. They showed a dermal papilla, which like the anagen papilla, was rich in ECM and this papilla was enclosed in a broad epidermal matrix. No sign of catagen or telogen characteristics were found in these follicles. Thus, I consider that if there were no such physical obstacles the plucked end bulbs were able to grow longer fibres.

Regeneration of new end bulb by the upper region of the follicle seen in the present work is in agreement with previous studies (Oliver, 1966a,b; Jahoda *et al.*, 1992a). This process emphasised the migratory and proliferative properties of both follicular dermal and epidermal cells. According to Jahoda *et al.* (1992a), this regeneration involves a sequence of dermal-epidermal interactions, which results in the

establishment of a new bulb at the lowest region of the follicle. The cells, which form the new bulb's dermal papilla most likely originate from follicular dermal sheath at the side of follicle. As a result of an unknown factor these cells are activated, and after proliferation they pass across the glassy membrane. The cells finally reside in the papilla-forming region. Epidermal cells are recruited from downgrowth of epithelial cells lining the residual hair shaft (Jahoda *et al.*, 1992a).

2.4.2 Multiple plucking

After repeated pluckings of growing fibres from individual follicles it was established that the histological and proliferative events following single and multiple depilations were essentially identical. Significantly then, the GE cells were providing the source of the new epidermal matrix each time. According to the bulge hypothesis (Cotsarelis *et al.*, 1990), these germinative cells are categorised as transient amplifying cells with limited potential for cell division. Therefore the duration of anagen and the length of any given fibre would be determined by the division potential of each germinative population. So, for example, if a follicle were plucked two thirds of the way through its anagen phase, the residual germinative cells would be expected to have the capacity to grow only the last third of the fibre length (Fig. 2.42). Taking advantage of the highly predictable growth of rat vibrissa, I have shown that the cumulative length of fibre produced from any given follicle position after multiple plucking could be up to three times the expected length. Any observed variation in the data was due to the procedure having been carried out on different sizes of follicles (see materials and methods). I included a number of safeguards in the protocol. Thus, it has been suggested that in the vibrissa follicle towards the end of each cycle the next generation of germinative cells might be mobilised and migrate from the bulge down the follicle early (Kobayashi *et al.*, 1993). By measuring hairs at short intervals just prior to plucking I was able to confirm that the follicles were in mid-growth and not entering the later (catagen) stages of hair growth prematurely. In other words there was little or no possibility that new germinative matrix cells had been recruited just prior to plucking. Moreover, it was also significant that hairs that were left to grow to completion after the final plucking attained expected terminal lengths and were of normal width and shape. If not, it could have been argued that the GE cells had produced cumulatively longer fibres



because the dimensions of the regenerated hairs were reduced so that their production required less cell division. Although I performed a maximum of six successive depilations in this study, there is no apparent reason to suppose that the process cannot be repeated further, perhaps for the lifetime of animal.

In short, while I cannot rule out the possibility that in the vibrissa follicle under normal circumstances, the germinative cells undergo some form of renewal, I have clearly shown that vibrissa follicle germinative cells have greater division capacity than that required for one normal anagen phase. This makes it highly improbable that hair growth normally ceases because transient amplifying GE cells reach the end of a phase of finite division. Even the idea that some of the proliferative activity in germinative cells following plucking could be a wound response implies a greater, rather than lesser role for these cells, since the ability to respond to injury is one of the proposed characteristics of stem cells (Potten & Loeffler, 1990).

Recent investigation performed by Ramirez *et al.* (1997) provided another reason to consider a greater role for GE cells in hair biology by localising expression of telomerase in different compartments of human hair follicles. Telomerase is a ribonucleoprotein enzyme capable of adding repeats of hexanucleotide onto the end of chromosomal DNA. It has been proposed that somatic cells in vertebrates are unable to completely replicate the ends of linear DNA molecules resulting in shortening of chromosomal ends (telomeres) with each round of cell division. According to this proposition, by adding telomeric repeats to the ends of the chromosomes and compensation of erosion, the telomerase enzyme prevents degradation or aberrant recombination of chromosomal ends. It has been documented that normal somatic cells do not express telomerase activity, but this activity is found in immortal cells, such as reproductive cells and primary tumour cells. Thus, presence of telomerase and subsequent maintenance of telomeres, implicates that this enzyme plays a role in preventing senescence in these immortalized cells (see Lee *et al.*, 1998).

Ramirez *et al.* (1997) showed that during anagen, hair follicle compartments expressed different levels of telomerase activity. The bulb region showed an exclusively higher level whereas this activity was low in the cells of the upper part of the follicle. Although low compared to the bulb, in the upper part of the follicle telomerase activity in the bulge region was also significantly higher than regions just above and below it.

Telomerase activity showed a general decline in the telogen follicle but bulb cells still exhibited a stronger expression compared to other follicle compartments. Although, this indicates that the bulb region cells are endowed with a property which enables them to replicate properly without limit the authors came to the opposite conclusion. They interpreted this evidence as meaning that cells in the bulge region are in quiescent state and cells in this situation do not need to express telomerase for complete replication of the chromosomal ends. However, if this is the point why do bulge cells express this activity at a higher level compared with cells in below and above? If the lower expression of this activity is associated with immortality and stemness, stem cells should be located in the lower intermediate region of the follicle not the bulge and if higher expression is correlated with this property, why not the bulb cells?

2.4.3 Timing of the hair growth cycle

Inhibitory influences have long been postulated as being involved in hair growth cycle control (Chase, 1955) and more specifically Bullough (1965) speculated that inhibitory influences affecting epidermal cell division (chalones) might be involved. More recently, Hebert *et al.* (1994) produced good evidence that FGF5 has a role in controlling events at the end of the growing phase of the hair cycle, and thus hair length. Knockout of the *fgf5* gene produces animals with hair that is around 50% longer than their wild type litter mates, and this phenotype mirrors that of angora, a natural mutation. FGF5 is localised to the lower epidermal outer root sheath of wild type follicles during late anagen, therefore one suggestion is that FGF5 acts to end anagen and initiate catagen, possibly by acting on dermal papilla cells (Rosenquist & Martin, 1996). If control of the hair cycle does not rest in the epidermis, it could reside in the follicle dermis. Oliver (1980) produced a model of cycle activity based on changing levels of positive growth signal by the dermal papilla. This component has well established inductive capabilities (Oliver, 1967b & 1970; Jahoda *et al.*, 1984 & 1993; Jahoda, 1992), and numerous growth factors and signalling molecules are localised to this group of cells during active hair growth (see Stenn *et al.*, 1996). The papilla cell population is also very stable and as I showed here no cell division was observed in these cells throughout the study. Papilla cells stain positively for bcl-2 throughout the growth cycle (Stenn *et al.*, 1994b), and they rarely, if ever divide in situ (Pierard & De La

Brassine, 1975). Interestingly, direct wound injury to the dermal papilla with a needle (with or without epidermal plucking) also produces increases in fibre length by the repaired follicles as a result of lengthening of the duration of the next growing phase (Jahoda & Oliver, 1984a). In the current study despite the trauma caused by plucking, no evidence of cell division was observed in the papilla except when labelling was seen in the location of capillaries. The overriding issue is that whatever molecular mechanism exists, and whether its origin is dermal, epidermal or both, underpinning it must be an exquisite cell-based clock capable of timing growth to within a day within a cycle of around 60 days (Ibrahim & Wright, 1975). For example, if the onset of catagen was being established by a build up of FGF5 in the lower follicle epidermis, perhaps to a threshold concentration, the timed release of this molecule still has to be regulated precisely. This point is underlined by the current work where individual follicles that were plucked had their timings "reset" midway through the cycle, yet retained their individual and intrinsic cycling pattern.

An interesting point observed in this study was that 3-4 days post plucking the papilla condensed down and began to display a catagen-like morphology, which corresponded to a decline in the number of dividing GE cells. The question that arises from this observation is whether this degenerative process was first initiated in the DP or GE cells? The plucking did obviously damage the epidermis but it did not appear to cause a severe trauma to the DP except for a small injury at its top. Therefore, it would be reasonable to consider that epidermal cells triggered this process which led to transformation of the anagen-like papilla into the catagen/telogen-shaped papilla. However, if we accept this by inference this means that epidermal cells control the behaviour and morphology of the DP and this is against the current ideas that the papilla controls the behaviour of the bulb epidermal cells. Nevertheless, another observation made in this study deepened this problem. During the time that the papilla exhibited a catagen/telogen-like morphology cell division was persistently observed within GE cells and this suggested that to some extent the epidermal cells do not follow the changes of the papilla. If it is the papilla that controls cell proliferation in matrix epidermal cells and dermal signalling molecules are responsible for terminating this activity at catagen why in my work did it not stop cell division in epidermal cells? Perhaps although the papilla influences the behaviour of epidermal cells the epidermal cells themselves have a degree

of independence. If it is not the case and dermal papilla truly controls cell proliferation in epidermal matrix cells it will suggest that the recession seen in follicles 4 days post-plucking may have not been a real catagen or telogen.

I have shown here that the cessation of growth at the end of each follicle growth phase cannot be attributed to limited cell division potential in the GE cells of the follicle base. However, this by no means rules out an epidermal input in timing events, or diminishes the importance of the GE cell population. Recently, my colleagues in this laboratory demonstrated that adult GE cells have powerful developmental influences in that they can alter dermal cell attributes (Reynolds & Jahoda, 1996) and this property singles them out among adult epidermal cells. In spite of the fact that upper ORS or bulge region cells play an important stem cell role in some contexts, my study shows that during follicle cycling GE cells do not have to be derived from the follicle bulge region and in the rat vibrissa follicle are unlikely to do so. Moreover, in this model at least GE cells have a capacity for cell division that greatly exceeds that required for a normal growth phase of the cycle. In the stem cell context and in relation to dermal-epidermal interactions they therefore remain a key target for future investigations.

Chapter 3

Hair follicle epidermal cells *in vitro*

3.1 Introduction

3.1.1 Proliferative capability of hair follicle epidermal cells

Tissue culture techniques have been used in laboratories for more than 100 years (Paul, 1975) although they did not greatly influence scientific research until the 1940s when new techniques allowing the dissociation of cells from one another were developed (Green, 1991). This allowed greater growth of cells than had been previously possible using tissue fragments. However, even today not all of the estimated 200 cell types of the adult animal body can be grown *in vitro*. Of the few cell types that can be cultured from tissues, most are derived from connective tissue including fibroblasts, myoblasts, and lymphatic cells (Green, 1991). In addition, epithelial cells, such as urothelial cells and mammary epithelial cells can also be successfully cultured. Among these epithelial cells, epidermal cells (keratinocytes) have been shown to possess a good ability for cultivation (Rheinwald & Green, 1975; Liu & Karasek, 1978; Boyce & Ham, 1983 & 1985; Barrandon & Green, 1985 & 1987; Normand & Karasek, 1995). When keratinocytes are dissociated with an enzyme and placed into a culture vessel, in the presence or absence of a supporting 3T3 feeder layer, the resultant single cells attach, proliferate and initiate colonies of cells. They not only grow rapidly but they also retain their proliferative capacity for a long time and therefore they can be repeatedly subcultured. By way of illustration, keratinocytes derived from foreskin of newborn humans have been cultivated serially through a total of 50 cell generations (Rheinwald & Green, 1975). However, it is noteworthy that not all keratinocytes derived from the epidermis have the ability to be repeatedly passaged. Even under optimal culture conditions, only 1 to 10% of the cells of the epidermis will proliferate and form colonies. Moreover, of these colony-forming cells only about 30% can proliferate for a long period of time (over months) while most of the cells will cease their growth after a short while (in 2-3 weeks) (Barrandon & Green, 1987; Barrandon, 1992).

The hair follicle is a specialised epidermal appendage and contains a large

number of epidermal cells situated in the bulb and along the length of the hair shaft. In recent years various methods have been developed for establishing cultures of hair follicle epidermal cells. In 1981, Weterings and colleagues (see Limat & Noser, 1986) grew human hair follicle epidermal cells by implanting plucked scalp hair follicle fibres onto the bovine eye lens capsule. Subsequently, Wells (1982) succeeded in culturing the same specimens directly on tissue culture plastic. Imcke *et al.* (1987) plated the plucked hair follicles on collagen-coated dishes and Kuwana *et al.* (1990) cultured epidermal cells of human hair follicles on a floating membrane with a mixture of collagen type I and type IV. It has been shown that hair follicle epidermal cells can grow better when they are co-cultured with post-mitotic 3T3 fibroblasts (Limat *et al.*, 1989). Under these conditions the epidermal cells grow and form a well-organised stratified epithelium. The technique of using cultured cells for treatment of burn and other injuries had been previously developed with epidermal cells derived from skin epidermis (Bank-Schlegel & Green 1980; O'Connor *et al.*, 1981; Gallico *et al.*, 1984; Faure *et al.*, 1987; Kanitakis *et al.*, 1987; Compton *et al.*, 1989; Nolte *et al.*, 1994; see also Leigh, 1994). In these experiments when cultured epithelial sheets were grafted into full-thickness wounds, histological observation showed that within a week a stratified epidermis, similar to that seen in normal skin was established at graft sites. The establishment of organised stratified epithelium by follicular epidermal cells also encouraged several investigators to use this epithelium for implantation onto wounds. It has been shown that this epithelium can generate an epidermal layer resembling natural skin epidermis when grafted onto athymic mice or onto de-epithelialized dermis (Lenoir *et al.*, 1988 & 1993, Limat *et al.*, 1991).

In addition to the practical uses, the clonogenic ability of follicular epidermal cells has been a subject of great interest because of its perceived importance in the biology of the hair follicle itself. As mentioned in the previous chapter, an intrinsic characteristic of the hair follicle is that it undergoes continued cycles of growth, regression and regrowth (Chase, 1954). During the growing stage (anagen) the GE cells divide, giving rise to cells which differentiate into the hair fibre. After a period of time (which varies between species and different body sites) the matrix cells cease proliferation and subsequently the follicle enters a resting stage "telogen" via a transitional catagen stage. One of the major challenges of hair biology is to understand

what causes the highly proliferative matrix epidermal cells to suddenly cease proliferation at the onset of catagen. There is circumstantial evidence that suggests this process happens because of gradual accumulation of an inhibitor in the follicle during anagen which reaches its highest level at late-anagen (Chase & Eaton, 1959; Bullough, 1975). This idea is supported by some evidence (see chapter 2). However, as I have already mentioned in the previous chapter, as a part of the bulge hypothesis (Cotsarelis *et al.*, 1990) it has been hypothesised that the reason for termination of cell proliferation in epidermal matrix cells in catagen is that these cells cannot divide any more. According to this theory the matrix epidermal cells are transient amplifying (TA) cells, which by definition have a limited proliferative potential. After a given time they eventually exhaust their proliferative activity and undergo terminal differentiation. This theory suggests that true epidermal stem cells are situated in the bulge area and that these cells supply new epidermal matrix cells which start the next cycle.

The bulge hypothesis implies that the length of the anagen phase is an intrinsic property of the matrix cells and duration of anagen varies due to this difference of proliferative potential among follicle types (Lavker *et al.*, 1993). However, in practice, proving this idea has always been difficult as it is hard to assess the proliferative ability of cells directly *in vivo*. Hence, with improving techniques for the cultivation of follicular epithelial cells, researchers began to evaluate the proliferative ability of these cells *in vitro* by serial cultivation or determining their colony-forming ability (Kobayashi *et al.*, 1993, Yang *et al.*, 1993). In these experiments epidermal cells from different regions of the hair follicle were dissected, cultured (either after dissociation or without it), and the proliferative ability of cells compared (Kobayashi *et al.*, 1993). Although, there are few of these investigations (Wells & Sieber, 1985; Reynolds, 1989; Yang *et al.*, 1993; Kobayashi *et al.*, 1993; Rochat *et al.*, 1994; Moll, 1995 & 1996) it has been generally reported that cells located in the bulb region of the follicle have a lower clonogenic potential in culture compared to cells that reside in upper part of follicle. For example, Yang *et al.* (1993) found that after repeated subculturing of cells from different regions of human hair follicles, cells from the upper follicle (containing mainly the isthmus area and bulge) showed the longest life span, whereas those of the bulb (containing the matrix cells) had the shortest among all follicular epithelial cells. Moreover, they showed that the growth of bulb epidermal cells, even in primary cultures, is relatively poor, and that

they form only small colonies. Similar results were also established in human and rat by others (Moll, 1995 & 1996; Kobayachi *et al.*, 1993) who drew the following conclusions from their observations; i) most bulb cells are unable to grow in culture, ii) even when they grow their potential for forming colonies is low, iii) within the hair follicle, cells in the mid or upper part of the follicle possess the highest growth capacity in culture.

However, despite the progress in culturing follicle epidermal cells the real properties of some subpopulations and particularly the matrix cells have never been clearly identified *in vitro*. Many investigations in hair follicle epidermal cell culture have been exclusively performed on ORS cells prepared from plucked or whole follicles (Ward, 1976; Wells, 1982; Limat *et al.*, 1986; Vermorken & Bloemendal 1986; Lenoir *et al.*, 1988; Schaart *et al.*, 1990; Detmar *et al.*, 1993). In some other experiments the matrix cells have been cultured mixed with ORS cells, therefore it has not been possible to determine the growth capacity of the matrix cells alone (Limat & Noser, 1986). Moreover, among investigations performed on cultivation of matrix cells different results are observed. For example, Kobayashi *et al.*, (1993) reported that compared to bulge region cells, matrix cells have a low ability for colony formation. They fragmented rat vibrissa follicles into several segments from which epidermal cells were later dissociated. When single cells were cultured on a lethally irradiated feeder layer of 3T3 cells they found that approximately 95% of the total colonies were from the bulge region and fewer than 4% were located in the matrix region. The same authors later reported similar observations in human hair follicles (Rochat *et al.*, 1994). However, Jones *et al.*, (1988) demonstrated that epidermal matrix cells of human follicles (with or without dispersal) are able to grow when placed on previously irradiated mouse dermal 3T3 cells or mitomycin C-treated human dermal fibroblasts. They showed that cells on a feeder layer increased in number and formed colonies. However, this experiment was performed with material prepared from the base of plucked hairs and did not include any cells left within the follicle after plucking. Considering the fact that epithelial cells left behind in the follicle after plucking are germinative epidermal (GE) material and differ largely in cellular composition from the material attached to plucked fibre, Reynolds & Jahoda (1991b) compared the characteristics of GE cells with ORS cells in rat vibrissa follicles. They showed that the GE cells do not grow at all even using the most sophisticated epidermal culture methods when they are cultured alone. Initially they did flatten and

attach but they did not show typical epithelial cobblestone patterning, or proliferation. These cells remained small, round, undifferentiated and inactive, whereas under the same conditions ORS cells from other parts of the follicles established colonies. Nevertheless, when GE cells were recombined with dermal cells (dermal papilla) they divided and produced outgrowths containing tightly packed cells (Reynolds & Jahoda, 1991b). This group (Reynolds *et al.*, 1993a) produced similar results using GE cells of human hair follicles. Moreover, there is another report that shows that human matrix cells are able to grow in culture similar to that seen in ORS cells (see also Fig. 2 in appendix).

What became clear from the above experiments is that to date it is not possible to draw a definite conclusion about the ability of matrix cells to grow in culture. We do not know whether these differences came about through different media, substrate, feeder layer, species, etc or reflect the specific behaviour of the matrix cells. Therefore, in this part of my thesis I attempted to compare the growing ability of matrix cells with ORS cells (from the upper part of the follicle) from the well-studied rat vibrissa follicle, but in the absence of any supportive layer.

To investigate this, rat vibrissa follicles were cut in sections and two populations of follicular epithelial cells, the matrix and the upper part of the follicle (UF) were dissected. The cells were dispersed into single cells and cultured in an epidermal cell growth medium on plastic. As soon as cells began to proliferate their behaviour was observed in detail for extended periods of time. During this time the adhesive behaviour, colony-forming abilities and proliferative rates of these two cell types were studied.

3.1.2 Pluripotency of adult epidermal cells

Sebocytes of the sebaceous gland are epidermal in origin (Xia *et al.*, 1989) and are formed during morphogenesis of the pilosebaceous unit from pluripotential epidermal cells. The question still arises as to whether during post-natal life the epidermal stem cells are committed to the production of only one cell lineage, or in contrast they conserve their embryonic pluripotentiality. During wound healing the damaged sebaceous gland is repaired as well as skin and other skin appendages, however, it has been difficult to establish whether these new sebaceous cells are derived from nearby epidermis or sebocyte cells. With recent advances in the development of techniques for the isolation and culture of cells it would seem possible to investigate the

pluripotency of epidermal cells in maturity, however literature research suggests that there have been few or no investigations in this area. If pluripotential stem cells reside in the bulge or upper regions of the follicle then it would be postulated that there may be differences in the lower and upper follicle epidermis in relation to sebocyte generation. As a complementary investigation to epidermal cell culture, part of this study was concerned with the examination of sebocytogenesis in cultured epidermal cells of the bulb and upper follicle.

3.1.3 Major histocompatibility complex (MHC) and epidermal cells

A piece of tissue taken from one individual and transplanted to another is destroyed by the recipient immune system because the cells in the donor tissue express molecules on their surface that are different from those expressed by the recipients. These molecules act as antigens which stimulate the recipient lymphocytes. The antigens thus determine whether the tissue will be immunologically compatible with the recipient—they act as histocompatibility antigens, encoded in histocompatibility genes. The way that a transplanted tissue is destroyed depends on the nature of histocompatibility antigens. Most antigens are weak (minor) and elicit slow and delayed tissue destruction. There is, however, a set of antigens that elicits rapid destruction, these are the major histocompatibility antigens encoded in a set of clustered loci termed the major histocompatibility complex (MHC) (Klein, 1990).

Immune privilege was first described more than a century ago, and is the property that allows tissues to be grafted on recipient sites without rejection. This property manifests itself in two forms; immune-privileged tissues and immune-privileged sites. Immune-privileged sites are the places (e.g. the eye, testis and brain) that tissues can be grafted into without rejection, whereas the immune-privileged tissues are the tissues that can be grafted onto any tissue without destruction. The immune-privileged tissues are characterised by one or more following features: intra-tissue structural barriers such as extensive tight junctions among cells, elaborate surface expression of hyaluronic acid, release of soluble class I molecules, secretion of immuno-suppressive cytokines (for example, TGF- β), corticosteroids and constitutive expression of FasL on paranchymal cells (see Streilein, 1995).

Histocompatibility complex molecules (MHC I and II) are also one of the factors

most likely to be involved in immune-privileged reactions, however there is conflicting evidence about the nature of their role in this phenomenon. Chitilian & Auchincloss (1997) demonstrated that MHC deficiency of cells offers no protection against natural killer (NK) cells. Hoglund *et al.* (1990) presented evidence demonstrating that cells with deficient MHC class I are sensitive to NK cells whereas it is reported that the deficiency in MHC class I produces a resistance against NK cells (Coffman *et al.*, 1993; Osorio *et al.*, 1994; Streilein, 1995).

The process of hair follicle cycling is modulated by numerous endogenous and exogenous factors (see Paus, 1996). One of the exogenous factors is the perifollicular environment which influences the cycling of hair follicles through different ways (see Paus *et al.*, 1994). It has been demonstrated that during the transition of follicles from anagen to telogen, which in most follicle types leads to regression and resorption of most of the lower part of the follicle, an increase is seen in the number of macrophage cells in the follicle bulb (Parakkal, 1969). Because it has been shown that granulocyte-macrophage colony-stimulating factor and tumour necrosis factor both induce expression of class II MHC antigens on tissues macrophages, it has been suggested that macrophages, through MHC antigen expression which is mediated these mediators, may have a role in catagen development (Westgate *et al.*, 1991). In rat pelage follicles in anagen, most of the lower and transitional parts of follicles do not express MHC class I while the upper and permanent parts of follicle do express this molecule (Westgate *et al.*, 1991). During regression in catagen a dramatic change is observed in expression of MHC-I. It is expressed in all the follicular epithelium while large numbers of activated macrophages aggregate around the follicles (Harrist *et al.*, 1983; Westgate *et al.*, 1991). In agreement with this evidence, Limat *et al.* (1994a) also showed that in anagen human hair follicles, cultured epidermal cells from lower portions of the follicle (including the matrix) express markedly lower levels of MHC-I than interfollicular epidermal keratinocytes. Based on this data it has been hypothesised that the difference in expression of MHC class I may provide a basis for the specificity of follicle regression in catagen in a such way that cells that express high levels of MHC-I survive but cells with a low level of MHC-I are absorbed by macrophages during regression (Limat *et al.*, 1994a)

Chondroitin 6-sulphate (C6S) proteoglycan, a component of the ECM of

connective tissues is highly expressed in anagen follicular dermal tissues but markedly diminishes during catagen. Westgate *et al.* (1991) put forth the theory that during anagen the high level of C6S in the ECM acts as a protective screen. This screen which surrounds the follicle prevents macrophages recognising the lack of self-histocompatibility antigen on the bulb cells of the growing follicles. The fact that during catagen, when C6S diminishes, an increase is observed in the number of activated macrophages in and around the follicle is put forward as the evidence in support of this concept (Westgate *et al.*, 1991).

To date there has been no report on the comparative expression of these surface antigens in cultured epidermal cells of the lower and upper follicle. Thus, in line with a previous study carried out by Limat *et al.*, (1994), the expression of MHC class I in these two cell populations was compared here using flow cytometry techniques.

3.2 Materials and Methods

3.2.1 Follicle isolation and tissue dissection

A diagrammatic representation of the main steps in the establishment of rat vibrissa epidermal cell culture is shown in Fig. 3.1. Briefly, anagen vibrissa follicles were isolated from the mystacial pad of inbred PVG hooded rats as outlined previously in section 2.2.3.1. Follicles were immediately placed in sterile Eagle's MEM supplemented with conventional antibiotics (ABS) (see section 2.2.3.4). Under a dissecting microscope (Nikon SMZ-10, Japan) the follicles were cleared of surrounding connective tissue using fine scissors. Using a scalpel blade number 11 the follicles were then transected into three fragments by two transverse cuts made just above the end bulb and below the nerve entry level into the collagen capsule. The central or middle fragment was discarded and the lower (bulb) and upper fragments (UF) were transferred into separate 35-mm plastic dishes (Falcon, Becton Dickinson, UK) containing the same medium.

In order to dissect the epidermal compartments of the UF the sharp edge of a syringe needle was used to make a longitudinal cut in the collagen capsule. Then using two pairs of watchmaker forceps the edges of the cut were held and pulled away gently to reveal the internal tissue that contained an epithelial core surrounded by a thick dermal sheath. The internal tissue was separated from the collagen capsule by needles and moved to a new 35-mm dish containing MEM. After dissection, the internal tissues were transferred into a dish containing 2-ml collagenase/dispase (50/50) at a concentration of 1 mg/ml (Collgenase was supplied by Sigma, Aldrich, UK, Dispase was obtained from Boehringer Mannheim) and incubated for 20 minutes at 37°C, according to the protocol of Kobayashi *et al.* (1993). This treatment resulted in digestion of the dermal-epidermal junction and made it feasible to detach the epidermal cores easily from the overlying dermal sheath using two pairs of sharpened watchmaker forceps. The dermal sheath was then discarded or cultured for other purposes and the epidermal core transferred to a 30-ml conical tube (commonly is called the universal) (Sterilin, UK) for trypsinisation.

To isolate the epidermal component of the bulb region, parallel cuts were made in the collagen capsule with syringe needles. The bulb was held carefully by sharpened

watchmaker forceps and its base was gently pushed upwards resulting in inversion of the bulb. This in turn resulted in isolation of the epidermal matrix from the bulb while the base was still attached to the dermal sheath and the dermal papilla. In order to dissect the maximum number of germinative cells, great care was taken when detaching the dermal tissues from the base of the epidermal matrix which was then transferred to a universal tube for subsequent trypsinisation. Overall for each 35-mm dish, about 12-15 vibrissa follicles were transected and the epidermal components of the bulb and UF were used.

3.2.2 Cell separation and culture

A solution of 0.05% crude trypsin (Sigma, Aldrich, UK) was added to each universal containing the separated follicle epidermis and the tubes were incubated at 37°C for 1 hour. After blocking the process of digestion with 20% foetal calf serum (FCS) in MEM, the cells were liberated by gentle agitation with pipettes for 2-3 minutes. The universals containing the cell suspensions were then centrifuged at 1800 rpm for 3-5 minutes, the supernatants discarded and the pellets of cells were resuspended in epidermal culture medium (section 3.2.5). Cells were counted under a phase contrast microscope (Nikon TMS, Japan) using a haemocytometer and an equal number of cells (2.4×10^4) were plated on every dish. Epidermal cells from both fragments were cultured on 35-mm or 55-mm primaria dishes (Falcon, Becton and Dickinson, UK) without using a 3T3 feeder layer. Dishes were incubated at 37°C in the presence of 95% O₂ and 0.5% CO₂ for 4-6 days before any examination.

3.2.3 Colony growth measurements and cells subculturing

Cultures were examined under a phase contrast microscope between 4-6 days after seeding. After that the medium was changed every 4-5 days and the cultures were regularly examined in order to measure the size of epidermal colonies. The measuring was conducted by selecting and labelling (for future identification and observation) between 5 to 8 colonies on the base of each petri-dish at day 4-7 post-seeding. The limit of growth of all selected colonies (for both bulb and UF) was observed and photographed regularly (at 4-5 days intervals) under a Nikon TMS inverted microscope (Japan) with x10 phase objective. At the same time the size of the colonies was

measured with an eyepiece equipped with a measuring grid graticule. The measuring was continued until the perimeter of colonies merged together which took between 30- 80 days in different cultures. After this stage, cells were subcultured and transferred to a 25-cm² flask (Falcon, Becton and Dickinson, UK). This process, otherwise known as passaging or subculturing was carried out in the following manner. The culture medium was first withdrawn and discarded. The cells were washed with a solution of PBS/EDTA (0.2 mg/ml) designed to remove traces of serum that would inhibit the following trypsinisation action. The PBS/EDTA solution was discarded and a solution of 0.25% trypsin in PBS/EDTA pre-warmed to 37°C was then added to the culture, just enough to cover the cell monolayer completely. Cells were incubated at 37°C in this solution until they rounded up and could be lifted off the substrate with gentle agitation. In order to terminate the process of trypsinisation, 3 ml of medium {(MEM+ ABS+ 20% foetal calf serum (FCS))} was added and the medium was then pipetted up and down sufficiently to disperse the cells into a single cell suspension. The cell suspension was transferred to a universal and centrifuged for 4 to 5-min at 1800 rpm. The supernatant was removed and the residual pellet of cells resuspended in MEM + 10% FCS by gentle pipetting. The cell suspension was transferred into a 25cm² flask, which was immediately returned to the incubator. After the first subculture the cells are termed passage 1.

3.2.4 Cell attachment

In order to evaluate the adhesive properties of the cells to the substratum, on one occasion, primary cell cultures of both regions (bulb and UF) which had grown for 30 days were trypsinised and resultant single cells were plated on 35-mm primaria culture dishes, 1x10⁴ cells per dish. Three days after plating, 2 dishes of UF cells and 2 with bulb cells were taken, media were removed and cultures gently washed twice with MEM. This resulted in the removal of all floating and unattached cells. After that fresh epidermal medium was added and the number and shapes of attached cells assessed and photographed.

3.2.5 Epidermal Culture Medium

The medium used for growing epidermal cells in vitro was made based on that used by Reynolds (1989) with some modifications. It consisted of Calcium Free Medium

(Kyokuto, Japan) containing antibiotics and supplemented with 20% FCS, 10 µg/ml insulin, 5 µg/ml transferin, 0.4 µg/ml hydrocortisone, 0.01 µg/ml epidermal growth factor, 10^{-9} M cholera toxin (all supplied by Sigma) and 140 µg/ml foetal rat pituitary extract. The pituitary extract was prepared according to Reynolds (1989). Briefly, pituitary glands were dissected and removed from foetal or new born (up to 3-day-old) PVG hooded or white rats and transferred into a universal tube. The pituitary tissue was weighed and a solution of 0.15M NaCl was added to the specimen, in the proportion 1.25 ml NaCl per 0.5 g pituitary tissue. Using a 3-ml plastic pipette the tissue was agitated for few minutes until it dispersed and then was homogenised. The homogenised solution was left at 4°C for 1.5 hours after which the agitation was repeated for another 10 minutes. The mixture was spun down at 10000 rpm for 25 minutes at 4°C. Supernatant was removed and placed in a 1.5 ml centrifuge tube and the pellet discarded. A small aliquot of the supernatant was used to measure the protein concentration using the Bradford assay (Bradford, 1976). The supernatant was used either immediately or stored at -20°C for further application.

3.2.6 Staining of the epidermal colonies

In order to provide macroscopic observations and measurement of colonies, cultures were stained with Giemsa stain (Sigma, UK). The medium was removed and the cells washed with PBS. Cells were first fixed in 50% methanol for 10 minutes and then 100% methanol at room temperature for 10 minutes. The cells were covered with 0.5 ml neat Giemsa stain for two minutes after which the stain was diluted by the addition of 3 ml distilled water. The cells were left in the diluted stain for 2 minutes and then washed in running tap water vigorously until any pinky background had been removed. The cells were then allowed to dry completely before taking photographs. Photographs were taken using a Contax 167MT camera (Japan) and processed and handled as explained earlier in chapter 2 (section 2.2.3.4).

3.2.7 Immunolabelling of epidermal colonies with BrdU

To investigate the rate of cell proliferation in the colonies, cultures were first labelled with BrdU. Briefly, after discarding the culture medium, 2 ml MEM supplemented with conventional antibiotics and containing 10-µmol/l BrdU was added

to the culture dishes. The dishes were incubated for 1 hour at 37°C. To remove any unincorporated label, the MEM/BrdU solution was discarded and 2 ml fresh MEM was added. Dishes were again incubated for 10-15 minutes at the same temperature and the MEM again discarded. The cells were then fixed in 70% ethanol (containing 50-mmol/l Glycine, PH=2) at -20°C for 30-45 minutes. To detect the label, the dishes were processed through the same protocol as described previously (section 2.2.3.4) using a monoclonal anti-BrdU antibody.

3.2.8 Oil Red-O staining of epidermal cells

Epidermal cells at passage 2-3 were utilised for lipid staining according to the method described by Doran *et al.* (1991). Growth medium was removed and the cells washed with PBS for 3 minutes. The cells were then fixed in 4% buffered formalin saline for 30 min followed by 3x5 minutes washes with PBS and stained with a saturated solution of oil red O in an isopropyl alcohol/water (3:2) mixture for 30 minutes. The cultures were rinsed with 60% isopropyl alcohol until the reddish background had disappeared. Subsequently, the cells were stained in Mayer's haematoxylin for 5 min before they were washed in distilled water and dehydrated through a series of 70%, 95% and 100% alcohol's (3 minutes each). Cells were coated with DPX mounting medium (Sigma, UK) and photography was carried out as described in section 2.2.3.4.

3.2.9 Flow cytometric analysis of MHC class I on epidermal cells

Monoclonal antibody to MHC class I (Mouse anti rat Rta class I), anti mouse IgG (Goat anti mouse) and an isotype negative antibody (Mouse IgG negative) were all purchased from Serotec (Oxford, UK).

For flow cytometric analysis, primary cultured epidermal cells (35 days after inoculation) from both regions (bulb and UF) were trypsinised, centrifuged and resuspended at 1×10^6 cells per ml in FACS medium (PBS containing 0.1% bovine serum albumin and 0.1% NaN_3 , pH=7.4) at 4°C. To completely ensure a single cell suspension (cell agglutination may give rise to inaccuracies in analysis) the medium was syringed through a syringe needle Microlance-3 (25G1 0.5x/25 Nr. 18, Becton Dickinson Drogheda, Ireland). Cells were then counted under a phase-contrast microscopy using a haemocytometer, and aliquots containing 2×10^5 cells were transferred into 12x75-mm

centrifuge tubes (Greiner Labortechnik Ltd., UK) for immunohistochemistry. Cells were incubated with 50 μ l MHC I antibody (diluted 1/50 with FACS medium) or negative isotype antibody for 20 minutes at 4°C. After washing twice with 0.5 ml FACS which every time was followed by centrifuging at 1800 rpm for 10 minutes, cells were incubated with polyclonal goat anti-mouse antibody for 15 minutes. The cells were then washed twice as above and suspended in 0.5 ml FACS medium for analysis. The cells were analysed on a Coulter Epics flow cytometer. The ELITE software that runs the Coulter Epics XL-MCL cytometer and analyses the produced data, allows analysis of the data, both at the time of acquisition of the data and also at a later point if the data is saved onto computer disk. For each cell population, markers were set to exclude maximum of stained cells with control reagents from positive analysis. Gates were also set by forward and side scatter to exclude dead cells from the analysis.

3.3 Observations and Results

3.3.1 Comparative behaviour of the bulb and UF epidermal cells in culture

3.3.1.1 General observation

The dissociation of epidermal cells from the upper follicle (UF) and the bulb regions always yielded widely differing cell numbers with the UF generally producing more cells than the bulb (2-3 fold). Therefore, in order to seed equal numbers of cells in dishes, after counting, the suspension containing UF cells was diluted with epidermal medium. Primary cultures of epidermal cells were established by plating a concentration of 2.4×10^4 cells on 35-mm Primaria dishes. In total, 12 pairs of dishes were set up for this experiment, and the behaviour of cells in these plates was subsequently followed. In both cultures, cells attached to the substrate and produced colonies. Over time, certain colonies from both sources stopped growth, and their cells underwent terminal differentiation. The remaining colonies, however, continued their growth until finally their perimeters overlapped. The colonies in UF cultures had overlapped 25-35 days after seeding, while the bulb cultures took 70-80 days to reach this stage. Both bulb and UF cells could be subcultured and grown to confluence (Figs. 3.2 and 3.3).

3.3.1.2 Colony examination- comparison of growth and behaviour

Examination of the cultures 4 days after plating of the cells revealed that in both cultures the majority of the cells failed to attach to the substrate and were seen floating in the medium. In UF cultures less than 1% of the cells attached to the plastic while the proportion of bulb cells that attached was even lower. At 4 days the attached cells from the UF had already started to proliferate and had produced colonies containing 2-8 cells (Fig. 3.4). However, cells from the bulb that had attached were still rounded and had not yet undergone any cell division (Fig. 3.5). After seven days, UF cells had produced relatively large colonies (1-2 mm), but the colonies displayed different morphology. Some were rounded and cells within these colonies were small and closely packed displaying a typical epidermal pavement pattern, whereas others displayed irregular shapes and contained large and flat cells (Figs. 3.6A-B). In contrast, at this time only some of attached cells from the bulb had started to proliferate, and those that had

divided had only undergone 1-3 cell divisions, the rest remaining as single cells (Fig. 3.7).

Regular observation of UF cultures throughout the next four weeks showed that there were generally three differing colony types. I) Short-lived colonies (25-30%): Colonies that at 7 days contained only flat and large cells (Fig. 3.8). These colonies soon became differentiated and gradually detached from the culture. II) Intermediate colonies (30-35%): Until day 25-30 the perimeter of such colonies was smooth, and they consisted only of small, closely packed cells. After this time the cells at the edge of these colonies flattened, giving them a wrinkled appearance (Fig. 3.9). Although many cells in these colonies were seen to progressively become involved in terminal differentiation, the colonies as a whole remained visible even when cultures were subcultured at 30-35 days. III) Long-lived colonies (35-40%): These colonies consistently showed a smooth perimeter and were composed of only small and dense cells (Fig. 3.10). The diameter of these colonies was constantly increasing. After 15-20 days, cells at the centre of these colonies stratified and the upper cells differentiated. While these differentiated cells remained at the surface in mid-colony as a dead and flat layer, cells at the edge of colony were always found to be small, dense and migrating over the substrate (Fig. 3.11).

To evaluate the proliferative profile of epidermal cells within the colonies, in three cases the cultures were processed for BrdU labelling at 25-30 days after culturing. Examination of the labelled colonies under a fluorescence microscope showed that the BrdU marked cells were only found in colonies that had small cells, no cell labelling was observed in colonies where all of the cells had undergone or were undergoing terminal differentiation. In positively labelled colonies the distribution of dividing cells was similar to that described by Tseng & Green (1994) for epidermal keratinocytes. The BrdU marked cells were essentially restricted to the peripheral regions of the colonies (Figs. 3.12A-B). The number of labelled cells was much reduced toward the centre to the extent that the marker was rarely detected in centrally localised cells (Fig. 3.12A)- particularly where the central region had keratinized and was covered by a mass of dead material.

The UF cultures were passaged at 30-40 days post-culture by which time colony boundaries had overlapped. The passaged cells were transferred into 25-cm² flasks. After subculturing the cells displayed more or less similar morphology and behaviour to

the primary cultures in terms of proportion of the three colony types and the pattern of their growth. The UF cultures had the capability to be subcultured multiple times. In one case they were subcultured serially on occasions over a period of over 7 months.

Regular observations of the bulb cultures revealed that 3 types of colony were also produced from this region but their proportion clearly differed from that of UF cultures. I) Short-lived colonies, constituted at least 50% of the established colonies (Fig. 3.13A). Cells in these colonies continued dividing until 12-17 days in culture after which they began to differentiate. The cells then keratinized (Fig. 3.13B) which resulted in their detachment from the substratum, and such colonies were no longer visible by around day 20. II) Intermediate colonies, in this case comprised 40% of the whole population (Fig. 3.14). These colonies grew in culture for 15-20 days after which cells at the interior part of the colony were still dividing but proliferation had ceased in cells that were situated at the perimeter of the colony so that the colony size no longer increased. After 25-30 days, cell division finally halted in all areas of the colony and cells were seen to undergo terminal differentiation and keratinization. These defunct colonies gradually detached from the dish and generally were no longer visible at day 35-40, although a few remained for a longer period of time (45-50 days). Before describing the third type it is essential to note that because the majority of colonies (type I and II) in the bulb culture stopped their growth after a relatively short time, it was generally feasible to follow the morphology of the third colony type in bulb cultures for long periods before the colonies overlapped. For example, in one case a primary bulb culture was maintained for 80 days before subculturing and without colonies overlapping. III) Long-lived colonies, which composed the remaining 10% of colonies behaved completely differently in culture compared with the two other colony types. The long-lived colonies were composed of numerous small and closely packed polygonal cells which were densest near the colony perimeter. For example, in one case a single colony at 45 days post culturing had approximately 7000 cells. The colonies were round and without any ridges at their perimeters, and this shape was maintained throughout primary culture. However, the interior of the colonies became gradually stratified and differentiated so that masses of terminally differentiated cells were visible in this area at around 30-35 days post-cultivation (Figs. 3.15A-C).

In examination of BrdU labelled cells at 25-30 days post-culturing, the BrdU

marked cells were only found in the long-lived colonies. Again, like UF cultures, cell proliferation (Fig. 3.16A-B) was generally confined to cells that were situated at the edge of the colonies and towards the centre, the density of positive stained cells was considerably reduced. In colonies where the central cells had already produced a mass of dead material virtually no cell division was seen in the centre, but in colonies that had not yet produced this dead material, some scattered cell division could be observed. In order to compare the intensity of cell proliferation between colonies of bulb and UF: pairs of colonies with similar morphology and size were selected and marked before immunolabelling. In observation of these colonies it appeared that density of labelled cells in the UF colonies was slightly higher compared to their counterparts from the bulb, although in some pairs of colonies it was not possible to distinguish any difference in labelling density between the two colony types.

In order to characterise the number and morphology of existing colonies in cultures, in several different cases both cultures (UF and bulb) were fixed and stained with Giemsa stain at various times post-seeding. In general, the number of colonies from the UF was much higher than the number from the bulb cultures. In bulb cultures, most of the colonies were small (these were the colonies that had stopped dividing) but a few large colonies were also observed (Figs. 3.17-18, see also Figs. 3-5 in appendix). On average the number of colonies seen in the UF cultures was approximately 3 times higher than the bulb cultures. The number of colonies in individual cultures is listed in Table 2 in the appendix.

The cells in the bulb cultures were normally subcultured 60-70 days after cell seeding and transferred to 25-cm² flasks. The resulting single cells again produced colonies, which had the same characteristics as the primary cultures in terms of the numbers of attached cells, the types of colonies, their proportions and the pattern of their growth. Like UF cells the bulb cells were also able to produce a confluent culture but compared to the former cells that reached a confluent stage 10-15 days after passaging, the bulb cells reached this stage after about 50 days.

3.3.1.3 Growth rate of epidermal colonies

The growth of epidermal colonies was obtained by taking regular consecutive measurements of the diameters of individually labelled colonies. Measurement of colony

diameter in 8 pairs of dishes revealed an interesting difference in growth rates between UF and bulb cultures. Although some variations were observed among pairs of dishes, the measurements generally revealed that UF colonies grew faster than bulb colonies. Figures 3.19-20 represent results from a pair of UF and bulb dishes in which colony size was measured regularly for the maximum time, namely, 44 and 72 days respectively. Apart from some variations similar results were observed in other cases. Three colony types were visible in cells from both regions, however, there were differences in growth rates between the same colony type from the separate regions- particularly in the case of long-lived colonies. In the bulb, not only did these colonies start their growth later than their UF counterparts but they also grew more slowly. This is illustrated in Fig. 3.21, which shows that 15 days after seeding the diameter of a long-lived colony from the UF was 1.4 mm whereas its counterpart from the bulb had a diameter of 0.6 mm. This difference increased with the time so that the same colonies at day 20 had diameters of 2.8 mm and 1.0 mm respectively (see also Figs. 6-7 in appendix).

3.3.1.4 Attachment of cells to the substrate

To determine the initial attachment characteristics of cells from both regions, cells were plated and cultured for three days. Microscopical observations of UF revealed that cells had already attached to substratum. Some of these had remained single while others had produced colonies containing 2-16 cells (Fig. 3.22). However, on examination of the bulb cultures no colonies were found on the plastic but a few cells had begun to attach to the substratum, since they did not wash off (Fig. 3.23). These cells were small and round but they had not flattened. This showed that although a period of 3 days had been enough for attachment, spreading and division of UF cells, bulb cells had attached but not spread on the substratum.

3.3.2 Examination of lipid production by cells

In all the above experiments a difference in morphology between cells of the UF and the bulb was constantly observed. In confluent cultures from the UF, a number of cells always displayed vesicular-type morphology (Fig. 3.24A) but this characteristic was not found in cultures from the bulb. Vesicles were found in larger cells, which were aligned in straight lines and in areas of densely packed cells. To clarify the nature of

these vesicular cells the cultures were stained with Oil Red-O which converts the lipid into a red colour. Lipid droplets were observed within these vesicles in the same areas as in pre-stained specimens (Fig. 3.24B). Additionally, lipid was observed within clumps of keratin-like material which formed at the surface of dense epidermal monolayer (Figs. 3.24C-D)

3.3.3 Flow cytometric analysis of MHC class I expression by epidermal cells

When analysed on the flow cytometer cells from both populations were scattered widely in both the horizontal and vertical directions indicating that both populations contained a broad range of cells in terms of granularity and size. Cells from both the bulb and the UF stained strongly with the MHC-I antibody and the overall staining pattern was similar, with 86-88% of the bulb cells (Fig. 3.25A) expressing the antibody, compared with 83-86% of cells from the UF (Fig. 3.26A). When the gates were set up on different cell populations it was found that more granular cells expressed a higher percentage of MHC compared with the less granular cells. In the bulb epidermal cells the MHC antigen was expressed approximately in 97% of highly granular cells whereas this was reduced to 42% in less granular cells (Figs. 3.25B-C). A similar relationship was also observed within the UF epidermal cells where the antigen was found in 96% and 73% of cells with high and low granularity respectively (Fig. 3.26B-C). An attempt was also made to investigate the relation between the size of cells and percentage of MHC expression. It was found that in both the bulb and UF cells the larger cells expressed the MHC antigen at a slightly higher level compared with the smaller cells. In the case of the bulb epidermal cells the percentage of positive cells was 96%, while 97% of the larger sized UF cells expressed the antigen (Figs 3.25D and 3.26D).

Fig. 3.1) Microdissection of a vibrissa follicle for cultivation of the epidermal cells.

A) A follicle after it was transected into three fragments. The cuts were made one just above the hair matrix and another just below the nerve entry point. The follicle bulb (B) and upper section (UF) were employed for cultivation of epidermal cells, the middle fragment (MF) was discarded. Scale bar 200 μm .

In order to dissect out the epidermal tissue from the upper follicle, after removal of collagen capsule the inner structure (B) composing of dermal sheath (DS) and epidermal outer root sheath (ORS) was placed into dispase/collagenase (C/D) solution. The outer dermal sheath was then separated from the inner ORS tissue (C). The ORS tissue was trypsinised (T) to liberate single cells which were later cultured in epidermal growth medium (D).

To isolate the epidermal cells from the bulb, the collagen was removed (E) and the epidermal matrix (m) was separated from the dermal sheath (DS) and papilla (p). F) The epidermal matrix was then trypsinised (T) and the resultant cells were cultured in epidermal growth medium (G).

Fig. 3.2) Culture of the bulb epidermal cells at passage 1. Forty days after subculturing the epidermal cells produced a confluent culture. Phase contrast, scale bar 140 μm .

Fig. 3.3) Culture of epidermal cells from the upper part of the follicles at passage 1, fifteen days after seeding. Phase contrast, scale bar 70 μm .

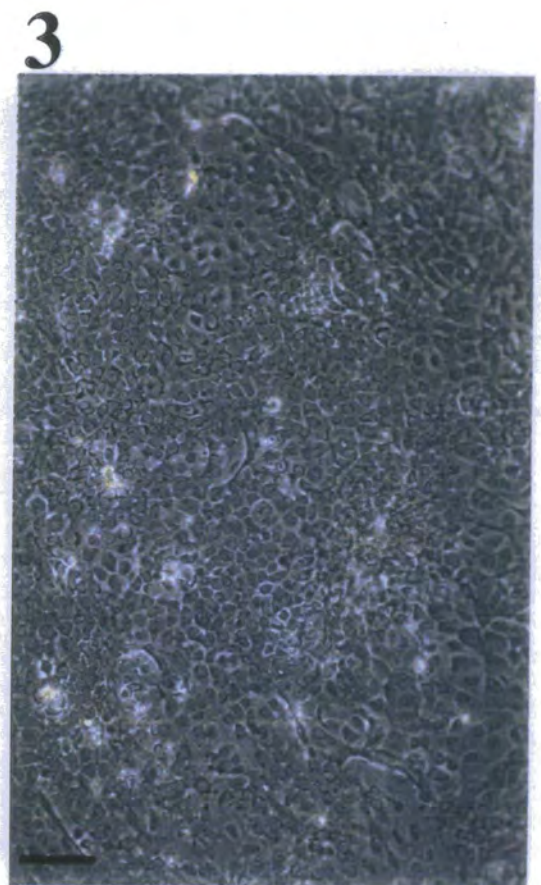
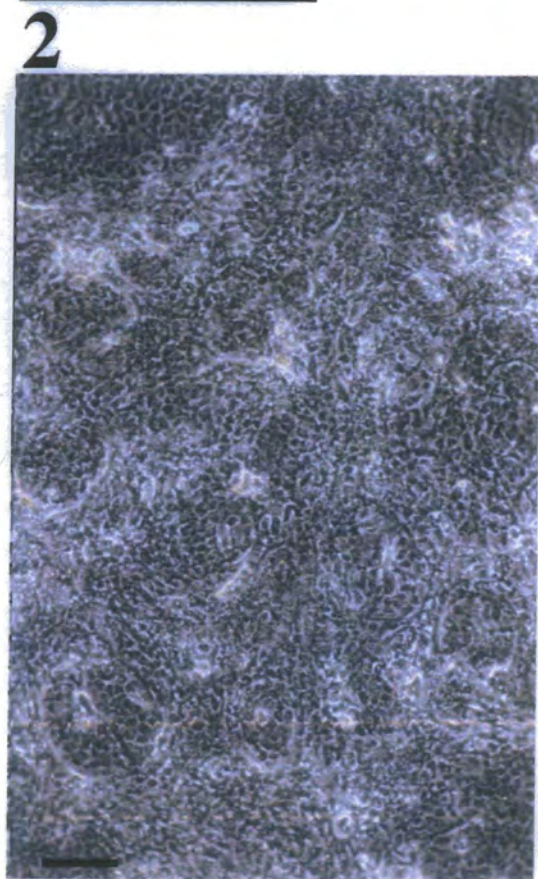
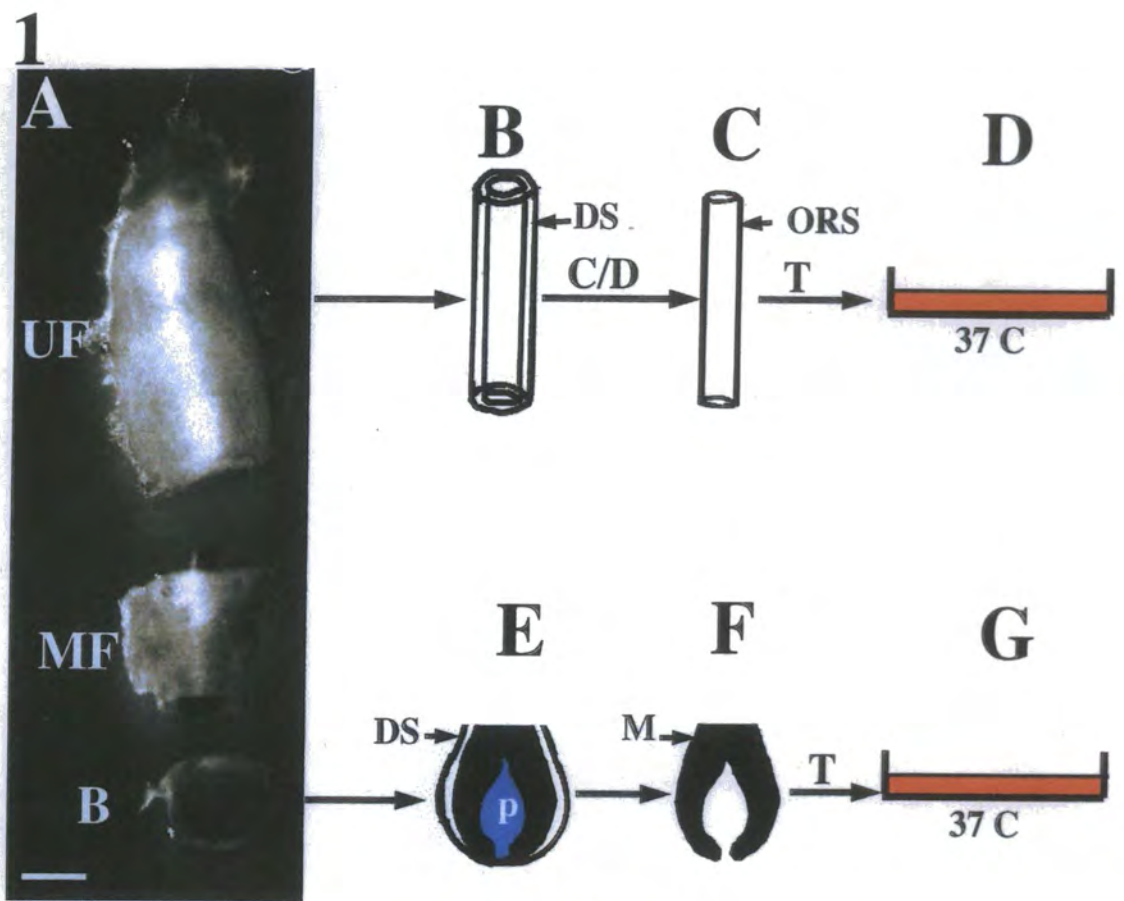


Fig. 3.4) A colony of cells established from epidermal cells of the upper part of vibrissa follicle, 4 days after plating. By this time the cells within the colony have undergone a few rounds of cell division. Phase contrast, scale bar 36 μ m.

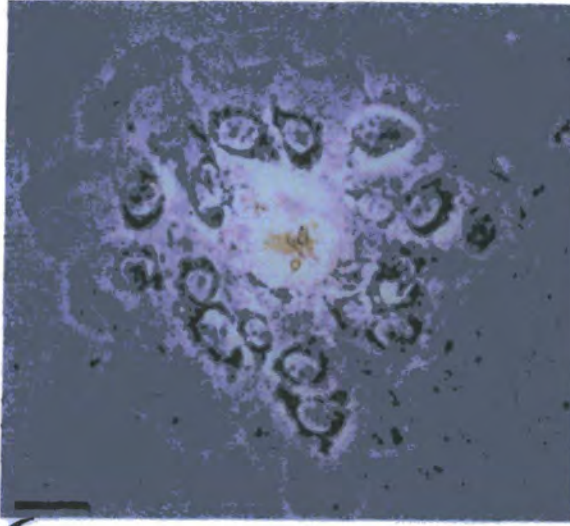
Fig. 3.5) Culture of bulb epidermal cells 4 days after seeding. At this time the cells had attached to the plastic dish (**A** and **B**) but they have not undergone any cell division. Phase contrast, scale bars 50 μ m.

Fig. 3.6) Morphology of the UF epidermal colonies at 7 days after plating. Different colony types are observed in the culture. **A)** The colony is round and contains only small and packed cells. **B)** A colony which displays a polygonal morphology and is composed of some small cells in the centre and large and flat cells at its perimeter. In this colony the large and flat cells shortly differentiate and transform into dead cells. Phase contrast, scale bars A 100 μ m and B 80 μ m.

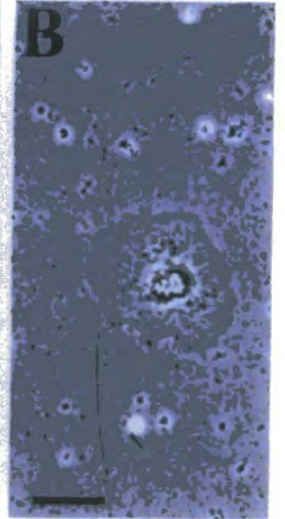
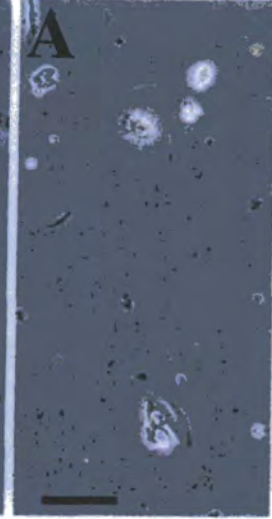
Fig. 3.7) Bulb epidermal colonies 7 days post-inoculation. The colonies by this time have only undergone a few rounds of cell division. While some colonies (**A**) contain only small cells in some others it appears that cells are differentiating (**B** and to some extent **C**) Phase contrast, scale bars A and B 100 μ m, C 50 μ m.

Fig. 3.8) A phase contrast morphology of the primary UF epidermal colonies 15 days cultivation showing a short-lived colony (left) beside a long-lived colony (right). At this time the short-lived colony is composed of mainly large and flat cells. The size of this colony does not increase any more and after a short time its cells all differentiate and die. Phase contrast, scale bar 90 μ m.

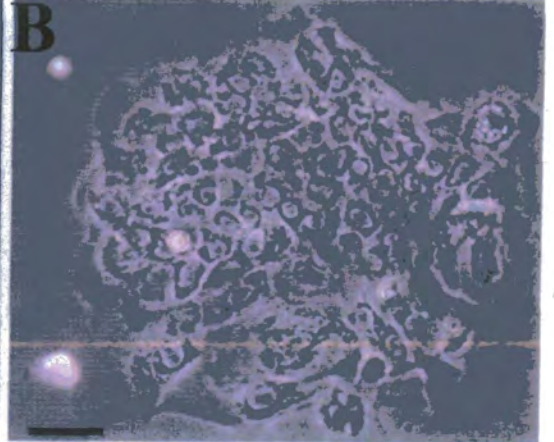
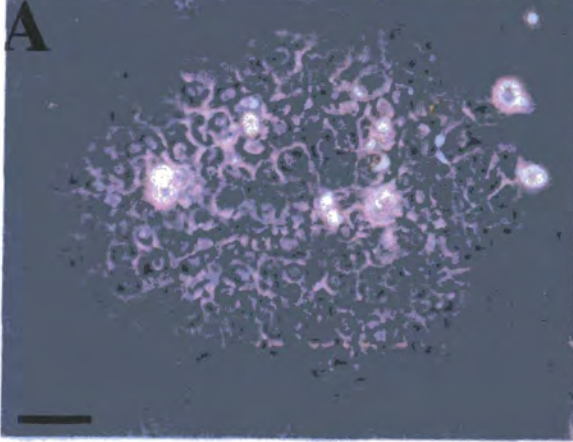
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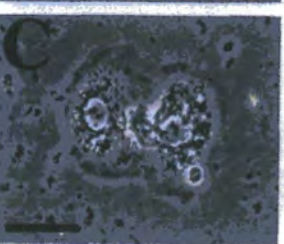
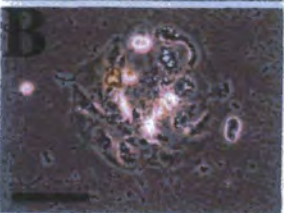
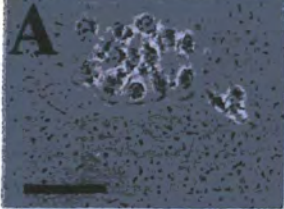
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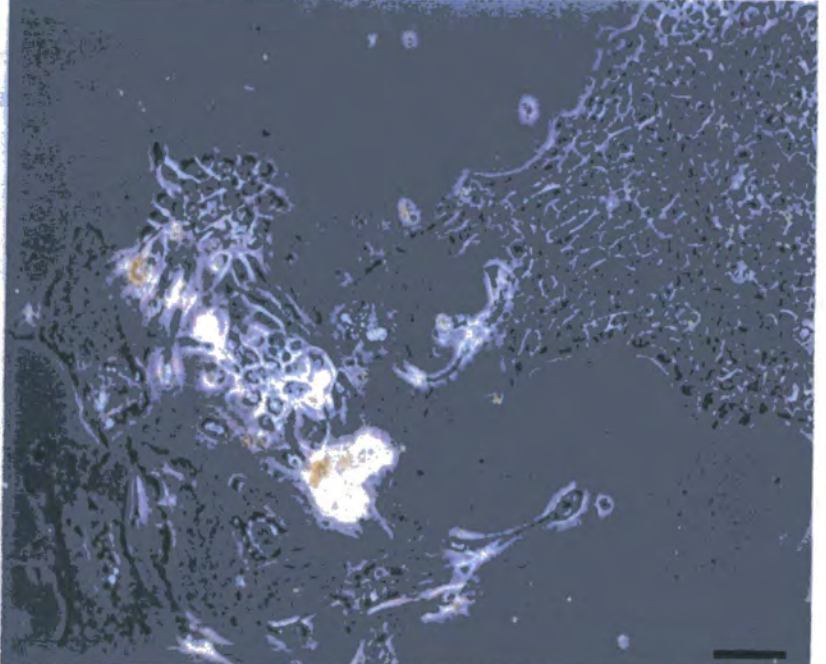


Fig. 3.9) Morphology of a primary intermediate UF colony 25 days after inoculation. The colony displays a polygonal shape. It is composed of small and dense cells at the centre, but cells at the edges of the colony are large and flat. Although this colony will grow for a while, after about two weeks all of the cells differentiate and transform into dead cells. Phase contrast, scale bar 75 μ m.

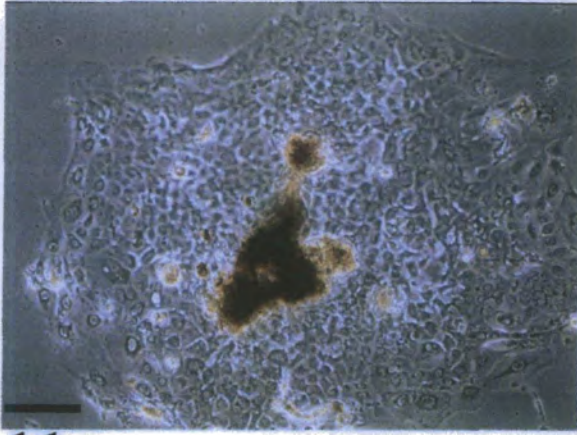
Fig. 3.10) A long-lived UF colony 12 days after plating. The colony displays a round morphology with small and well-packed cells, both in the centre and at the perimeter. Cells in this colony divide steadily and invade the surrounding substrate. At the centre of the colony a mass of dead material has started to form. Phase contrast, scale bar 150 μ m.

Fig. 3.11) Micrographs of a UF culture 20 days after seeding showing the left (A) and right (B) sides of a long-lived colony. Cells at the edges of the colony are still small and display projections indicating they are actively invading the substrate. The mid-region the colony has become stratified and a thin layer of dead material is present above these cells. Phase contrast, scale bars 200 μ m.

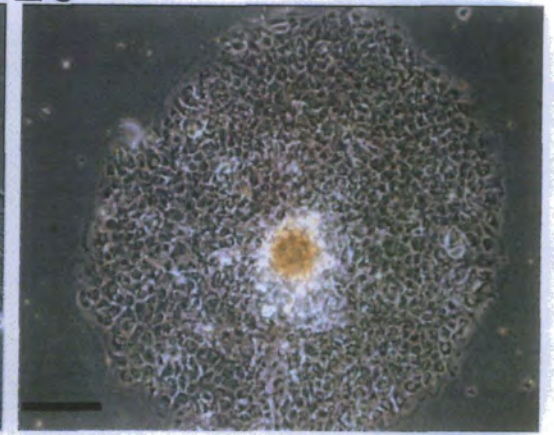
Fig. 3.12) Low (A) and high power (B) micrographs of the BrdU labelling of the UF colonies 28 days post-plating. The label is observed in nuclei of many cells located in the peripheral regions of the colony. Towards the central regions the BrdU marking decreases, and in the centre (c) of the colony no labelling is observed. Scale bars A 100 μ m, B 50 μ m.

Fig. 3.13) Micrographs of the bulb short-lived colonies. A) A colony 10 days after cultivation. The colony displays an elongated morphology and contains large cells. A few cells have already begun their final differentiation. B) A colony 15 days after plating. The colony has lost its integrity and all cells are involving in terminal differentiation. Phase contrast, scale bars 100 μ m.

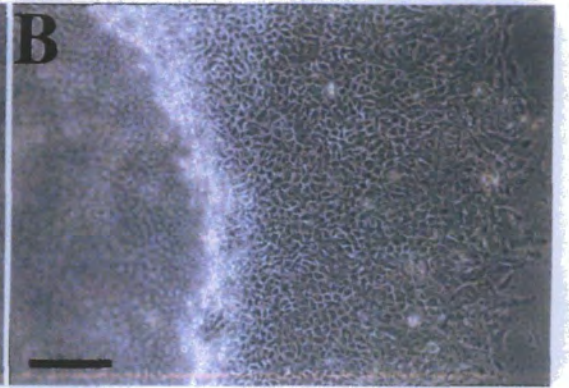
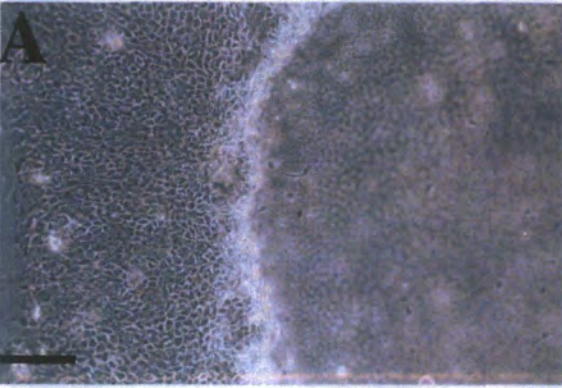
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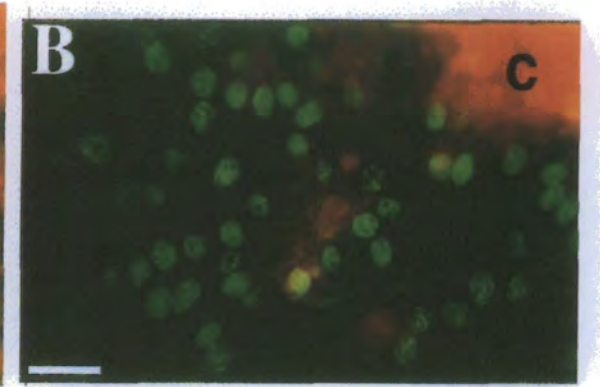
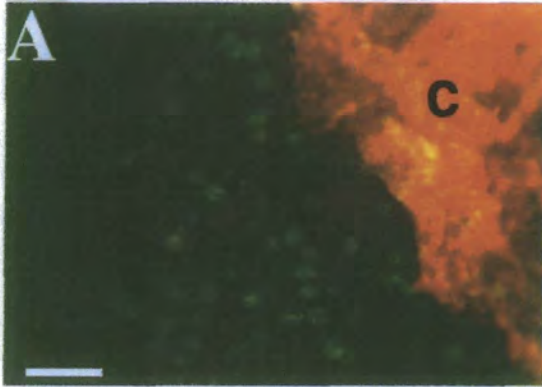
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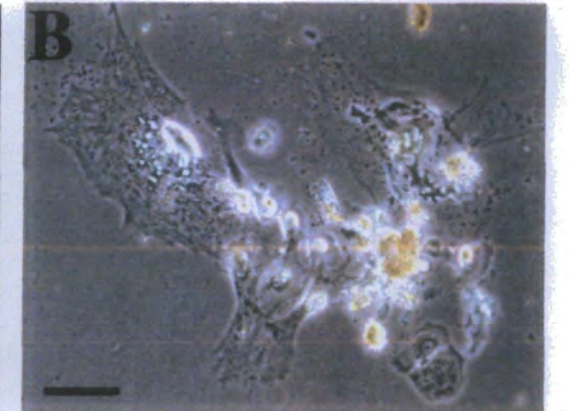
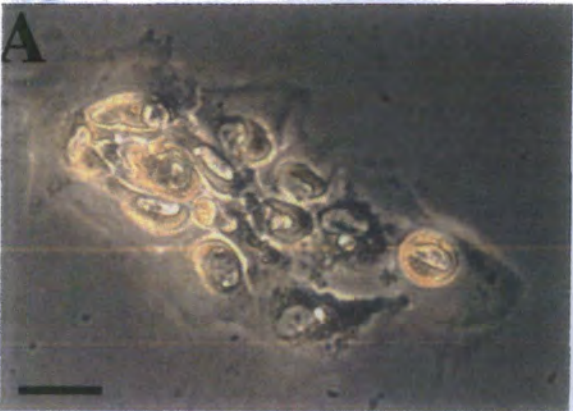


Fig. 3.14) Phase contrast micrographs of the bulb intermediate colonies. **A)** A colony 23 days after growing in culture. While the colony still contains some small and packed cells, most cells are large and flat. The colony at this stage does not grow in size any more but cells remain alive for a while before they finally die and detach from the substrate. **B)** Another colony 29 days post-plating containing mostly dead or dying cells but a few healthy cells are still present within the colony. Phase contrast, scale bars 150 μ m.

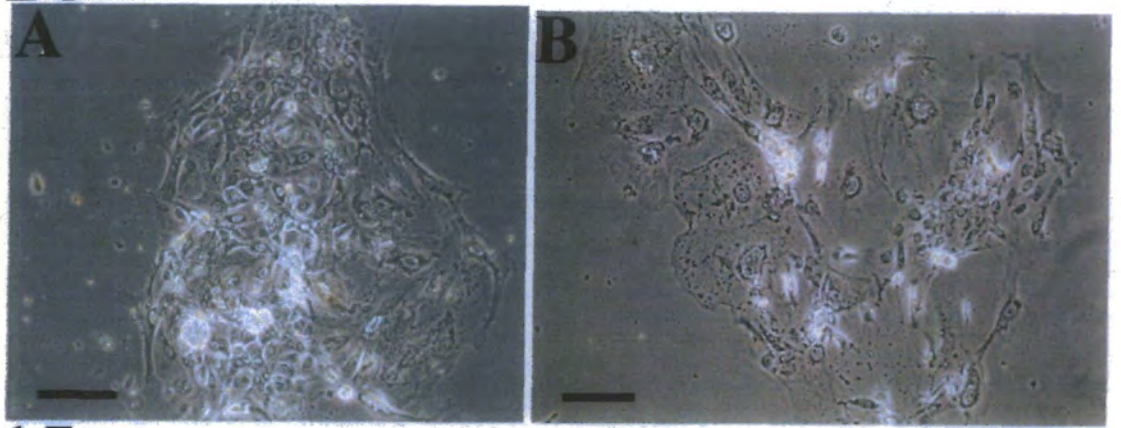
Fig. 3.15) Micrographs showing the long-lived colonies at 14 (**A**), 20 (**B**) and 33 (**C**) days after inoculation. The colonies always exhibit a round morphology with a relatively smooth perimeter and contain small and tightly packed cells at all regions. Due to continued proliferation of cells, the colony sizes progressively increase over time. **A** and **B** phase contrast, scale bars **A** 120 μ m, **B** 200 μ m and **C** 400 μ m.

Fig. 3.16) Low (**A**) and higher (**B**) power BrdU labelling micrographs of the bulb epidermal colonies 28 days after seeding. The labelled cells are densely observed in the peripheral region of the colonies while they decrease in number towards the central area (c). It appears that the density of labelling cells in the bulb is lower than that seen in those colonies from the UF (see Fig. 3.12B). Scale bars **A** 100 μ m and **B** 50 μ m.

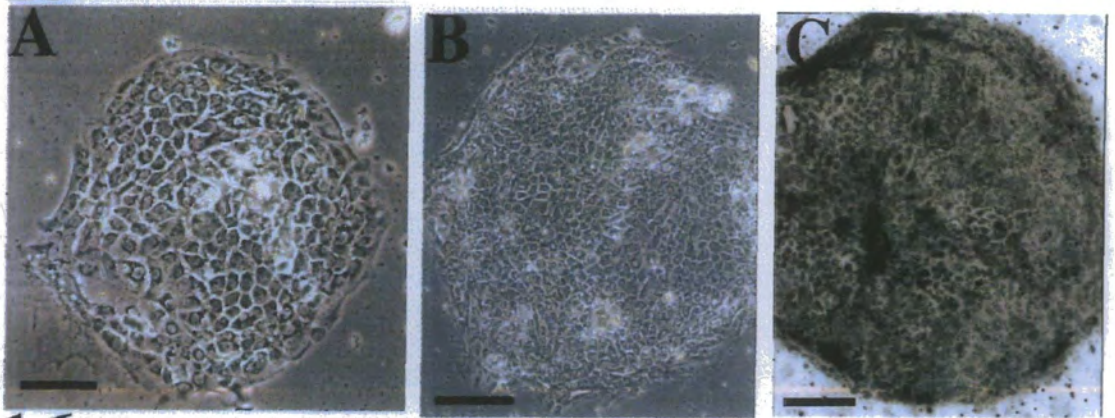
Fig. 3.17) Macroscopic comparison of the bulb (left) and UF (right) epidermal colonies formed from single cells after 15 days in culture. The number of colonies in the UF culture is more than the number of the bulb colonies and in general the UF colonies are bigger than the colonies in the bulb. Scale bar 6mm.

Fig. 3.18) Macroscopic comparison of the bulb (left) and UF (right) epidermal colonies formed from single cells after 27 days in culture. While the boundaries of several UF colonies have overlapped, in the bulb culture the colonies are still well spaced. Scale bar 6mm.

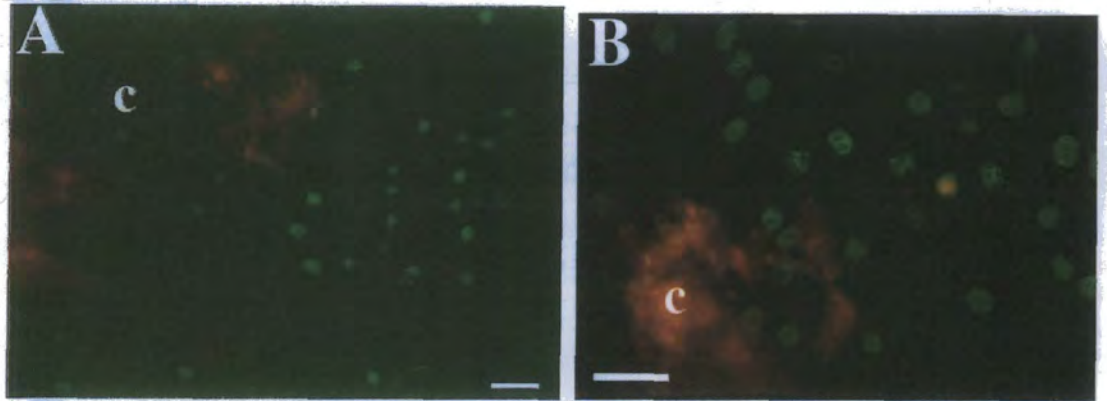
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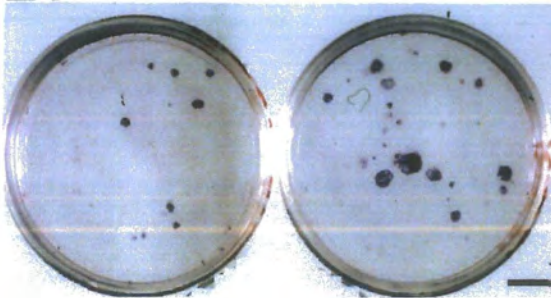
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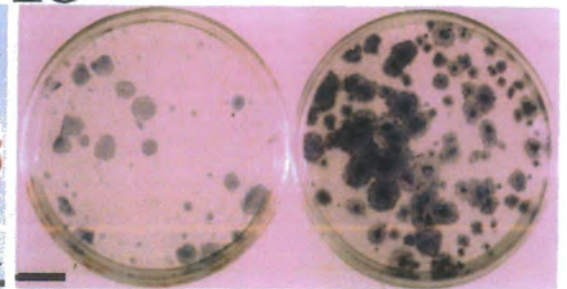
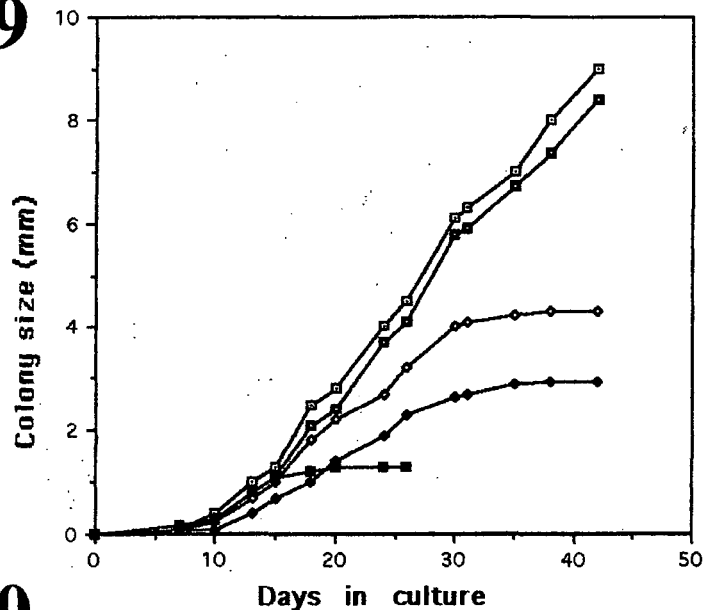


Fig. 3.19) Growth curves of different UF epidermal colonies derived from single founding cells. Each curve was derived from the same colonies measured consecutively. One colony-type (UF 2) grew slowly from the beginning and stopped its growth very early. The second type grew for a while but it also terminated its growth about 30 days after seeding (UF 4). The third type were growing quickly and continuously (UF 1 and 3).

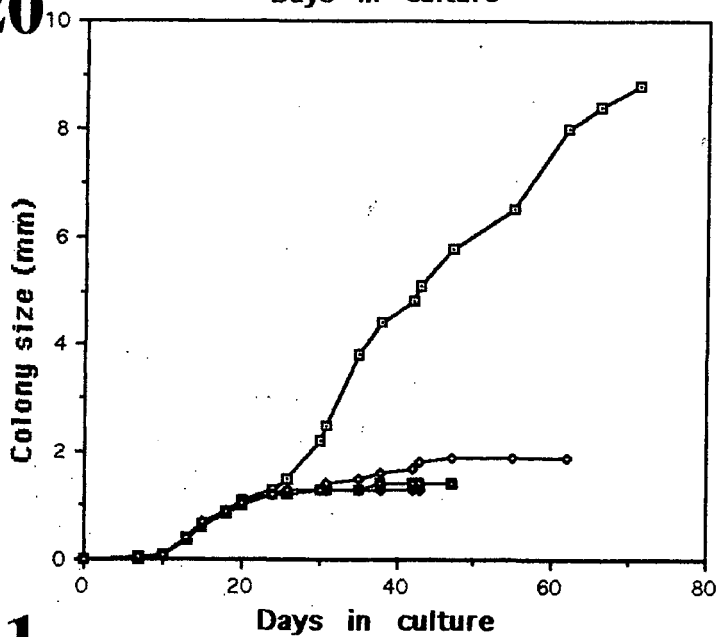
Fig. 3.20) Growth curves of different bulb epidermal colonies derived from single founding cells. As with Fig. 3.19 each curve was obtained from consecutive measurement of the same colony. Most of the established colonies in the bulb region stopped their growth shortly after formation (bulb 2,3 and 4) but a few colonies (bulb 1) grew continuously.

Fig. 3.21) Comparison of the growth rate of the bulb long-lived colonies with the colonies derived from the UF. Compared to the UF long-lived colonies (UF1 and UF3) their counterparts from the bulb region were established later and grew slower all the time.

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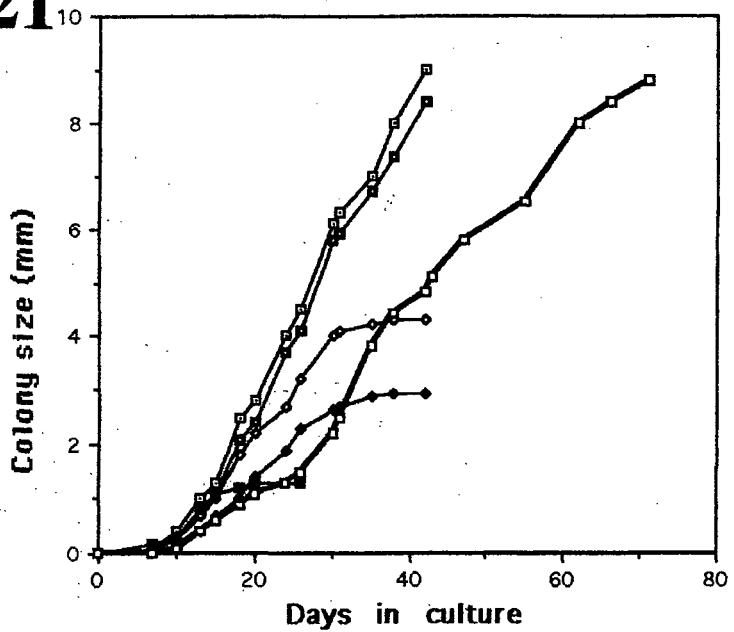
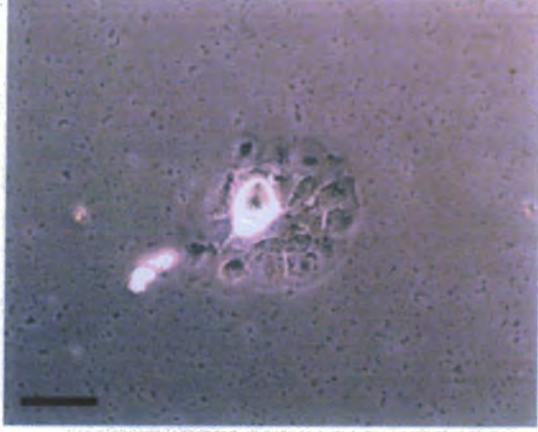


Fig. 3.22) Phase contrast micrograph of the UF epidermal cells 3 days after plating. The cells have already attached to the plastic and started proliferation. Scale bar 100 μ m.

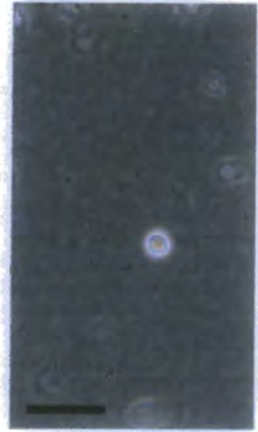
Fig. 3.23) Phase contrast micrograph of the bulb epidermal cells 3 days after seeding. The cells have attached but not spread and divided yet. Scale bar 120 μ m.

Fig. 3.24) Phase contrast micrographs of cultured epidermal cells derived from the UF. **A)** Confluent monolayer of epidermal cells at passage 2. Note the presence of large vesicles (arrows) within the cells. **B)** Another confluent monolayer of epidermal cells stained with Oil Red. Lipid droplets (arrow) are observed within cells. **C-D)** Lipid droplets are also seen within clumps of keratin-like materials. Scale bars 60 μ m.

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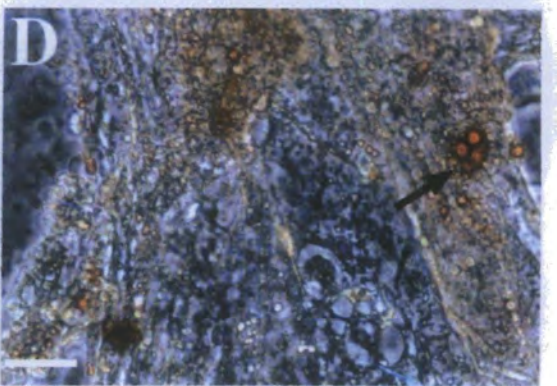
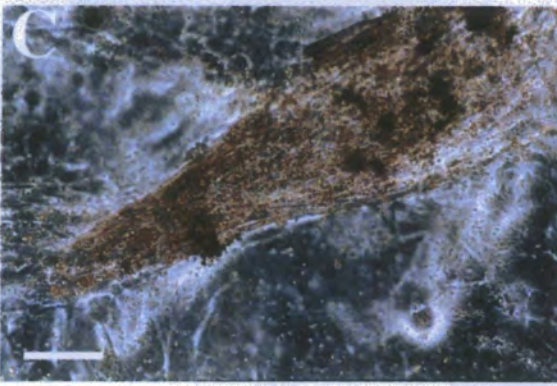
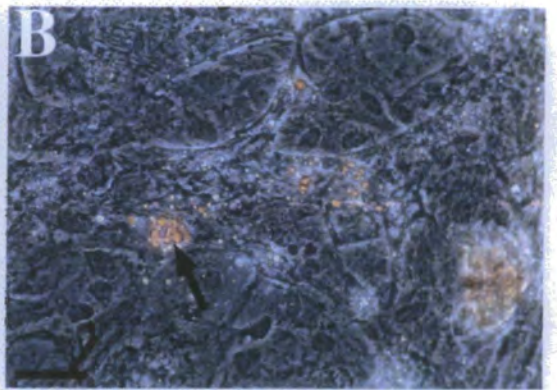
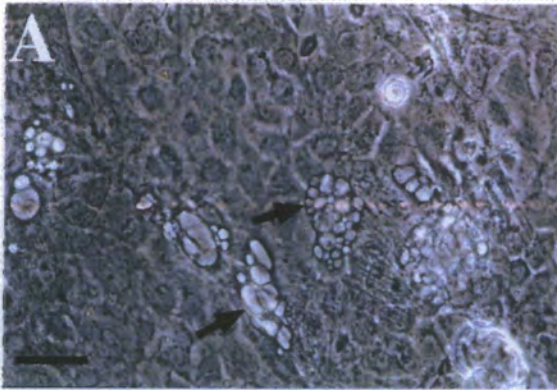


Fig. 3.25) Flow cytometric histograms identifying the distribution pattern (left) and expression of MHC-I by the bulb epidermal cells (right) after 35 days in culture. **A)** When gating is set up to cover the main population of cells, the percentage of positive-stained cells is 88. This percentage is lower (42) for non-granular cells (**B**) but higher (96) for highly granular cells (**C**).

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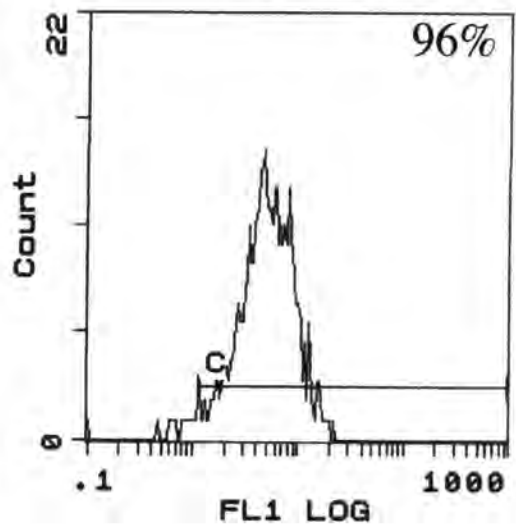
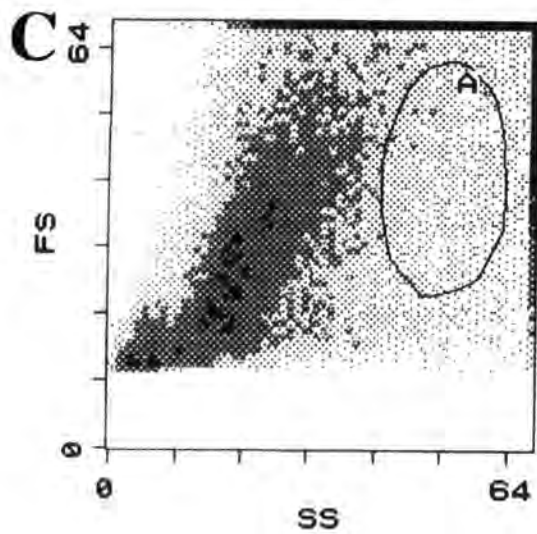
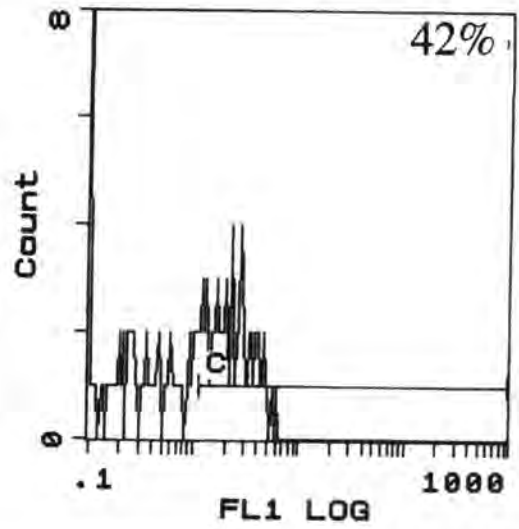
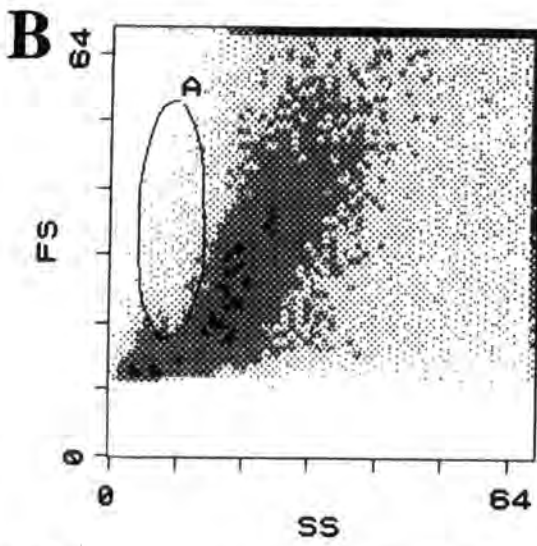
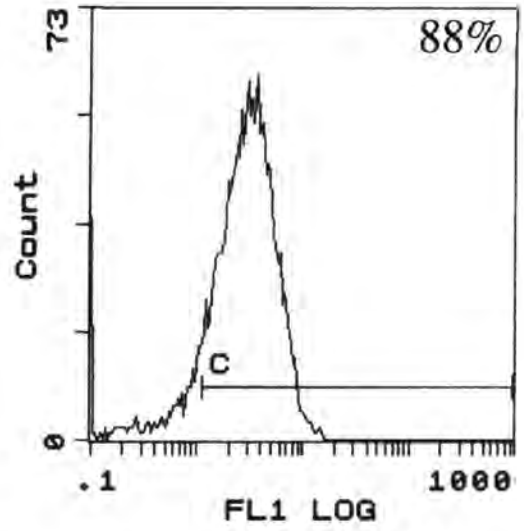
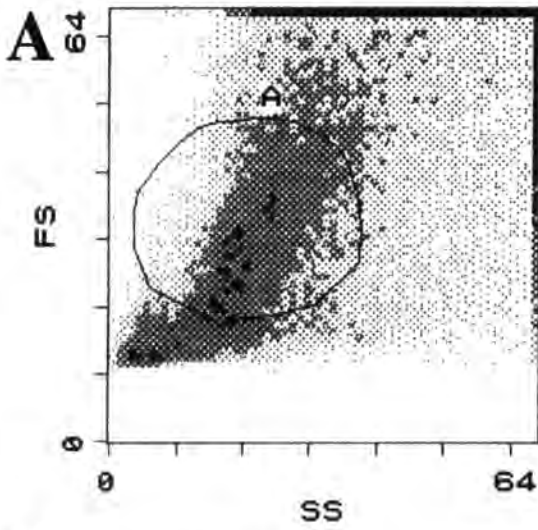
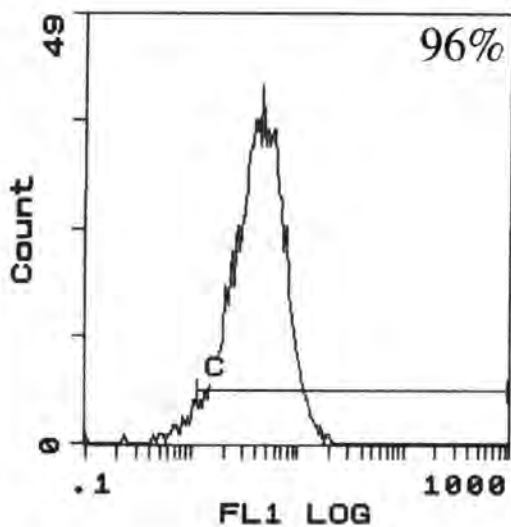
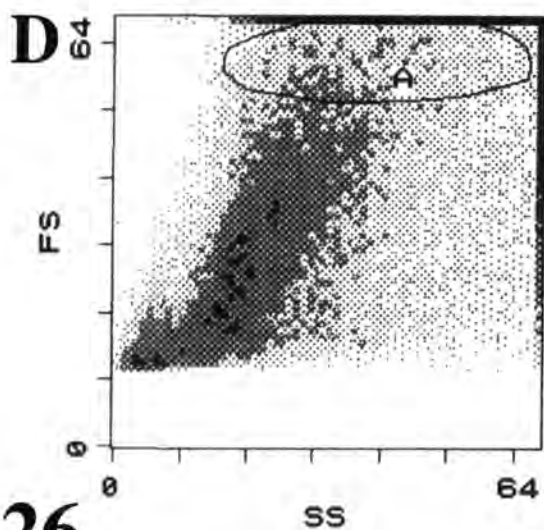


Fig. 3.25D) Continued from previous page

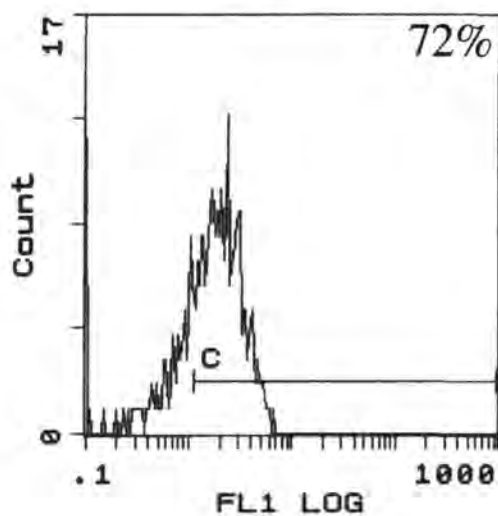
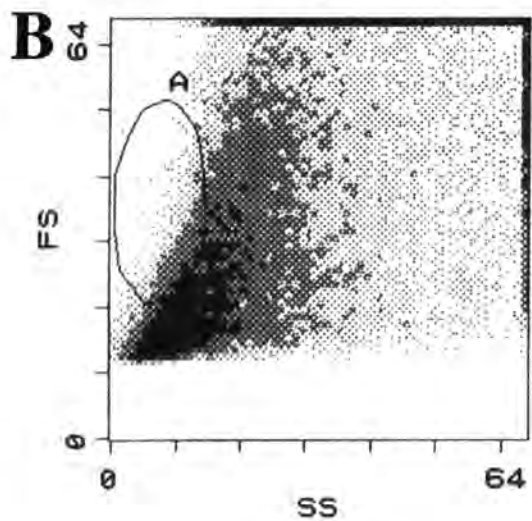
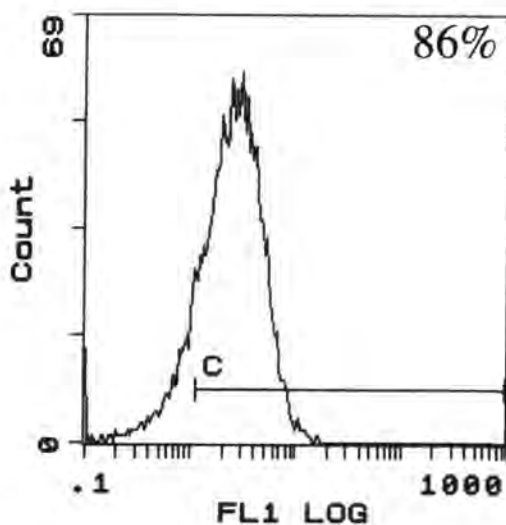
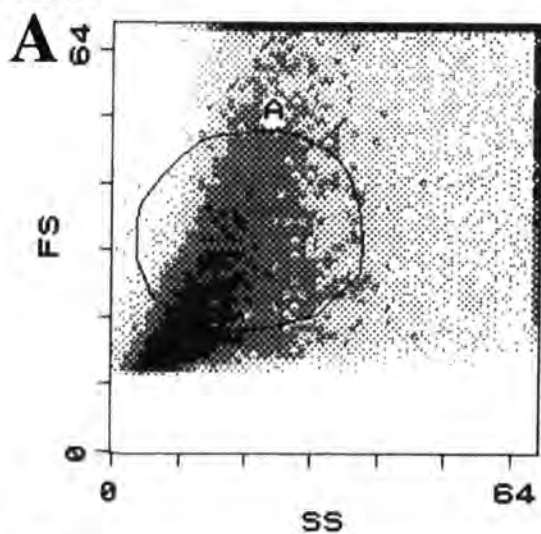
The larger cells are more positive for MHC-I so that 96% of these cells express the antigen (**D**). FS= cell size and SS= cell granularity.

Fig. 3.26) Flow cytometric histograms showing the distribution pattern (left) and the expression of MHC-I in the UF epidermal cells (right) after being grown in culture for 35 days. These cells basically behaved like the bulb cells in terms of the percentage of MHC-I expression within the main body of cells (**A**) and within less granular cells (**B**). Compared to the population as a whole, a lower percentage of less granular cells expressed the MHC-I.

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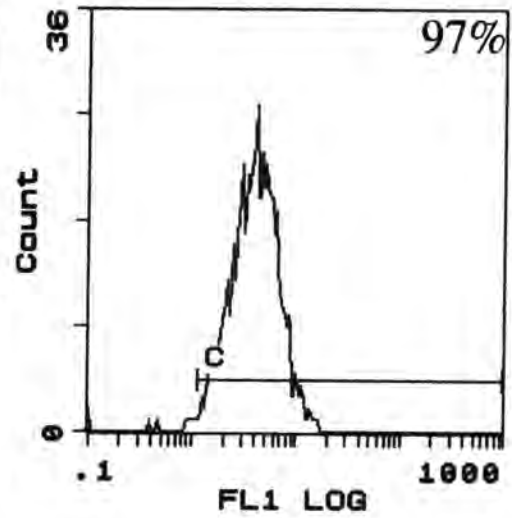
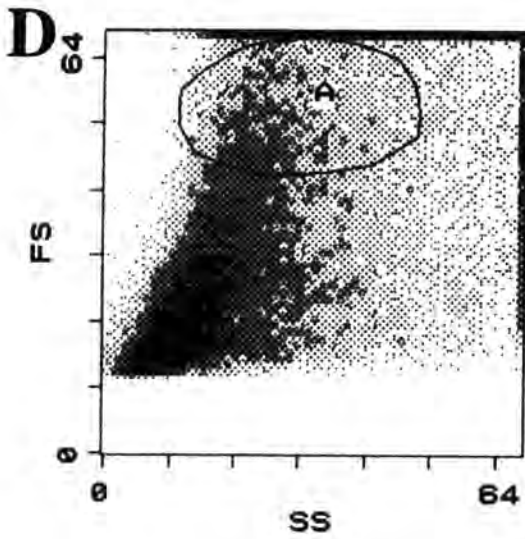
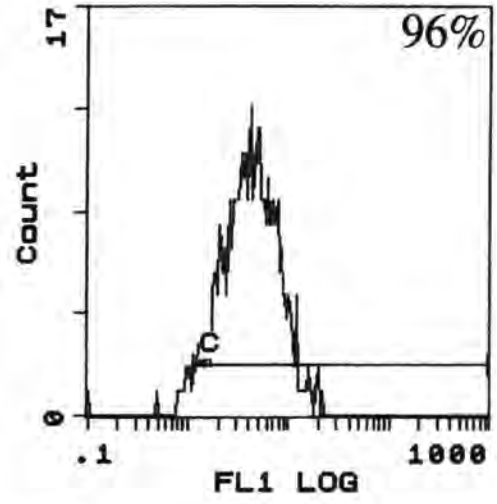
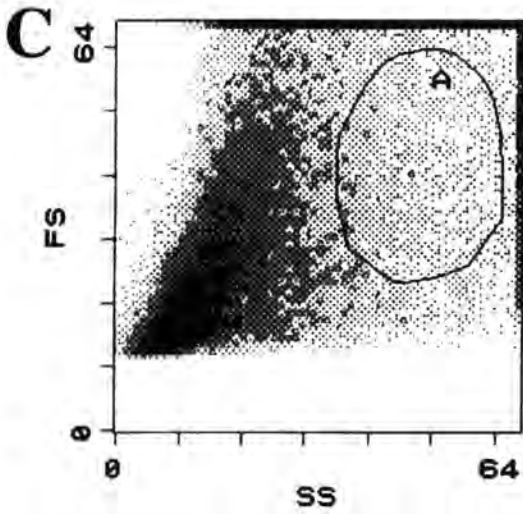


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Figs. 3.26C-D) Continued from previous page

Like the bulb epidermal cells, MHC-I was expressed more within the highly granular (C) and large (D) UF epidermal cells. The percentage of positive-stained cells for each group of cells is shown in the right corner of each individual graph. FS= cell size and SS= cell granularity.



3.4. Discussion

3.4.1 Hair follicle epidermal cells and colony-forming ability

The results presented in this study are essentially in agreement with previous investigations that have been performed on the colony-forming ability of hair follicle epidermal cells but with some notable exceptions which have not been reported to date. The first assay that established a relationship between morphological characteristics and clonogenic ability of epidermal cells was performed by Barrandon & Green (1985). They demonstrated that different cell types can be obtained from the epidermis and that these cells display different capabilities as regards colony formation in culture. The same authors later showed that the cells capable of forming colonies produce three types of colony (Barrandon & Green, 1987). These are classified as paraclones which contain cells with a short replicative lifespan, after which they uniformly abort and terminally differentiate; meroclones, whose cells grow for some time but then they also differentiate; and holoclones (long-lived colonies) which have a perimeter that is nearly circular (evidence of colony growing) and contain mainly small cells concentrated near the perimeter (Barrandon & Green, 1987).

The hair follicle is mainly composed of epidermal cells which are situated all through the length of the follicle. The colony forming ability of epidermal cells from different regions of the follicle has been investigated *in vitro* by a number of workers (Kobayashi *et al.*, 1993; Yang *et al.*, 1993; Rochat *et al.*, 1994; Moll, 1995 & 1996). It has been generally shown that cells residing in the upper part of follicles have a better ability to form colonies *in vitro* than bulb derived cells. Moll (1996) reported that of a total of 34 explants from the upper central ORS of human follicles 13 explants produced cell outgrowth, whereas none of 34 bulb explants displayed such outgrowth. One of the most comprehensive studies on the clonogenic ability of rat vibrissa follicle was carried out by Kobayashi *et al.* (1993). After culturing epidermal cells from different regions of the follicle on feeder layers they reported that epidermal colony-forming cells are highly clustered in the cultures which derive from ORS that includes the bulge region. According to this report about 95% of the total colonies formed in the culture were from the bulge region and fewer than 4% were located in the bulb area (Kobayashi *et al.*,

1993). They showed that colonies from the bulge region mainly contained small cells which were highly proliferative, and that cells could be passaged several times with no apparent sign of senescence. However, the bulb colonies appeared to have a more differentiated appearance than colonies from the bulge region, as large squame-like cells were observed within the colonies. Nevertheless, they stated that the bulb cells could give rise to a few progressively growing colonies, which could be passaged several times (Kobayashi *et al.*, 1993). My results in general are in line with this study as I have shown that the number of colonies established from UF cells was three times more than from the bulb, and that cells from both regions can be subcultured several times. However, there are some findings which differ from those of Kobayashi *et al.* (1993). First, in my work the average proportion of colony forming cells of the bulb to the upper follicle was 1/3 compared to 1/19 for Kobayashi *et al.* (1993). This discrepancy can be explained by differences in i) the number of cells which were seeded, ii) the culture medium that was used, and most importantly iii) not using feeder layers in the present study. Secondly, microscopically I did not observe any consistent or specific differences between the long-lived colonies from the two regions in terms of their morphological appearance- both colony types basically consisted of small but not squame-like cells. In fact in some occasions I noticed that the process of stratification and the appearance of dead material in the central region of colonies started later in bulb derived colonies compared with counterparts from the upper follicle. However, the most important observation made in this study which was not examined by Kobayashi *et al.* (1993) was the growth rate of colonies. I have evidence that the bulb colonies grow slower than the UF colonies and this slow growth was consistent throughout the period that cells were growing in culture. Although I have not quantified the proportion of proliferating cells in the different colonies, immunostaining of colonies with a monoclonal antibody against BrdU sometimes showed a lower level of cell proliferation in the bulb colonies again to some extent supporting the observation that the bulb colonies were growing slower than the UF colonies. Indeed, a proper quantitative study of cell proliferation would be an important part of any future study. In this study I was able to maintain the bulb cells in a proliferating state for more than five months which showed they have the ability to divide for a long period. This was also demonstrated by Kobayashi *et al.* (1993) who stated that bulb cells can be passaged several times.

Therefore, my observations clarified two points. First, it was confirmed that there are cell types with different replicative and differentiative abilities in the bulb, as different colony-types formed in the bulb culture, and secondly I showed that although the vast majority of these cells have a low potential for colony formation, within these cells there is a small population of cells which show a high capacity for colony formation. It is possible that cells with low growth potential are the cells that have originated from the upper part of the matrix while the small population cell with high colony forming capacity are the cells that derive from the GE region in the lowest part of the bulb. In fact the latter, are the cells that have always been missed in studies that have used plucked fibres for culture of epidermal cells (e.g. Wells & Sieber, 1985; Limat & Noser, 1986; Jones *et al.*, 1988; Moll, 1995 & 1996). Although in only one single case cell growth has been seen around the bulb region of plucked fibres (Jones *et al.*, 1988) it has been repeatedly stated that the bulb cells have low or no ability to grow in culture (Moll, 1995 & 1996). However, in every study where lower bulb cells have been present in culture and under suitable conditions, these cells have been shown to be able to grow and produce colonies (Reynolds & Jahoda, 1991b; Detmar *et al.*, 1993; Rochat *et al.*, 1994). Consistent with the concept of using the whole human follicle, Rochat *et al.* (1994) reported that although the number of colony-forming cells in the bulb is low, their growth potential is similar to that of colonies from a region of the upper follicle that shows extensive proliferative capacity. Moreover, when human matrix cells were isolated by microdissection of the hair bulbs and cultivated in serum-free medium on collagen-coated dishes they displayed a similar morphology to ORS cells and interfollicular keratinocytes (see Detmar *et al.*, 1993). Furthermore, the clonogenic bulb cells have been able to form a pluristratified epithelium when grafted onto athymic mice (Rochat *et al.*, 1994). Based on the results presented here, along with those mentioned above I believe that the small population of long-lived colonies in the bulb are the GE cells which, since they showed a long lifespan with continuous proliferation, are unlikely to be TA cells. Even considered as TA cells they should be very unusual TA cells as they showed immense capacity for division, compared with an approximate 15 division for TA skin cells in culture dishes (Barrandon & Green, 1987). In fact the observation that they grew slower than the UF colonies suggests that they might be candidates for follicle stem cells, a point that will be dealt with later. However, as I to some extent implied in

chapter 2, because it is believed that stem cells are present permanently in the follicle, the idea that stem cells are located in the bulb region raises the question as to what happens to the GE cells during catagen and telogen. One possibility that has been put forward is the GE cells may migrate upward and stay above the papilla throughout these stages after which they come down and contribute in initiation of the next anagen. This suggestion is supported by the clonogenic ability of epidermal cells which are situated above the bulb at different stage of the follicle cycle. In the rat vibrissa follicle, the anagen cells in this region only produce very few colonies in culture, whereas at the late catagen this region produces more colonies (Kobayashi *et al.*, 1993). This cellular mobilisation can be viewed as a way of protecting and preserving GE cells from any possible damage which threatens them in the bulb during the regression stage.

The rate of a cell's division has been interpreted as an indication of the stem-like nature of cells. In this context one of the common elements that characterise stem cells is that they are slow-dividing and have a long cell cycle time *in vivo*. However, when placed in culture, these cells are believed to be actively cycling and clonogenic (Potten & Morris, 1988). The bulge hypothesis holds that hair follicle stem cells are located in the bulge area of the follicle because: first, a population of slow cycling cells has been localised in the bulge, when mouse follicular cells have been labelled with [³H]TdR *in vivo* (Cotsarelis *et al.*, 1990) and second, most of the colony-forming cells in the human hair and rat vibrissa follicle have been situated in the bulge or neighbouring areas (Rochat *et al.*, 1994; Kobayashi *et al.*, 1993). However, there is a contradiction between these two points because "rapidly proliferating" is one of the characters that has been attributed to colony-forming cells in culture. Hence, there is confusion here as it has not been fully explained why a change of behaviour should suddenly happen to cells *in vitro*. If slow-cycling is an intrinsic property of stem cells why then do these cells not maintain this property or a tendency towards this behaviour in culture? In the present study, by measuring the colony size at regular intervals, evidence was produced that the long-lived cells in the bulb region grow more slowly than upper colony-forming cells. This suggests that the clonogenic and slow-cycling cells are also present *in vitro*, specifically in the follicle bulb.

According to the bulge hypothesis the stem cells located in the bulge region divide only when they are activated by the dermal papilla (see Lavker *et al.*, 1993) and

the question remains unanswered as to why these cells show this high proliferative activity in the absence of a dermal papilla *in vitro*? In contrast, it has been shown that the bulb germinative epidermal cells both *in vivo* and *in vitro* need to be in close association with dermal cells to enable them to divide (Reynolds & Jahoda, 1991b). The fact that these germinative cells do not grow in isolation in culture could possibly be due to the fact that they are not placed in the special niche or environment which they need. Therefore the lack of this proliferative activity is unlikely to be a result of a deficiency in their growth potential but is possibly due to inappropriate conditions. In support of these concepts Rochat *et al.* (1994) showed that in human follicles when cells are cultivated either on culture dishes previously coated with collagen or on uncoated dishes, the number of colony forming cells in the of the bulb region in coated dishes is seven times greater than uncoated dishes. In contrast, they demonstrated that no difference is observed in the number of colonies initiated from cells from other parts of the follicle. The data not only strongly suggests that cells in the bulb region need additional requirements to initiate a colony in culture but also implies that the germinative cells are possibly not being provided with the required conditions to maintain their growth in culture.

One of the features of epidermal cells is that their size is closely related to their state of differentiation (Barrandon & Green, 1985). This is believed to be true not only for epidermal cells *in vivo*, but also for those which are growing *in vitro*. It was shown here that in colonies in which growth was ceasing, the majority of cells were large and flat implying that they were undergoing terminal differentiation. This was confirmed by the fact that no cell division was observed in these cells. However, cells within the slow growing colonies of the bulb, like some colonies from UF, were small in size and were tightly packed with cell division still clearly taking place. The small size of long-lived cells of the bulb suggested that, in terms of differentiation, these cells were in the very early stages. This point can be better evaluated by examination of these cells with specific differentiation markers such as filaggrin, involucrin, keratins and integrins.

In case of interfollicular epidermal cells it has been suggested that there is a relationship between adhesiveness of a cell to the basement membrane and its differentiative state. In simple terms, as the epidermal stem cells or cells with a high colony-forming efficiency lose their adhesiveness to the proteins in the basement

membrane they become committed to terminal differentiation (Watt, 1987; Adams & Watt, 1990). One component of the basement membrane, fibronectin, seems to play a vital role in inhibiting the differentiation process. Adhesion of cells to the basement membrane proteins is mediated by a family of surface receptors known as integrins, heterodimeric glycoproteins comprising an α and a β subunit (see Jones, 1997). There are reports suggesting that epidermal cells having a high level of integrin $\beta 1$ adhere more rapidly to culture dishes coated with fibronectin and possess a high colony-forming efficiency (Jones & Watt, 1993; Jones *et al.*, 1995; see also Jones, 1996). Cells that adhere to the substrate slowly have been shown to have a lower level of integrin $\beta 1$ and a low colony-forming capacity, so that in culture they undergo a limited number of divisions prior to terminally differentiating. During suspension induced differentiation of human keratinocytes, transcription is inhibited for the genes encoding the fibronectin receptors, $\alpha 5 \beta 1$ integrin, and concomitantly, the receptors lose their ability to bind fibronectin and subsequently are lost from the cell surface (see Fuchs, 1993). Therefore, it appears that loss of fibronectin attachment enhances the differentiation process of cells.

In this study no attempt has been made to grow cells on the basement membrane proteins, but when cells were grown on plastic for a short time before washing, it was confirmed that bulb cells attached to the substrate much later than upper follicle cells. Hence, based on the above idea because the bulb cells attached later than the upper cells to the substratum they would be expected to have a low colony forming capacity. However, in the present study it was shown that, although most of these slow-adhering cells have a low potential for division, some still have the capacity to form long-lived and large colonies, which can be subcultured. Because the expression of integrins on the cell surface was not measured in this study, the relation between adhesiveness and integrin expression remains undefined. Nevertheless, the present experiments raise the question as to whether the difference in speed of adhesiveness to the substrate could be used to determine the colony-forming efficiency of cells, at least in this model.

In summary, I have shown that a small population of the bulb epidermal cells has the ability to produce colonies which grow for long time without displaying a sign of senescence. Although, these colonies, like the UF colonies, were able to be subcultured several times they grew at a slower rate compared with their counterparts in the upper

follicle. These results support the idea that GE cells are unlikely to be TA cells with limited proliferative potential, but emphasise the significance of GE cells as a possible candidate for follicular stem cells

3.4.2 Pluripotency of epidermal cells

During embryogenesis pluripotent epithelial cells differentiate into different cell types which form the epidermis and various epidermal appendages. Moreover, due to the fact that also during post natal life the epidermis and its appendages are involved in continued renewal, as mentioned in the first chapter, it is believed that the epidermis and its appendages must contain a population of stem cells to replace the lost cells. Based on different studies including [³H]-TdR label and clonal analysis (Lavker & Sun, 1982; Morris *et al.*, 1985), it has been suggested, that in epidermis, stem cells are preferentially distributed in the basal layer of deep papilla ridges. The location of these cells in the hair follicle, as discussed earlier, still is a matter of debate.

Apart from the location of stem cells, a question still arises to whether the stem cell of follicular or interfollicular epidermis is committed to the production of only one cell lineage during adult life or whether, as with their embryonic progenitors, they are still pluripotent and consequently have equivalent potentiality. There are many reports that support the idea that adult epidermal cells possibly are pluripotent. In rat vibrissa it has been shown that adult epidermal keratinocytes from a glabrous site are able to differentiate into follicular epidermis when recombined with the follicular papilla (Reynolds & Jahoda, 1992). With regard to using cultured cells, there are reports that cultured keratinocytes have the ability to differentiate into hair follicular keratinocytes. For example, recently Ferraris *et al.* (1997a) demonstrated this potential in human cells. After recombining human cultured keratinocytes with rabbit embryonic dermis and implanting onto nude mouse they showed that human keratinocytes are found in the induced hair follicle structures. On the other hand, cultured follicular keratinocytes have also been shown to be able to regenerate a fully differentiated epidermis *in vitro* (Limat *et al.*, 1995). In line with these experiments and in a further step, in this study I provided evidence that cultured follicular keratinocytes might have the ability to differentiate into sebocytes in culture. This conclusion came from observations of vesiculated cells within the follicular epidermal cells, since positive staining of these cells suggested that these

vesiculated cells could be sebocytes which are normally seen in sebaceous gland. Because lipid-bearing cells were found only in the UF cultures and were absent in the bulb region colonies, it may be argued that these cells were present in the primary explants along with the epidermal cells. However this is unlikely to be the case as care was taken to ensure that the sebaceous gland cells were excluded during the follicle dissection. Moreover, it has been shown that like keratinocytes, isolated sebocytes also form colonies in culture (Xia *et al.*, 1989), hence if lipid-bearing cells were present in the explants you would expect to see colonies of both cell types and not single sebocytes within the epidermal cells. Therefore, there is a possibility that these cells originated and differentiated from the epidermal cells. These results would support the idea that even in maturity the epidermal cells possibly maintain a degree of pluripotentiality. Although at present I cannot explain why the bulb cultures did not show the sebocyte-like cells, the presence of these cells only in the UF cultures could be interpreted as the UF cells having more potency than bulb epidermal cells. Consequently, due to the fact that pluripotency is one indication of stem cells, it implies that the cells in upper part of the follicle are more likely stem cells. Nevertheless, if one were to differentiate stem cells from non-stem cells based on this idea the bulb epidermal cells could be the first candidates, since it is well known that the bulb epidermal cells during anagen have the potential to produce at least 6 differentiated progenies including hair shaft and root sheath cells (see Reynolds & Jahoda, 1993). In general, I believe that this observation supports the idea that the bulge region cells should be regarded as a population of stem cells which function in the epidermis and the epidermal structures located in its proximity, including sebaceous gland and the upper part of the hair follicle. In support of this hypothesis it has been shown that in the case of skin injury, follicular cells migrate upward to cover the wound, suggesting that they can serve as a source for epidermis (Lenoir *et al.*, 1988).

3.4.3 Hair follicle epidermal cells and MHC-I

In this study it has been shown that cultured epidermal cells from UF and the bulb regions of the vibrissa follicle express a similar level of the MHC class I antigen. This result is interesting in that a previous investigation demonstrated a low expression of the antigen in the bulb region cells (Limat *et al.*, 1994a). At first sight this difference

might be a culture effect as there is indication that cells show different level of MHC expression during growing *in vitro*. However, Limat *et al.* (1994a) employed the plucked fibre as a model, and as mentioned previously, this does not represent all of the bulb epidermal cells. Using the whole follicle, which included the lowest germinative cells, may account for the difference in findings. Moreover, this result is not in agreement with previous *in vivo* studies (Westgate *et al.*, 1991) wherein it has been shown that MHC class I is not expressed in the bulb region of the human hair follicle. A possible explanation is that the human hair follicle is different from the rat vibrissa follicle to some degree. According to Westgate *et al.* (1991), a lower level of this antigen in the lower region of the follicle (including the bulb) provides a basis by which macrophages recognise and destroy this region in catagen. Destruction of the lower region of follicle in turn leads to regression and retraction of the follicle. However, Paus *et al.* (1994) suggested that the lower level of MHC-1 expression may serve to sequester potentially damaging autoantigens from immune recognition. Hence due to these contradictory speculations about the relationship between MHC class I and immune-privilege, at the present time it is difficult to discuss or offer an explanation as to the role of this antigen in the hair follicle. Moreover, according to the results presented here the MHC-I is expressed more in large and granular cells rather than small and less granular cells. Since in keratinocytes cell size determines the differentiative status of the cell these results suggest that this antigen is expressed in more differentiated cells, however, due to lack of literature the exact relationship between expression of MHC-I and the differentiative status of cell remains to be clarified.

Chapter 4

Dermal-epidermal interactions in adult skin appendages

4.1 Introduction

4.1.1 Properties and interactive abilities of skin appendages dermal cells

All skin appendages are essentially composed of two cell types, dermal and epidermal. In addition to the critical role played by dermal cells in embryonic appendage development the dermal component is equally important in maturity, for the maintenance and direction of epidermal cell activities.

4.1.1.1 Dermal - epidermal recombinations

Interactions between adjacent tissues of different ontogenetic origin brought together by morphogenetic cell movements are important determinants during embryonic development. These interactions happen as precisely timed, bidirectional communications which cue the orderly, sequential development of adjacent tissues (Briggaman, 1982). It is well known that these interactions occur during the development of many organs such as the tooth, kidney, gut, lung and thyroid glands (see Sawyer, 1983; Kedinger *et al.*, 1987; Holbrook *et al.*, 1993). In addition, during embryonic development of skin these interactions lead to the transformation of embryonic mesenchyme and ectoderm into dermis and epidermis respectively. Epithelial-mesenchymal interactions have been widely investigated in relation to the development of skin (Billingham and Silver, 1963; Kollar, 1972; Sengel, 1976, 1986; Wessells, 1962, 1977; Sawyer, 1983; see also Mackenzie, 1994) and its appendages, including hair follicles (Lapiere, 1989; Holbrook *et al.*, 1993; Kaplan & Holbrook, 1994; Bitgood & McMahon, 1995; Paus *et al.*, 1997a), feather follicles (Dhouailly, 1975; Sengel, 1976; Dhouailly, 1977; Chuong *et al.*, 1996; Ting-Berreth & Chuong, 1996a,b), and mammary glands (Sakakura, 1983), in which the vital role of these interactions has been documented. In addition to the importance of these interactions during embryonic development, there is also data that suggests that these interactions are essential for regular growth and differentiation of epithelium in the adult organism (see Fusenig,

1994). Studies performed on cultured cells have revealed the crucial role of mesenchymal influences on the normal growth and differentiation of the epithelium (Limat *et al.*, 1991 & 1993; Smola *et al.*, 1993; see Fusenig, 1994) as well as the expression of basement membrane components (Marinkovich *et al.*, 1993) *in vitro*. These studies suggest that there are signalling factors which act between the epithelium and mesenchyme and regulate their homeostasis.

The study of these interactions, and their direct effects *in vivo* during the course of morphogenesis has been found to be difficult, because there are so many variables involved and experimental conditions cannot be easily regulated. Hence, to obtain a better understanding of the nature of these interactions, recombination techniques were established and developed (Rawles, 1963; Kollar, 1970; Dhouailly, 1975 & 1977; Sengel, 1976). In these experiments the developing dermal and epidermal tissues are detached from one another and recombined with their counterparts from heterotopic, heterochronic or heterospecific sources. The recombinations are then cultured *in vitro*, or on the chorioallantoic membrane or other suitable host environment. Such recombination experiments have not only revealed the chronology and cascade of events during the development of skin appendages, but they have also ascertained the role of each tissue in such events.

Using heterospecific recombinations (from animals with different zoological-class origins) between embryonic mouse and chick tissues it was first demonstrated that the species specificity of skin appendages resides in the epidermis (Coulombre & Coulombre, 1971). When mouse epidermis was recombined with chick dermis it was shown that mouse epidermis always differentiates towards hair follicle formation (Kollar, 1970). Similarly, in recombinations of chick epidermis with mouse dermis, feather follicles were developed (see Sengel, 1976). These experiments ascertained that the specific nature of an appendage is dependent on the epidermis- mouse epidermis cannot make anything else than hair, similarly chick epidermis cannot form structures other than feathers, or in some areas scales, irrespective of the specific origin of the dermis (Sengel, 1976). Moreover, in these studies it appeared that the dermis does not play a determinant role in development of skin appendages. However, it was found that a dermal influence is indispensable for this process. Dermis from the glabrous area of mouse or chick was unable to induce any appendage formation when recombined with

epidermis (Dhouailly, 1973), and this demonstrated that although the production of hair by mouse epidermis, or of feather by chick epidermis, is an intrinsic property of the epidermal tissue (Sengel, 1976), appendages can be only formed if the epidermis is induced by dermis.

In the recombinations described above, although the process of skin appendage (feather or hair) development began, it never proceeded to the completion of either feather or hair follicle morphogenesis. This suggested that although the development of a skin appendage in the epidermis can be initiated under a non-class-specific dermal message, its continuation is dependent on more specific dermal information (Sengel, 1976). Moreover, these experiments confirmed that the dermis not only initiates the development of skin appendages but that continuous interactions with the epidermis are crucial to achieve a functional appendage. Furthermore, using homospecific heterotopic recombinations, (from the same species but different regions of developing dermis and epidermis) in chick it has been established that the dermis also determines the morphology and pattern of skin appendages (see Sengel 1976). For example, in reptiles the pattern of scales of the dorsal region differs from that seen on the ventral area. When epidermis from the dorsal region is grafted onto dermis of the ventral region this epidermis will develop ventral-type scales (Dhouailly, 1975) and this rule is true for all appendages in birds and mammals. Therefore, whatever type of appendage is produced, the distribution pattern is, in most combinations, strictly controlled by the dermis.

In contrast to embryonic epidermal-dermal interactions, which have been widely investigated, little is known about the nature of these interactions during adult life. Nevertheless, there is evidence that suggests that the dermis continues to strongly influence the fate of the epidermis even in adulthood. This evidence has been derived mostly from recombinations involving vibrissa hair and feather follicles, both of which have proven to be excellent systems for investigating adult interactions. In these systems the dermal component consists of two cell populations, the dermal papilla at the follicle base and a sheath which surrounds the epidermal component along the length of the follicle. The important role of the dermal papilla during follicle morphogenesis, and its vital relationship with other follicular components has been clearly documented (see Jahoda & Oliver, 1990). While investigating the role of the dermal component of the feather follicle in adult life, Lillie and Wang (1941, 1943 & 1944) transplanted chick

feather papillae, to empty follicles of a different region (with a different feather pattern) where they became resurfaced by host follicle epidermis. It was found that this combination resulted in the induction of feather growth in the implanted follicles. Because in this experiment the induced feathers were always of the host type, it was concluded that the specificity of this appendage is determined by the epidermal component of the follicle. Cohen (1961 & 1964) reported similar findings with vibrissa dermal papilla grafts in rats. When vibrissa papillae were implanted into ear skin, ear-type hair follicles were induced in the implanted sites. He suggested that the vibrissa papilla is a non-specific inductor as when in contact with ear epidermal cells it induced the formation of hair follicles. However, he challenged the validity of the idea that appendage specificity is in the epidermis, arguing that it is more likely to reside in the local dermis (surrounding the papilla grafts) instructing the epidermis which type of appendage to produce (Cohen, 1965). Later, Oliver (1966a,b,c, 1967b, 1968), in a series of amputation and implantation experiments using adult rat vibrissa follicles, studied the dermal-epidermal interactions which occur in experimental hair follicle regeneration between the vibrissa dermal papilla and homeotopic and heterotopic epidermis. He first reported the regeneration of the follicle papilla after removal of the dermal papilla alone or after amputation of up to the lower third of the follicle incorporating the papilla (Oliver, 1966a). The same author also showed that isolated rat vibrissa dermal papilla are not only capable of inducing whisker growth in inactivated upper-follicles, but also have the ability to induce hair formation in heterotopic epidermis from ear, scrotal sac, and oral epithelium (Oliver, 1967a, 1968, 1970 & 1973). The isolated rat vibrissa papilla also displayed this ability in combination with embryonic skin (Pisansarakit & Moore, 1986). The area beneath the kidney capsule was used by Kobayashi and Nishimura (1989) as a suitable environment to test the hair follicle regeneration potential of various segments of mouse vibrissa follicles. When transplanted, the upper two-third segments of the follicles were not able to regenerate a bulb, but these segments induced formation of the bulb and hair growth in combinations with microdissected dermal papilla placed at the cut edge of the epidermis.

Following the pioneering work of Jahoda and Oliver (1981) in the cultivation of rat vibrissa dermal papilla cells *in vitro*, several laboratories have reported the successful propagation of dermal papilla cells in culture from rat pelage hairs (Reynolds & Jahoda,

1992), sheep wool (Withers *et al.*, 1986), sheep vibrissa (Pisansarakit *et al.*, 1991), human scalp (Messenger, 1984; Messenger *et al.*, 1996; Warren *et al.*, 1991 & 1992) and beard and pubic hairs (Randall *et al.*, 1991). As with freshly dissected dermal papilla, such inductive abilities have been demonstrated to exist even in cultured vibrissa dermal papilla cells (Jahoda *et al.*, 1984, 1987 & 1993; Horne *et al.*, 1986; Jahoda, 1992; Reynolds & Jahoda, 1992 & 1996; Ferraris *et al.*, 1997b), since the harvested cells induced hair follicles in deactivated follicles or ear wounds. In order to prove that the induced follicles were the result of the implanted cells, the incorporation of implanted cells into the dermal papillae of the induced follicles has been demonstrated by autoradiographic detection of [³H]-thymidine labelled dermal papilla cells (Jahoda *et al.*, 1984). The basic conclusions from these findings are that i) as in embryonic development, the dermis also has a profound influence on epidermal growth and differentiation in adult life, ii) the dermal component of the hair follicle (dermal papilla and lower DS) retains embryonic properties and can induce follicle morphogenesis and fibre growth, iii) these components also specify the type of follicle and thus the fibre type that develops, and iv) finally, they have the ability to interact even with heterotopic epidermis.

Despite these interesting findings there are still many unanswered questions within the context of dermal-epidermal interactions in adult skin. Hence, in this chapter, while investigating some general aspects of the activities of skin appendage dermal cells, I also directed experiments to answer three questions in relation to adult dermal-epidermal interactions. The first being whether the inductive ability of dermal cells in the hair follicle is restricted only to cultured papilla cells or whether dermal sheath cells also have the same property? Secondly, to investigate if hair follicle papilla cells (as whole papilla or cultured cells) show the same inductive abilities in a different location such as back skin? And thirdly to ascertain if papilla cells from other adult skin appendages retain the same inductive capabilities as hair follicular papilla cells? These questions will be expanded where they are discussed separately.

4.1.1.2 Properties and interactive abilities of hair follicular dermal sheath

The hair follicle, with the exception of its neck region, is surrounded by a mesenchymal sheath, termed the dermal sheath (DS) or connective tissue sheath, which

is continuous with dermal papilla at the follicle base (Melaragno & Montagna, 1953). During ontogenetic development of the hair follicle the dermal sheath arises from the population of mesenchymal cells that are deposited along the length of the down-growing hair peg (Holbrook *et al.*, 1989; Holbrook & Minami, 1991). Unlike the dermal papilla which is perhaps the most investigated group of follicular-derived cells, dermal sheath cells have been the subject of far fewer investigations. Nevertheless, the few experiments which have been carried out on these cells, suggest that they do have interesting capabilities which makes them a good target for future investigations. Oliver (1966a) showed that in the rat vibrissa follicle when the dermal papilla alone was removed, or the follicle end bulb containing the epidermal matrix and dermal papilla was surgically amputated, a new dermal papilla regenerated. In both cases, the new papilla was considered to have been formed by the dermal sheath cells. Surgical removal of the bulb with more than one-third of the lower vibrissa follicle has been shown to lead to permanent cessation of hair growth due to the inability of the tissue to regenerate a new dermal papilla (Oliver, 1966b). Nevertheless, when follicles inactivated in this manner are stuffed with intact dermal papillae, cultured dermal papilla cells; or tissues from dermal sheath derived from the lower third of follicle, a new dermal papilla is formed and hair production is restarted (Oliver, 1967b, 1980; Ibrahim & Wright, 1977; Jahoda *et al.*, 1984; Horne & Jahoda, 1992). Moreover, Oliver (1967a) reported that when lengths of the lower third of the vibrissa follicle (comprising epidermal and dermal sheath cells) were transplanted into the ear skin they regenerated new bulbs. These results all demonstrated that, like the dermal papilla, the follicular dermal sheath also has some capacity to induce hair growth. On the other hand it has been established that unlike cultured dermal papilla cells which are competent to induce hair growth in follicular and non-follicular sites, cultured dermal sheath cells lack hair-inductive potential (Horne *et al.*, 1986; Reynolds, 1989; Horne & Jahoda, 1992). However, recently, Reynolds and Jahoda (1996) utilising the rat vibrissa follicle showed that dermal sheath cells derived from the lower part of follicles in association with GE cells are able to induce follicle formation, an ability which was not observed when GE cells were combined with skin fibroblasts.

Based on this body of evidence it has been suggested that dermal sheath cells may represent a kind of stem cells for the follicular dermis (Oliver, 1991) and they may

act in the normal course of events to maintain follicle size. According to this suggestion in human vellus follicles to terminal transformation at puberty, dermal sheath cells could be possibly the source of the new large papilla, and that papilla cells are lost to the sheath to reduce papilla size during androgenetic alopecia (Jahoda & Reynolds, 1993). However, with respect to available evidence at the present time it is not possible to draw a definite conclusion about the possibility that these cells have stem-like abilities and verification of this requires much more investigation.

In the first section of this chapter follicular dermal sheath cells along with other dermal cell types were dissected and cultured to further elucidate their properties. In culture, the morphology and behaviour of these cells were investigated and compared with other follicular or non-follicular dermal cells. The cultured cells were later used for further investigations as follows.

i) Dermal sheath and expression of α -smooth muscle actin and ECM components

Actin is a cytoskeletal protein found in all eukaryotic species. This protein is the most abundant protein in many eukaryotic cells, often constituting 5% or more of the total cell protein (Alberts *et al.*, 1994). This protein in association with other proteins performs specific functions such as contraction, mechanical support, cell adhesion and cell movement (Ronnov-Jessen & Peterson, 1996, see also Furumura & Ishikawa, 1996). Within eukaryotic tissues there are three actin isoforms (alpha, beta, and gamma) that can be distinguished from each other by virtue of their different isoelectric points, as revealed by two-dimensional gel electrophoresis (see Reynolds *et al.*, 1993b). While among these, only beta-actin is ubiquitously expressed by all eukaryotic cells, alpha-cardiac, alpha-skeletal, and alpha-smooth muscle actin (ASMA) have been considered tissue specific (Garrels & Gibson, 1976; Vandekerckhove & Weber, 1978 & 1979). However, it has been realised that ASMA is also ectopically expressed in several other cell types (see Ronnov-Jessen & Peterson, 1996). The functional significance of the ectopic expression of ASMA in other cell types has not yet been clarified but a number of observations suggest that it may be involved in cellular locomotion or contraction. For example, ASMA is highly expressed in skin fibroblasts during wound healing which is associated with tissue contraction (Estes *et al.*, 1994).

ASMA is also expressed in normal skin and its appendages. In addition to skin

fibroblasts, follicular dermal cells have been shown to express ASMA, however, the level of the expression differs among different cell types and different regions of the follicle (Skalli *et al.*, 1986; Jahoda *et al.*, 1991; Reynolds *et al.*, 1993b, Sleeman, 1995). In adult rat vibrissa follicles ASMA expression is restricted to the lower half of the DS *in situ* (Jahoda *et al.*, 1991). The lowermost region of the DS, below and around the DP, the DP itself, and the upper half of the follicle, are ASMA negative. Despite being negative *in situ*, when the DP is cultured *in vitro* the cells that grow out express ASMA strongly (Jahoda *et al.*, 1991). Reynolds *et al.* (1993b) in a quantitative study demonstrated that while cultured DS cells from the lower/mid region of rat follicles expressed the highest level of ASMA (98%) among follicle-derived cells, SF cells displayed the lowest level (10%) among all skin-derived cells. In human hair follicles it has been shown that cultured DP and DS cells expressed ASMA but this protein has not been observed in cultured SF (Chiu *et al.*, 1996). In a part of this study I compared the expression of ASMA in hair follicle dermal sheath cells from the lower and upper part of rat vibrissa follicles in order to establish a possible role for this cytoskeletal component in the follicle.

On the other hand it is well documented that the hair follicle during its cycle is involved in continuous dermal-epidermal interactions that occur across the follicular basement membrane. As mentioned in the first chapter, basement membranes are assemblies of ECM components which include laminin isoforms, different types of collagen, fibronectin and proteoglycans (see Marinkovich *et al.*, 1993; Yamane *et al.*, 1996). It is known that in humans and rodents, follicle dermal papillae express the basement membrane components within their ECM *in vivo* and *in vitro* (Couchman *et al.*, 1979 & 1990; Westgate *et al.*, 1984; Couchman & Gibson, 1985; Couchman, 1986 & 1993; Katsuoka *et al.*, 1988, Messenger *et al.*, 1991a,b; Jahoda *et al.*, 1992b). These results suggest that the papilla possibly contributes to the formation of the basement membrane between the papilla and the outer epithelial matrix. There is also evidence that follicular dermal sheath cells have an ECM, which contains basement membrane elements. Expression of basement membrane proteoglycans has been shown in the dermal sheath of rat pelage hair follicles *in vivo* (Couchman *et al.*, 1990; Couchman, 1993). Evidence from the human dermal papilla shows that type IV collagen and laminin, two major basement membrane components are present at high levels in the

papilla throughout the hair cycle *in vivo*. The papilla cells have been found to produce type I and type III collagen and fibronectin *in vitro* but no type IV collagen (see Almond-Roesler *et al.*, 1997). The question arises consequently as to whether dermal sheath cells are able to produce these components *in vitro*? Therefore, in this part of the study I investigated the expression of three ECM elements in cultured dermal sheath cells.

ii) Dermal sheath in wound sites

As previously mentioned (section 4.1.1.1) there is much evidence that demonstrates that cultured rat vibrissa papilla cells are able to induce type-specific follicles in heterotopic sites (ear skin and foot pad) while this ability is not observed in DS cells (Reynolds & Jahoda, 1996). However, rat vibrissa DS cells also induced hair follicle formation in ear skin when they were associated with GE cells (Reynolds & Jahoda, 1996). In a part of this study in addition to dermal sheath cells other dermal cell types were implanted into ear wounds or dorsal skin biopsy punch sites to follow their subsequent fate and distribution.

iii) Dermal sheath in recombinations with epidermal cells

In chapter 3, I explained that the use of cultured skin substitutes (first suggested by Green *et al.*, 1979) in burn care and reconstructive surgery has attracted increasing interest. For this purpose one method has been to apply sheets of cultured keratinocytes onto wound sites and this was initially reported in 1981 by O'Connor and colleagues who used these sheets for the treatment of burn wounds. This method was also later used by several other researchers (e.g. Gallico *et al.*, 1984; Kurata *et al.*, 1994). Despite some success, however, grafting of epidermal sheets has not provided optimum results because several studies have reported that the healed epithelium is fragile and the skin is prone to breakdown and contraction (Kumagai *et al.*, 1988; Herzog *et al.*, 1988; Clugston *et al.*, 1991). It also has been noticed that in this healed skin there is a delay in basement membrane formation (see Hansbrough, 1995), so that full regeneration of wounds may take a few years (Compton, 1989). This delay is due to the fact that in full-thickness wounds the dermis is absent and because dermis regenerates very slowly it takes a long time for the new dermis to become established. As a result, during the first

1-2 years post-grafting, the graft site does not have a supporting dermis to furnish strength and durability to the overlying epidermis as seen in intact skin, so grafts are very fragile and prone to blistering (see Hansbrough, 1995).

For the above reason it appeared that for treatment of full-thickness wounds it is essential to establish a composite graft consisting of dermis as well as epidermis. To produce composite grafts cultured dermal cells have been inoculated into collagen gels (Bell *et al.*, 1979; Shahabeddin *et al.*, 1991) or acellular dermis (Prunieras *et al.*, 1979; Krejci *et al.*, 1991) as dermal analogues, which were then seeded with cultured epidermal cells (Bruke *et al.*, 1981; Bell *et al.*, 1983; Hansbrough *et al.*, 1989). When these composite grafts were implanted into wound sites, since greater physical and chemical support is provided by the underlying dermis, it has been shown that they result in the establishment of functional tissues quicker and more effectively than with the epithelial cells alone (see Hansbrough, 1995). Moreover, there are reports that by using this method basement membrane structures with specific proteins such as laminin and collagen type IV are expressed at the dermal-epidermal junction (Boyce & Hansbrough 1988; Bell *et al.*, 1983; Madden *et al.*, 1986; Bosca *et al.*, 1988; Nanchahal *et al.*, 1989; Mommaas *et al.*, 1992; Cooper *et al.*, 1993; Nolte *et al.*, 1994).

In spite of the considerable clinical achievements that have been gained by implanting these organotypic grafts a basic biological question had risen in this context. Since the grafts have been in direct contact and interact with the host tissues, the regenerated structures cannot be attributed simply to the composite grafts. In other words, if the composite grafts were positioned in a situation where they did not interact with host tissues directly, would they be able to produce a normal skin with dermis, epidermis and most importantly basement membrane, such as has been found in the above-mentioned studies?

In the past 10 years many investigations have been performed to try and answer this question. At least one special device, a transplantation chamber, has been designed for this purpose, to separate the host tissue from the organotypic grafts. There are reports that show that even under these circumstances, the cultured organotypic grafts are able to form normal dermis and epidermis (Rouabhia, 1996; Xu *et al.*, 1996). However, it has been evident that these tissues do not form a normal and complete basement membrane or if one forms its formation takes a long time and that hair follicle

morphogenesis is not observed in these grafts.

In regard to the basement membrane formation there are several reports describing the presence of basement membrane molecules at the dermal-epidermal junction of *in vitro* or *in vivo* reconstructive skin equivalents (Lenoir *et al.*, 1988 & 1993; Medalie *et al.*, 1996). Limat *et al.* (1995) demonstrated that organotypic cultures of human ORS cells with dermal fibroblasts developed a well-differentiated tissue resembling normal skin when grafted onto nude mice. Using immunofluorescence methods some basement membrane components including laminin, collagen type IV and integrins were found to be localised at the dermal-epidermal junction of this tissue. Similarly, in earlier work using histological and immunofluorescence methods (Bouvard *et al.*, 1992) demonstrated the presence of laminin and fibronectin at the dermo-epidermal junction of cultured human skin equivalents, but type IV collagen was absent. Also, Breitreutz *et al.* (1997) established skin-like structures in human organotypic grafts implanted onto nude mice and the grafts expressed basement membrane integrins at the dermal-epidermal junction. Nevertheless, despite this body of evidence, this question as to whether or not the full complement of basement membrane components are present at this junction, remains unanswered.

In the context of hair follicle formation there are a few accounts that show hair follicle formation in organotypic grafts. In a series of experiments Fusenig and colleagues demonstrated that an epithelial cell preparation enriched with hair follicle buds formed complete hair follicles in grafts when combined with fresh dermal cells (Fusenig and Worst, 1975; Fusenig *et al.*, 1980; Worst *et al.*, 1982; Mackenzie & Fusenig, 1983). Subsequent to similar grafting experiments carried out in other laboratories, it has become well-documented that normal haired-skins can be produced, using the transplantation of new-born mouse hair follicles mixed with cultured or uncultured dermal cells directly onto the graft bed of nude mice (Lichti *et al.*, 1993, Weinberg *et al.*, 1993). However, in the context of using adult follicle-derived cells for grafting experiments there is a single report that demonstrates hair follicle morphogenesis in organotypic grafts (Watson *et al.*, 1994). Nevertheless, in this experiment the hair follicle was induced, as result of implantation of whole papillae into the graft and not cultured cells.

It has been shown that the presence of dermis influences and improves the

reconstruction of a normal-like skin in organotypic grafts (Limat *et al.*, 1989, see also Fusenig, 1994). However, it could also be suggested that deficiencies in the formation of a normal basement membrane and the absence of skin appendages in skin equivalents might still be because of the type of dermal cells currently used. In other words, these cells, while functional, might be unable to provide the appropriate stimuli and other conditions for particular interactions. In all experiments of this kind researchers have used cultured skin fibroblasts to make the dermal analogous in organotypic grafts. However, there is evidence that follicular dermal sheath cells while showing some inductive properties like dermal papilla cells (Horne & Jahoda, 1992) share some characteristics with fibroblasts (Reynolds *et al.*, 1993b). Hence, it was considered that dermal sheath might provide a better substrate for the growth of keratinocytes in organotypic grafts. Thus, in this study, in order to investigate this property in follicle dermal sheath cells, instead of skin fibroblasts I have utilised rat vibrissa dermal sheath cells mixed with epidermal cells before culturing or grafting. The organotypic combinations were cultured *in vitro*, and either implanted onto granulation tissue beds in rats or onto the chick chorioallantoic membrane (CAM).

iv) Dermal sheath in recombinations with embryonic cells

Traditionally, the chick CAM has been widely used as a natural environment for growing cells or tissue grafts (e.g. Weiss & Taylor, 1960; Ferraris *et al.*, 1994). This membrane possesses a high degree of vascularisation which make it very suitable for supporting grafts. The CAM is formed at day 6-7 of development but the immune system of the embryo does not develop until day 14-15 of incubation, hence a foreign graft can be grown on this membrane for 7 to 8 days without risk of rejection.

It is well documented that embryonic cells of amniote organs even in advanced stage of differentiation, can be completely dissociated and are able to reorganise and form the same type of organ once again, in appropriate conditions without instructive outside intervention (see Weiss & Taylor, 1960). Dissociated and re-aggregated cells from 8-14 day chick embryo skin gave rise to skin-like structures with feather buds *in vitro* (Weiss & James, 1955), and to feather filaments or feather like structures when grown on the CAM (Weiss & Taylor, 1960). Similar experiments have also been carried out with embryonic mouse skin (Garber & Moscona, 1964; Moscona & Moscona, 1965;

Moscona & Garber, 1968; Garber *et al.*, 1968; Yuspa *et al.*, 1970) where the skin of 13 to 15-day mouse embryos was dissociated and grafted onto CAM. It was found that this graft reconstituted a skin with normal keratinizing epidermis, hair follicles and sebaceous glands. In accord with these results, Ihara *et al.* (1991) showed that dissociated cells obtained by trypsinization of day-15 embryo rat upper lip were able to form hair follicles in culture. In line with these investigations, in a part of this study, by recombining cultured DS cells of adult follicles with dissociated embryonic skin I aimed to find out whether follicular DS cells are able to be involved in the process of the reconstruction of skin and its appendages?

4.1.1.3 Inductive abilities of follicle dermal papilla

The follicular DP is a specialised structure of fibroblastic-like cells situated at the base of the hair follicle and together with the DS, is derived from a condensation of mesenchymal cells seen initially beneath the epidermis at the start of hair follicle morphogenesis (Davidson & Hardy, 1952; Holbrook *et al.*, 1989; Serri & Cerimele, 1990; Holbrook & Minami, 1991). There is now a large body of investigation concerning the properties of the DP and its importance in hair follicles (see Jahoda & Oliver, 1990). It has been established that the DP is a vital component of the follicle for hair growth (Geary, 1952; Chase, 1955) and that it determines many of the physical characteristics of the hair produced (Van Scott & Ekel, 1958; Van Scott *et al.*, 1963). Without the dermal papilla and its intimate contact with surrounding epidermal matrix cells hair growth cannot be sustained (see Nutbrown & Randall, 1995). Moreover, although this has not yet been unequivocally proved, much circumstantial evidence suggests that the DP also plays a significant and central role in follicle cycling (see Stenn *et al.*, 1994b; Yu *et al.*, 1995; Randall, 1996; Paus, 1996). This cycling is closely associated with several changes in the DP itself, in a manner that not only the size of DP changes (Young & Oliver, 1976; Young, 1977 & 1980) but these changes are also reflected in its extracellular matrix (Montagna *et al.*, 1952; Jahoda *et al.*, 1992; duCros *et al.*, 1995; Handjiski *et al.*, 1994; Yu *et al.*, 1995; Messenger *et al.*, 1991a,b), and its vascular system (see Lachgar *et al.*, 1996).

Much of the current knowledge concerning the properties of DP cells has come from dermal-epidermal recombination experiments. These experiments have revealed

that, in addition to its importance in follicle maintenance and cycling the DP displays a high level of inductive ability in terms of stimulating hair growth and follicle formation. An inductive role for the DP in hair growth was first directly documented by implanting whole DPs into the base of rat vibrissa follicles, that had been inactivated by amputation of their lower halves (Oliver, 1967b). Moreover, as mentioned previously the rat vibrissa DP is also able to induce follicle formation in association with epidermis from ear and scrotal sac skin (Oliver, 1970, 1973; Jahoda, 1993). However, because these implanting sites are hairy, the above experiments leave unresolved the question of which epidermal cells (follicular ORS or interfollicular cells) have been induced to form the epithelium of induced follicles? In the current study to further investigate the inductive ability of the DP, I examined the ability of the papilla to induce follicles in ordinary back skin, a site that has not been tried yet. For this purpose small punch biopsy devices were employed to create cavities, into which various cell and extracellular combinations were implanted in such a way that dermal-epidermal interactions could occur.

4.1.1.4 Properties and interactive abilities of dermal cells in other skin appendages

i) Mammalian claw unit

As mentioned previously, recombination experiments involving hair follicle tissues have revealed that cultured dermal papilla cells are not only able to induce dermal-epidermal interactions in association with homeotopic epidermis, but they also show a capacity to interact with heterotopic epidermis (Jahoda & Reynolds, 1993). This property has also been reported in case of adult rat tooth dermal papilla cells which were able to induce the formation of skin appendages in heterotopic ear wound skin (Reynolds, 1989).

Nails in humans or claws in non-primate animals are also skin appendages which develop during the embryonic stages as a result of dermal-epidermal interactions. The structure of claws and their relationship to human nail have been well reviewed by Spearman (1978). Although, unlike the hair follicle, claws and nails grow continuously, with no resting or transitional periods, throughout life there are similarities between these epidermal structures. It has been suggested that the nail or claw unit could be seen as an unfolded form of hair follicle producing a hair with no cortex, just hard cuticle (see Dawber *et al.*, 1994). There is also a considerable amount of comparative biochemical



work that has been undertaken on nail and hair which confirms their common background (Dawber *et al.*, 1994). Moreover, like the hair follicle, the nail or claw unit contains a dermal component which induces the epidermis above it to give rise to the claw plate. To my knowledge as yet there have been no experiments to investigate the inductive properties of the dermal component of the claw unit. Hence, in this study, in addition to examining the properties of claw dermal cells in culture, I aimed to investigate a) whether claw papilla cells have the ability to induce the formation of skin appendages in association with heterotopic epidermis (hair follicle) and b) if so what type of appendage will form?

ii) Bird feather follicles

It has previously been demonstrated that the feather-forming dermis from embryonic chicks is capable of initiating hair development in the developing mammalian epidermis and *vica versa* (see Sengel, 1976). As in the hair follicle it is well documented that the dermal papilla is a vital component of the feather follicle (Lillie & Wang, 1941, 1943 & 1944; Wang, 1943). Wang (1943) reported that the adult feather papilla is also able to induce feather growth in adult life, in a similar manner to that seen with the hair follicle papilla (Oliver, 1967b). It was shown that when a dissected feather papilla was implanted into a heterotopic deactivated feather follicle the implanted papilla induced feather growth in the follicle. The question remains as to whether the adult feather papilla is able to interact with epidermis from a adult hair follicle and induce any appendage growth? Hence, in a part of this study I aimed to clarify this question by the recombination of feather follicle papilla with hair follicle epidermis. However, chick is not an ideal experimental model for such experiments, as it does not have functional feathers for flight. Moreover, feathers in chick do not molt regularly and thus the follicles are not cycling. As a result, the dermal papillae in these follicles are usually in the resting stage and are therefore less likely to have the same level of inductive ability as the papillae of flying birds. Therefore, in this study, pigeon feather follicles were employed to investigate the interactive abilities of the feather papilla cells in association with epidermis from adult mammals.

4.1.2 Organ culture of hair follicles

The hair follicle is a highly complex system, which not only lends itself to *in vivo* studies but it is one of the few mammalian tissues with the ability to grow as an organ culture *in vitro*. The growth of the embryonic or adult hair follicle *in vitro* has been reported by a number of authors in various species like rat (Philpott *et al.*, 1992), mouse (Jindo *et al.*, 1994; Kamiya *et al.*, 1995; Robinson *et al.*, 1997), sheep (Hynd *et al.*, 1992; Hynd & Nancarrow, 1996; Bond *et al.*, 1994; Williams & Stenn, 1994) and human (Philpott *et al.*, 1990 & 1991; Li *et al.*, 1992; Harmon & Nevins, 1994). Historically, the first attempts on hair follicle organ culture were performed on embryonic hair follicles. Murray (1933) demonstrated that vibrissa follicles from rat embryos could produce hair when they are cultured *in vitro*. Later Hardy (1949) cultured skin from the trunk of mouse embryos and hair follicles developed and produced keratinized hairs. However, in further experiments it has been shown that the ability of follicles to sustain prolonged hair growth decreases with both time in culture and with the increasing age of the embryo from which the skin was removed- so that by birth no *in vitro* hair growth can be observed (see Philpott *et al.*, 1996). Due to this fact, it was thought for some time that post-embryonic hair follicles are not able to grow as organ cultures *in vitro*. One of the first successful attempts at growing post-embryonic follicles in culture was performed by Frater and Whitmore (1973) who cultured new-born mouse haired-skin (up to 5-day-old) using rat tail collagen as a substrate. They demonstrated that hair growth occurred over 4-5 days during which the follicles produced approximately 0.2-0.4 mm of fibre, but they reported that hair growth ceased at this point.

The culture of individual microdissected follicles was first attempted on mouse by Uzuka *et al.* (1977) who showed that follicles increased in length over 10 hours in culture. Later, using morphological criteria Frater (1980) demonstrated that rat hair follicles continued hair production for over 48 hours when they were cultured in medium containing serum from young rats. Recently, due to progress in establishing better culture systems, it has been shown that follicles can grow for longer periods of times in culture. Buhl *et al.* (1989) reported that new-born rat vibrissa follicles maintained their growth for 3 days *in vitro* and later Philpott *et al.* (1990) demonstrated using autoradiography that cell proliferation was maintained in human hair follicles for 9-10

days and this was consistent with the synthesis of hair keratins. The same authors later showed that although most follicles stopped growing after 10 days, individual follicles were frequently seen growing for up to 17 days (Philpott *et al.*, 1996). Utilising sponge gel, Li and Hoffman (1991) reported that mouse skin hair follicles grew at rate close to that seen *in vivo* rate, reaching over 3 mm by day 10. Tobin *et al.* (1993) cultured human hair follicles in a serum free medium and using ultrastructural and histological observations showed that follicles continued to be viable in culture for 12 days. One of the most interesting studies in this area was reported very recently by our group (Robinson *et al.*, 1997) who showed that mouse vibrissa follicle can be maintained in culture, with continued hair growth for up to 22 days. In this study the follicles produced fibre at average rate of 0.3 mm/day (similar to growth rates *in vivo*) for 15 days.

As I showed in chapter 2 it is well documented that hair follicles are able to regenerate a new hair after plucking *in vivo*. Despite all attempts at growing follicles *in vitro*, to my knowledge there has, however, been no investigation of the ability of plucked follicles to grow in culture. Hence, in conjunction with earlier experiments on the regenerative ability of the plucked follicle *in vivo*, in this part of the study I examined whether plucked follicle bulbs would grow *in vitro* and if so for how long?

4.2 Materials and Methods

4.2.1 Procedures involving rat skin-derived dermal cells

4.2.1.1 Cell culture

Unless indicated otherwise inbred PVG hooded rats were used either as a source of cultured cells or as recipients of cultured cells, which in case of any operation prevented an immune system-mediated rejection. Initially primary cultures of rat vibrissa follicular dermal cells (dermal papilla, lower and upper dermal sheath) and skin fibroblasts were established. Dissection of the dermal tissues was performed under a low-power (x10) stereomicroscope.

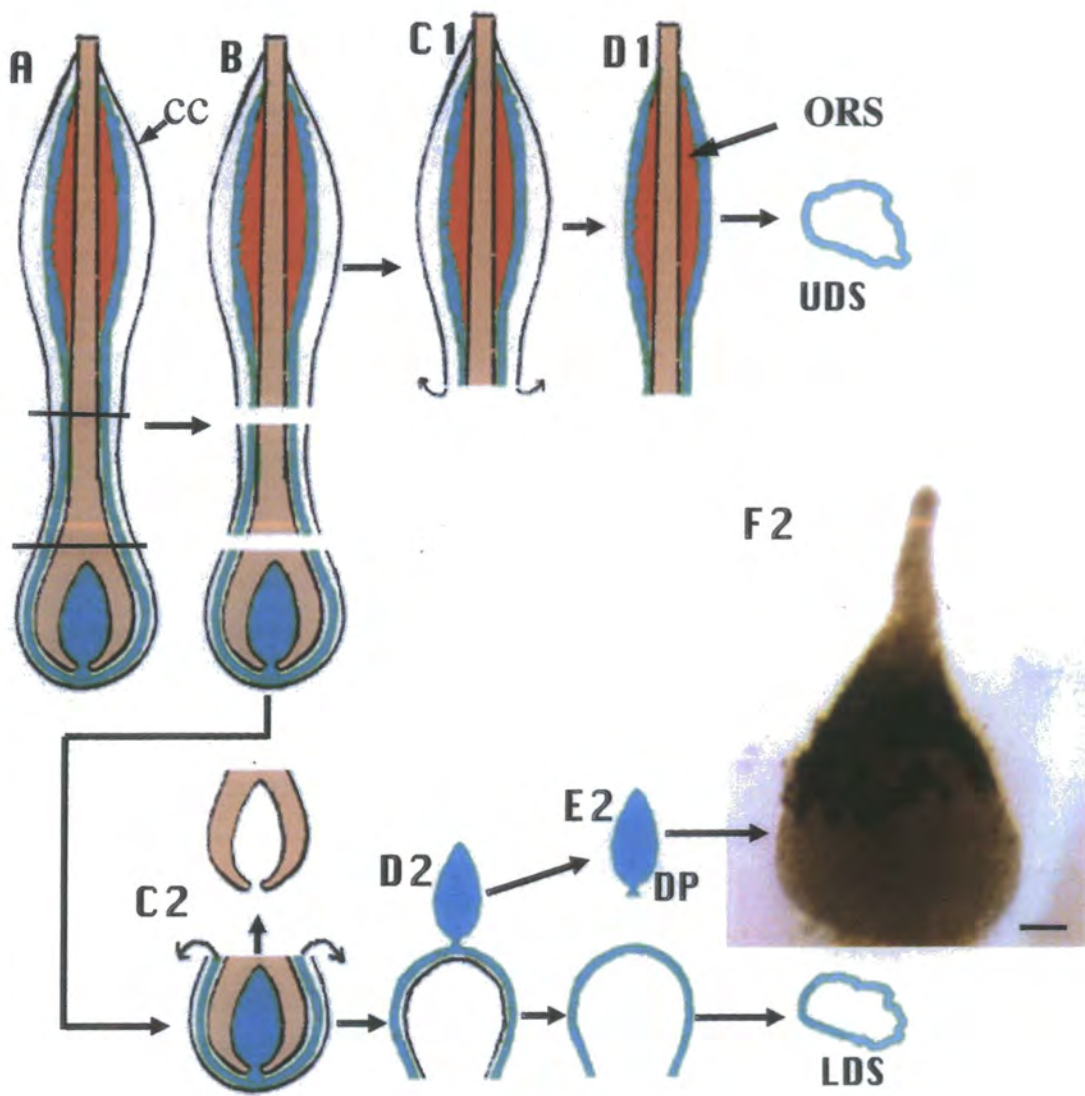
i) Follicle isolation and transection

Vibrissa follicles on the upper lip were dissected and transected into three fragments as described previously in section 3.2.1. The middle fragments were discarded and the lower (bulb) and upper fragments were transferred into separate petridishes containing MEM and antibiotics (section 2.2.3.4). The bulb was used for the isolation of dermal papilla (DP) and lower dermal sheath (LDS) whereas the upper fragment was employed for the dissection of the upper dermal sheath (UDS). A schematic representation for isolation of follicular dermal cells is shown in Fig. 4.1.

ii) Isolation and cultivation of dermal papilla

Follicular dermal papillae were isolated according to the method described by Jahoda and Oliver (1981). In brief, using a syringe needle, parallel cuts were made at the sides of the bulb in the enveloping collagen capsule. A pair of forceps was used to immobilise the bulb while the bottom of the capsule was pushed up to expose the papilla and epidermal matrix. Often the epidermal component automatically detached from the papilla, but if not it was easily removed using fine forceps. The papilla was cleaned of any remaining epidermal tissue and transferred to a culture dish containing 20% growth medium (section 4.2.1.2). Approximately 15 dermal papillae were placed in each dish to initiate the culture.

Fig. 4.1) Diagrams illustrating isolation of dermal tissues from a rat vibrissa follicles. The follicle (A) was transected into three fragments (B). C1-D1) The upper fragment was employed for dissection of the upper dermal sheath (UDS). C2-E2) The lower fragment was used to isolate the lower dermal sheath (LDS) and dermal papilla (DP). F2) Displays a pear-shaped papilla dissected from a pigmented rat vibrissa follicle. The dark strip of material around its mid-region contains melanocytes which have remained attached to papilla during dissection, scale bar 150 μ m. (CC) collagen capsule, (ORS) outer root sheath.



iii) Isolation and cultivation of lower dermal sheath

Lower dermal sheath (LDS) tissue was isolated from the region of vibrissa follicles as described by Reynolds (1989). After inverting the bulb and removing the epidermal matrix and dermal papilla as described above the dermal sheath tissue was the only tissue attached to the inner face of the collagen capsule. It could then be teased from the capsule using sharpened forceps. The isolated lower dermal sheaths were transferred to a culture dish containing growth medium (section 4.2.1.2) for cultivation. In some cases to help the initial tissue attachment to the substratum, these explants were cultured under sterile glass coverslips for one week.

iv) Isolation and cultivation of upper dermal sheath

The upper dermal sheath (UDS) was isolated from the upper follicle fragments as outlined in section 3.2.1. The isolated UDS was subsequently cultured as described above for LDS.

In one case to examine the approximate growth rates of LDS and UDS, both cell types at passage 4 were separately trypsinised, harvested and resuspended in 10% MEM as described above. The cells were counted as outlined in section 3.2.2 and for each cell type 1.92×10^5 cells were inoculated into 35-mm culture dishes, 3 replicate dishes being used for each cell type. After 24 hours the medium was removed and fresh medium added after which the cells were incubated for another 24 hours. At this time the cells were photographed before counting (section 3.2.2).

v) Isolation and cultivation of skin fibroblasts

A small area (5x5-mm) of skin from the dorsal region of the animal was utilised as a source of fibroblasts. External hair fibres were shaved using a scalpel blade before the area was swabbed with 70% ethanol. A patch of clean skin was then dissected out using a scalpel blade and immediately placed into MEM containing antibiotics (section 2.2.3.4). The underlying muscular and fat layers were trimmed using iridectomy scissors and the most superficial layers of the epidermis (stratum corneum) also detached from the skin using fine watchmaker forceps. Using a number 10-scalpel blade the remainder of the skin was chopped into small pieces and transferred into 35-mm culture dishes. The explants were encouraged to adhere to the culture dishes by partially drying the tissue.

This was achieved by leaving the tissues without medium for 5-10 minutes to dry and adhere to the plastic. The skin explants were then gently covered with 20 % culture medium (section 4.2.1.2).

4.2.1.2 Cell maintenance

i) Cell feeding and subculturing

After isolation, all tissues were grown in 1 to 2 drops of culture medium (20% FCS in MEM). Cultures were incubated at 37°C in the presence of 95% O₂ and 5% CO₂. At day five, cultures were observed under a phase contrast microscope (Nikon, Japan). If the explants attached or showed signs of cell outgrowths, 2 ml of culture medium was added. The old culture medium was removed and replaced every 4 days to ensure sustained growth. When the cells had reached confluence (cells occupied all available substrate) cultures were subcultured into 25-cm² flasks as described in section 3.2.3.

ii) Cell storage

When long term storage of cells was required, the cells were harvested as described above. The pellet of cells was then resuspended in 1 ml freezing mixture (FCS and dimethyl sulphoxide (DMSO) in the ratio 3:1) and transferred into 1 ml plastic ampoules which were then placed at -80°C in a freezing container (Nalgene Cryo, -1°C/min) and allowed to cool slowly using ice isopropanol. After 24 hours they were transferred to liquid N₂ for long term storage.

Cells were recovered from the freezer by thawing quickly at 37°C and the suspension being transferred to a 25-cm² flask and gradually diluted with culture medium. The flask was then incubated at 37 °C and as soon as the cells attached the medium was changed.

4.2.1.3 Immunohistochemistry of dermal cells

i) Flow cytometric analysis of MHC-I on dermal cells

The analysis was carried out with SF, DP, LDS and UDS cells (all at passage 4-5) as described for epidermal cells in section 3.2.8. For this part rat spleen lymphocytes were utilised as a positive control. Animals were killed and their spleens were dissected out via an incision to the abdomen and transferred immediately to a 35-ml petridish

containing FACS medium. Single cells were liberated into the medium by teasing the spleens apart using two pairs of forceps. The supernatant now containing a large number of cells was transferred into a 30-ml universal tube and left for a few minutes so that the cell clumps settled out. The cell suspensions were then carefully moved into 12x75-mm tubes where they were shaken for a few minutes to establish free, single cell suspensions. The single cells were later used for analysis.

ii) Labelling of LDS and UDS cells with ASMA antibody

Cells at passage 2 were employed for this experiment. Cells were trypsinised, harvested and resuspended in 10% MEM as outlined elsewhere (section 3.2.3). Cell suspensions were placed on sterile glass coverslips in 35-ml culture dishes. Once the desired stage of confluence had been reached, normally 3-4 days after seeding, the cells were washed several times with PBS, fixed in acetone for 5 minutes at -20°C, and then rinsed for 3x5 minutes with PBS. A 1/10 dilution of ASMA monoclonal antibody (the antibody was kindly provided by Dr. G. Gabbiani, Geneva) in PBS was applied to the cells for 1 hour at room temperature. The cells were again washed three times with PBS and then incubated in fluorescent conjugated secondary antibody (Goat anti-mouse Ig) in the dark for one hour at room temperature. After washing the cells as described above, the coverslip was mounted onto a glass slide with citifluor (Agar aids), and observed with an Axiovert microscope (section 2.2.3.4). In the control specimens the first antibody was replaced with PBS but the second antibody was applied to the cells as normal.

iii) Labelling of LDS cells with antibodies against laminin, types I and IV collagen

Antibodies to laminin and types I and IV collagen were raised and characterised as previously described by Jahoda *et al.* (1992). Cells were grown on glass coverslips, labelled with the antibodies and processed for immunofluorescence observation in exactly the same manner as described above (section 4.2.3) for ASMA, but with their specific first and secondary antibodies {fluro-isothiocyanate (FITC)-labelled goat anti-rabbit Ig}.

4.2.1.4 Implantation of DiI-stained dermal cells into wound sites

i) Implantation of LDS, UDS and SF into ear wounds

A: Staining of cells with FM-DiI

All staining was performed using passage 2 cells. Staining with DiI was carried out according to the manufacturer's instructions (Molecular probes, Inc, USA). Subsequent to harvesting and pelleting (section 4.2.1.2), the cells were resuspended in 1 ml MEM containing 5 μ M FM-DiI (Molecular probes, USA). They were incubated at 37°C for 5 minutes and then for an additional 15 minutes at 4°C. The cells were then centrifuged as normal and the DiI solution removed. The pellets were washed by resuspending in 5 ml MEM. The cells were again pelleted and finally resuspended in fresh culture medium (MEM + 10% FCS), inoculated in 35-mm dishes, and incubated at 37°C in darkness for 3 days. The medium was then removed and fresh MEM without serum was added to the dishes. The cells were incubated in this medium for 24 hours after which they were used for implantation in the following manner.

B: Operation procedure

Adult rats were lightly sedated with fluothane (section 2.2.4.2) and their ears wiped with 70% ethanol. Each ear was held by hand and a small incision of 2-3 mm in length was made in the centre of the ear using a number 11 scalpel blade. The tip of a syringe needle was then passed through the dermis to create a pocket for the implantation of cells. Any resultant blood or plasma was removed using sterile cotton wool or tissue paper just prior to implantation.

At this point medium was removed from a confluent (approximately 2×10^5 cells) culture dish. The cells were rapidly scraped from the base of the dish with a rubber policeman and harvested as small clumps. A pair of sharpened watchmaker's forceps were used to transfer these clumps of cells into each pre-prepared ear wound pocket. The wound was observed until the upper layer of cells/blood had dried. Normally, only one incision was made per ear.

Seven to ten days post implantation the animals were killed and skin containing the wound areas was immediately snap frozen in tissue-tek embedding fluid (Miles Inc, USA) using liquid nitrogen. Sections of 6- μ m were cut using a cryostat microtome (Bright, UK) and transferred onto glass slides. Sections were then immediately examined under a fluorescence microscope (section 2.2.3.4) and the location of the DiI was

determined using a rhodamine fluorescence filter. This experiment was repeated for three separate occasions.

ii) Implantation of LDS, UDS and SF into punch biopsy sites

In addition to the implantation into ear wounds, DiI labelled cells were introduced into collagen gels which were later implanted into punch biopsies in the dorsum of host rats.

A: Staining and preparation of cells for implantation

Three cell types with the same specifications (in terms of passage number) were stained as described above (section 4.2.1.4) with the following exception. Immediately after staining, the cells were counted and introduced into 300 μ l of collagen gel prepared as described below.

Gels were prepared using type I collagen solution previously made from rat tail tendons in the laboratory according to the method of Michalopoulos and Pitot (1975) and stored at -20°C . In a 7-ml bijou plastic container on ice the collagen and x10 MEM (Gibco) solutions were mixed in the proportion of 8 to 1 according to Bell *et al.* (1979). While agitating a buffer of 0.15M NaHCO_3 was added dropwise to the collagen and MEM mixture. As soon as the solution turned a pink/red colour, it was inoculated with the stained cells. The cell solution was placed at 37°C incubator for gel formation to take place. The gels were maintained at 37°C for 2 days, after which they were implanted into rats as following.

B: Operation procedure

Rats were anaesthetised as described elsewhere (section 2.2.4.2) and hair in the mid-dorsum area was shaved using an electric shaver. Any remaining external hair stubs were removed with depilatory cream and then was later swabbed with ethanol. A 3-mm diameter punch biopsy (Stiefel, Germany) was used to create 3 full-skin thickness cavities or "punches" in each rat. The cut pieces were removed, the wounds cleaned using sterile cotton wool pellets, and the punches filled immediately with collagen gel containing cells of one type (LDS, UDS, or SF). After swabbing the around of the operation sites with 70% ethanol, strips of Gypsona plaster of Paris bandage (Smith and Nephew Ltd, UK) were loosely but securely wrapped around the trunk. After recovery, the rats were kept for two weeks, then killed and the punch areas were removed. The

specimens were processed for sectioning and the location of DiI was determined as outlined above (section 4.2.1.4).

4.2.1.5 Organotypic recombination of LDS cells with epidermal ORS cells

i) Recombination *in vivo*

Cultures of ORS epidermal and LDS cells were recombined within small silicon chambers and implanted under the back skin into a (glass disk-induced) granulation tissue site as outlined below. A schematic representation of the main stages in the preparation of an organotypic recombination is shown in Fig. 4.2.

A: Host site preparation: granulation tissue

Animals were anaesthetised and hair was removed from the mid-dorsal region as described in sections above. After swabbing the area with 70% ethanol, a horizontal incision was made across the lumbar region using a scalpel blade. A 1.5-cm diameter sterile glass disc with a roughened surface was then implanted into the incision and the wound was sutured with 5/0 coated Vicryl sutures (Ethicon). The glass disc was secured to prevent any movement using 5-6 stitches around its perimeter. Animals were allowed to recover, and then kept in isolated cages for a period from 10 days to 2 weeks prior to the main operation.

B: Preparation of silicon chambers

The chambers had two sections, a lower (main section) with an inner diameter and height of 3 and 5-mm respectively, and an upper section, which fits over the top and protects the lower contents from possible contamination. Prior to an experiment both parts of the chambers were sterilised by autoclaving. A porous filter (0.22 μm , Millipore S.A, Molshem, France) was then attached to the base of the lower part using MF Cement (Millipore Corporation, USA) and sterilised under UV light for 48 hours before use. At this point the collagen solution was prepared as described above (section 4.2.1.4). Prior to setting, the solution was pipetted into the lower third of the silicon chamber (lower section). The chamber was then left at 37°C for 10 minutes until gelation was completed.

C: Cell preparation

Cultured LDS cells between passage 3 and 7 were employed for this experiment. Epidermal cell cultures from the upper part of vibrissa follicles (ORS) were established

as described in section 3.2.2. Recombinations were performed using ORS cells between passage 3 and 6. Normally, 24 hours before recombination the normal growth medium was removed from both culture dishes (LDS and ORS) and replaced with serum free media.

D: Addition of cells into the chamber

LDS cells were harvested and centrifuged as described elsewhere (section 4.2.1.2) and the cell pellet was resuspended evenly in 30 μ l MEM without FCS. The cell suspension containing 1×10^5 cells was transferred into the chamber onto the layer of collagen gel that had been previously poured onto the filter. The chamber was then returned to the incubator and left for 2 hours, allowing the dermal cells to attach to the collagen and any extra liquid to diffuse out from the bottom of the chamber. At this point, the epidermal ORS cells were prepared and introduced into the chamber on top of the LDS cells in the same manner. The chamber was then incubated at 37 °C for another 3 hours. Subsequently, the chambers were implanted either into the granulation tissue site in the dorsum of the host rat or onto the chorioallantoic membrane of chick embryos.

E: Implantation of organotypic chambers into granulation tissue

The host animal was anaesthetised as described in section (section 2.2.4.2) and the area covering the granulation site swabbed with 70% ethanol. Using a pair of scissors an incision was made in the skin directly above the glass disc which was then removed by gripping its edge with artery forceps. The lower portion of the chamber containing the dermal sheath-epidermal cell recombination was held by a pair of forceps and gently positioned inside the subdermal granulation tissue pocket that had formed. The cut edges of the incision around the chamber were then stitched together, and the upper portion of the chamber placed over the lower section. The wound area was then wrapped with strips of Gypsona plaster of Paris bandage as described (section 4.2.1.4) but in a manner that left the top of the chamber exposed to the air. Animals were allowed to recover and subsequently kept in isolated cages. The animals were regularly observed, not only to ensure that the chambers had not moved but also to check the grafts for possible contamination. In general, the chamber provided an effective barrier to migratory incursion from local skin or hair follicles, since the edge of the host skin was held well outside the chambers. However, in a few cases the host tissues underneath

the chambers or the specimens inside the chambers became infected. In all cases as soon as any infection was observed the experiment was terminated and the specimens discarded. At 3, 7 and 20 days post operation, animals were killed and the chambers were removed. The resulting structures inside the chambers were photographed, fixed and then processed for electron microscopy. A total of 14 recombinations were grafted, and examined in this manner.

ii) Recombination on chorioallantoic membrane

In some cases the chorioallantoic membrane (CAM) of the 7-day chick embryo was used as a host site for implantation of the organotypic chambers. The fertilised eggs were purchased from a hatchery (Premier Poultry Thirsh, Yorkshire) and incubated at 37°C for seven days. Host eggs were prepared approximately 1-2 hours before implantation. The eggs were placed on plasticine nests, blunt end up and after swabbing the eggs with 70% ethanol, a rectangular outline was cut in the shell using a hacksaw blade. The shell was then broken off with forceps. In order to expose the CAM, the external membrane was held and peeled off using fine forceps. To allow the embryos to recover from this trauma, the eggs were sealed with sellotape and returned to an incubator for 1-2 hours. Subsequently, the eggs were reopened and the organotypic chamber gently placed on the CAM. The host eggs were resealed with sellotape and transferred to the incubator where they were left to develop for 6-7 days. Following this, the chambers were removed and the specimens fixed and processed for histological observation.

iii) Recombination *in vitro*

This procedure was performed using three different combinations. In three sterilised cylindrical containers (7-ml bijou), 0.5-ml of collagen solution was prepared as described above (section 4.2.1.4). In one case (combination 1), prior to setting, the solution was directly transferred into culture chambers (Iwaki, USA) which were later incubated at 37 °C for at least 20 minutes before the next stage. In the other 2 cases (combinations 2 and 3) and again before setting the gel, a 200 µl cell suspension containing approximately 1×10^5 LDS cells (prepared as section 4.2.1.4) after 4-5 passages were evenly incorporated into the collagen gels. The gels were immediately

pipetted into similar culture chambers and placed in the incubator. All three gels with or without the LDS cells were set completely after a maximum of 10 minutes.

In combination type 1, cell suspensions containing 1×10^5 LDS and epidermal ORS cells (passage 3-6) were added to the gels as described for the *in vivo* recombination in section 4.2.1.5. For combination 2, shortly after the gel containing LDS cells has set a 200 μ l cell suspension containing 1×10^5 ORS cells was placed onto the gel. In combination 3, the gel incorporating LDS cells was incubated at 37 °C for two days to allow contraction to take place after which time the contracted gel again had a 200 μ l ORS cell suspension added. After preparation, all three kinds of cocultures were placed in 24-well microplates (Falcon, Becton Dickinson, UK) and cultured submerged in epidermal culture medium (section 3.2.5) for 4 days. Thereafter, the cocultures were exposed to the air-liquid interface with medium changes every 3 days. The specimens were harvested after 7 or 14 days post-culturing and processed for histological observations as described elsewhere (section 2.2.3.3).

4.2.1.6 Recombination of LDS cells with embryonic skin cells

Embryonic PVG rats at gestation day 15-16 were used as skin donors. After the mother had been killed by cervical dislocation, the uteri were aseptically excised intact, and placed in MEM. The embryos were removed and their heads and limbs were amputated. Under a dissecting microscope the skin was separated from the rest of the body using fine forceps. Isolated skins were piled in 35-mm dishes containing Ca^{+2} and Mg^{+2} free PBS. Following washing in the same PBS the skins were transferred into a 30-ml conical tube containing a solution of 0.25% crude trypsin in PBS and incubated for 20 to 30 minutes at 37°C. The digestion was terminated by adding 3-4 ml MEM containing 20% FCS. Single cells were liberated from the trypsinised skin manually by vigorously flushing the tissues using 3-ml plastic pipettes. The resulting cell suspension was filtered through a sterile nylon strainer to remove any residual clumps and the filtered cell suspension was kept at 37°C (for up to 1 hour) before being associated with LDS cells.

LDS cells at passage 3 were harvested as a pellet as described in section 4.2.1.2. The pellet was suspended in 3 ml MEM (without serum) and the suspension mixed evenly with the cell suspension from skin embryos. The mixture was spun down at 1800-

rpm for 3 minutes and the supernatant discarded. To remove any remaining trypsin, the pellet was again washed in MEM and the suspension centrifuged for a second 3 minutes. After removing the supernatant a small amount of MEM (50 μ m), without FCS, was added to the pellet to make a mushy aggregate. The aggregate was then deposited on the CAM of a 7-day chick embryo, which had already been prepared as outlined in section 4.2.1.5. The graft was left to develop for an additional 6-7 days, at which point it was removed and processed for routine histology. In control experiments the embryonic skin was dissociated, treated as described above and implanted on the CAM in the absence of LDS cells.

4.2.1.7 Recombinations involving hair follicle DP

Dermal and epidermal cells were combined in collagen gels and implanted into punch sites that had been prepared in the dorsum of host rats in the presence or absence of intact dermal papillae as described below.

A: Cell and tissue preparation

Vibrissa follicle LDS, SF, DP and epidermal ORS cell cultures were established as described previously in sections of 4.2.1.1 and 3.2.2 respectively. This study was carried out with cells at passages 2 or 3. In order to form a cellular dermal matrix, dermal cells (either LDS or SF) were harvested and seeded separately into a collagen gel that was allowed to contract as described previously in section 4.2.1.2. In 2 cases, prior to inoculation into the gel, the LDS cells were first combined with equal numbers of cultured DP cells (only passage 2).

The collagen solution was made as described in section 4.2.1.4. Before setting the gel, 250 μ l MEM containing 3×10^5 dermal cells were mixed into the collagen which was then maintained at 37°C for 4 days. At the day of operation and shortly (1 hour) before that, epidermal ORS cells (3×10^5) were also introduced into 200 μ m of collagen gel which was then allowed to set at 37 °C. In addition, vibrissa dermal papillae were dissected as outlined in section 4.2.1.1 a few hours before the recombination. The isolated papillae were placed in a 35-mm petridish containing MEM and antibiotics but in the absence FCS until use.

B: Experimental design

On the dorsum of each host rat 4 punches were prepared as described in section

4.2.1.4. The punches were filled with dermal and epidermal cells in four different combinations (COM).

COM 1: A gel populated with LDS was first placed at the bottom of the punch biopsy hole and the gel containing ORS was placed on top (LDS + ORS).

COM 2: Was performed in the same manner as COM 1, however before adding the gel containing the ORS cells, 8 to 10 isolated dermal papillae were placed inside the gel containing the LDS cells (LDS + ORS + intact DPs).

COM 3: The gels containing SF and epidermal ORS cells respectively were positioned in the punch in the same manner as for COM 1 (SF + ORS).

COM 4: Performed as COM 2, but instead of intact dermal papillae, cultured papilla cells at passage 2 were mixed with LDS cells in the collagen solution (LDS + ORS + DP).

The operation sites were then bandaged as described in section 4.2.1.4. Five to seven days post-operationally the bandage was removed and the punch areas were routinely observed under a stereo microscope (section 2.2.2). The animals were killed 30-35 days after the operations and the implantation areas dissected out using fine scissors. The internal and external sides of the punch regions were photographed, and the specimens then processed for routine histology. This experiment was repeated 4 times.

4.2.2 Procedures involving other skin appendages

4.2.2.1 Rat claw unit

i) Histology

To establish a greater knowledge about the morphology and exact location of the dermal papilla in the rat claw, a new-born rat (4-day of age) was utilised. The animal was killed by an overdose of CO₂, and its fingers and toes amputated using a scalpel blade. The specimens were fixed in 4% formalin saline and processed for histology as described in section 2.2.3.2.

ii) Isolation and cultivation of claw dermal cell

Rats aged between 4 and 30 days were used in this part of the study. Animals were killed and their limbs washed with 70% ethanol. Using a scalpel blade, the last joint

of each digit was amputated and the pieces immediately transferred into a petridish containing MEM and antibiotics. Using a dissecting microscope and sharpened scissors a longitudinal cut was made through the skin to expose the internal structures. The skin was then removed and discarded. The internal structures comprising terminal phalanx and claw tissues were transferred into fresh MEM, and the claw plate smoothly detached from the phalanx bone using forceps before discarding. The claw papilla residing between the claw plate was now the only tissue left attached to the phalanx surface. The claw papilla was peeled off from the phalanx using fine forceps and transferred to a 35-mm culture dish. The papillae explants were cultivated in the same manner described for other dermal cells in section 4.2.1.1.

iii) Immunolabelling of dermal cells with ASMA antibody

Cells at passage 2-3 were harvested, cultured onto glass coverslips and processed for ASMA detection as outlined elsewhere (section 4.2.1.3).

iv) Implantation of DiI-stained dermal cells into the amputated vibrissa follicles

This experiment was set up to investigate the inductive abilities of claw cells in the formation of hair follicles *in vivo*. Operations were performed with papilla claw cells after the second or third passages. Cells were stained with DiI, transferred into dishes and prepared for implantation as described in section 4.2.1.4.

Two rats at 4 months of age were used as hosts for this experiment. Anaesthesia was induced and maintained as described in section 2.2.4.2. The method employed to expose follicles was essentially the same as described previously in section 2.2.3.1. After exposure, individual follicles were cleaned of the surrounding connective tissue and their position recorded. This was achieved by gentle manipulation of the follicles, which resulted in the visual movement of the fibres and thus identification of the corresponding whisker position on the skin surface. Operations were performed on the larger follicles of the most posterior dorso-ventral row. Follicles were prepared for implantation according to Horne *et al.* (1986). Using a pair of forceps the follicles were carefully held at the level where they joined to the skin surface and then using fine scissors, lengths of follicle were amputated with a transverse cut. The cut was made above the level of nerve entry point into the capsule wall and resulted in the removal of the lower region of the

follicle. The cut vibrissa shafts were plucked from the upper segments, and the base cleared to receive cultured cell implants (or act as non-implanted controls).

When the recipient follicles had been prepared, the media was removed from the culture dishes and the cells were harvested in small clumps as described in section 4.2.1.4. The clumped cells were then transferred into the opening at the base of the follicle in order to insert cells into the hair shaft cavity.

A total of 9 follicles were successfully implanted in this manner. After cell implantation the skin flap was sutured back in place. The animals were killed at 20 days post implantation and the amputated follicles removed, and processed for histological observation.

4.2.2.2 Feather follicles

Adult birds including 2 pigeons and 1 sparrow that were all suffering from serious natural injuries with no possibility of recovery were obtained from a local veterinary surgery.

i) Follicle isolation

The birds were killed with an overdose of CO₂ and their wings detached from the rest of the body. All wing feathers were plucked manually except the contour (large flight feathers) and small newly emerged feathers. Using scissors, the shafts (rachis) of the remaining feathers were cut just above the skin surface. The wings were then washed with 70% ethanol and transferred into a petri-dish containing MEM with ABS. Fine scissors were now utilised to separate the skin along with its feather follicles from the underlying forelimb tissues. Since the base of the follicles were in close contact with the limb bones, great care was taken to isolate the follicles without damaging their proximal ends. The skin, along with the follicles, was moved into another dish with fresh MEM in which the follicles were detached from the skin using forceps. Isolated follicles were either processed for routine histology or employed for papilla cell culture.

ii) Immunolabelling of feather follicle with ASMA antibody

Newly emerged pigeon feather follicles, which were in a growing state, were processed for immunohistochemistry with ASMA antibody. In brief, feather follicles

were dissected and snap frozen in tissue-tek embedding fluid using liquid N₂. Sections of 6- μ m were cut and collected on poly-lysine coated slides (section 2.2.3.4). The sections were air dried and then fixed in acetone for 10 minutes at 4°C. The specimens were washed twice with PBS and then incubated in the first antibody (section 4.2.1.3) for one hour at room temperature. Sections were washed again as above and incubated in fluorescent conjugated secondary antibody (section 4.2.1.3) for one hour at room temperature. After final washing specimens were mounted and observed as described in section 2.2.3.4.

In addition, pigeon papilla cell cultures at passage 2 (see below) were labelled with ASMA antibody as described for other cell types elsewhere (section 4.2.1.3).

iii) Papilla dissection and cell culture

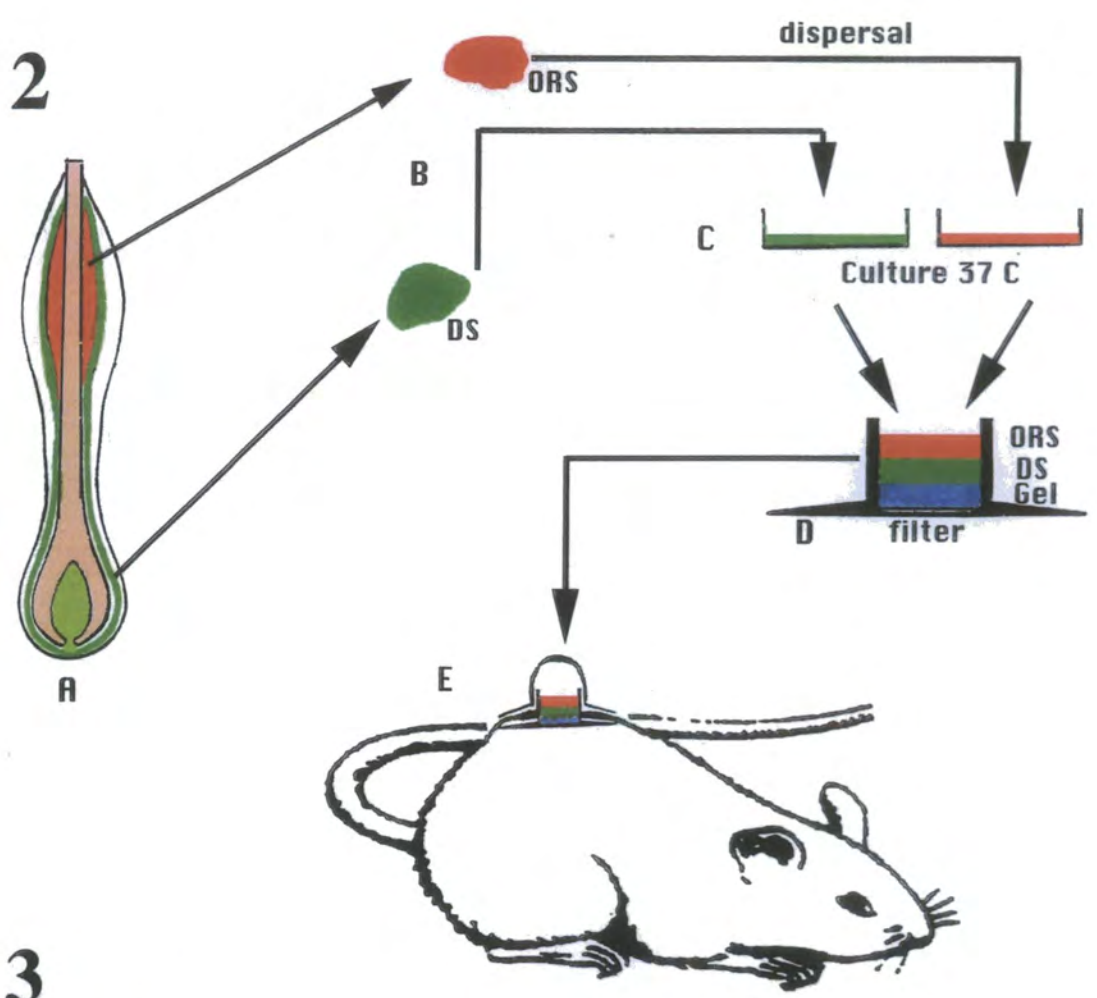
The main stages in the dissection of the feather papilla are shown in Fig. 4.3. In order to remove the corneous connection (the wall of the follicle) covering the lower portion of the feather follicle, a pair of forceps was used to hold the upper part while another pair was used to pull the wall down until it was separated from the follicle (Fig. 4.3). Using a scalpel blade a longitudinal incision was then made through the follicle sheath to expose the tissues inside the feather cavity. The tissues comprising the external epithelium and internal connective tissue-papilla were detached from the follicle sheath, and using fine forceps the epithelial part surrounding the papilla was detached and discarded. Because the feather dermal papilla is tightly connected with its surrounding epidermal cells, before culturing, the papilla was placed in dispase solution (1 mg/ml) for 15 minutes to digest the junction between dermal and epidermal cells. After removing and discarding the epidermal compartment attached to the papilla, the intact dermal papillae were cut into small pieces and transferred to plastic dishes for cultivation as described for hair follicle papillae in section 4.2.1.1. In one case feather papillae were dissected from sparrow contour feather follicles and processed for electron microscopy as outlined in section 2.2.3.3.

iv) Implantation of pigeon papilla into athymic rat hair follicles

Whole pigeon dermal papillae were dissected as described above and implanted into the upper section of vibrissa follicles as below.

Fig. 4.2) Diagram of the procedures involved in dermal sheath-epidermal cell recombinations. Rat vibrissa follicles (A) were used to dissect out both dermal sheath (DS) and epidermal outer root sheath (ORS) tissues (B). The dermal sheath cells were cultured as tissue explants, but ORS cells were dissociated by trypsin. Cells grew separately in special media (C) until they reached desired the stages, when they were harvested and recombined in a collagen gel in a silicon chamber (D). The chamber was then implanted to a host rat (E).

Fig. 4.3) Stages of the dissection process (A-F) used in the isolation of a dermal papilla from a pigeon's growing feather follicle. Scale bars A 1mm, B 400 μ m, C and D 380 μ m, E 330 μ m and F 270 μ m.



A: Preparation of the vibrissa follicles stuffed with feather papilla

Adult athymic rats (HsdHan. NZNU-rnu^N, Harlan, UK) used for another experiment were killed and the vibrissa follicles were isolated as outlined previously in section 2.2.3.1. The vibrissa follicles were transferred into MEM and the lower half of the follicles removed with a transverse cut some way above the level of nerve entry point into the capsule wall. The hair fibres were finally plucked from the remainder of the follicle. Now pieces of cleaned, isolated pigeon feather papilla (obtained from newly emerged follicles and maintained in MEM for up to 24 hours) were introduced into the hair shaft cavity of amputated rat vibrissa follicles. Of 12 amputated follicles 8 received the implants and the remaining 4 were used as controls. The follicles (both experimental and controls) were then kept in MEM with antibiotics at 4°C overnight.

B: Implantation of the tissue

One male and one female nude mouse (HsdOla. MF1-na, Harlan, UK) both 3 months of age were used as recipients. Animals were kept in a pathogen-free facility until the operation, which was performed using sterile instruments. The mice were anaesthetised as described in section 2.2.4.2 and the backs were cleaned with alcohol wipes. An incision was made in the skin at the lumbar region and at the side of the vertebral column just above the kidney. The incision was made through the subcutaneous musculature until the kidney was exposed. Using a pair of towel clamps the kidney was held and carefully moved to the skin surface where it was rested during the operation. Utilising a scalpel blade number 11 a shallow incision was carefully made in the surface membrane of the kidney. The edges of the membrane were lifted up and a pocket was made between the membrane and the underlying tissue using a syringe needle. Four experimental rat follicles containing feather papilla were then implanted into the pocket. After implantation, the kidney was returned to its normal position and the skin incision sutured using 4 or five 5/0-coated vicryle sutures (Ethicon). The other kidney in the same mouse was used for implantation of control follicles as described above. Animals were killed 4 weeks post-operation, and the part of kidneys containing the implanted follicles were cut and processed for routine histology (section 2.2.3.2).

4.2.3 Organ culture of plucked follicle end bulbs

Rats were killed and vibrissa follicles were immediately plucked (any abnormal plucking was noted and recorded). The follicles were then dissected from the face as described in section 2.2.3.1 and unplucked or abnormally plucked follicles (still containing hair matrix) were discarded. The plucked follicles were transferred into MEM containing antibiotics, cleaned, and transected just above the bulb as described in section 2.2.3.4. The bulbs were immediately submerged individually in 1.5 ml of prewarmed growth medium {William's medium E with Glutamax, supplemented with 100-U/ml penicilin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 10 ng/ml sodium selenite (all supplied by Gibco, Life Technologies, UK) and 11.1 mM glucose (Sigma, Aldrich, UK)} in 24-well microplates (Falcon, Becton Dickinson, UK) and incubated at 37°C. At intervals of 7 and 15 days, follicles were removed, fixed, and processed for morphological examination according to Karnovsky (1965).

4.3 Observations and Results

4.3.1 Characteristics and interactive abilities of rat SF and hair follicle dermal cells

4.3.1.1 Morphological characteristics in culture

i) Skin fibroblast (SF)

Skin fibroblasts grown from rat dorsal skin explants displayed morphology and behaviour similar to that described in rats and other species (e.g. Horne *et al.*, 1986). Cell outgrowths consisting of epidermal cells and dermal fibroblast cells emerged from skin explants 7 to 8 days after cultivation. Epidermal cells remained in close proximity to the explants, adopting a typical pavement-like morphology (Fig. 4.4A), whereas fibroblast cells could be observed at the peripheral region of the cell outgrowths. Fibroblast cells exhibited a ruffled membrane structure. The leading edge of the cell was expanded into a thin, broad fan, with lamellipodia. As confluence was approached, the epidermal cells ceased growth, while the fibroblasts displayed a gradual change to a bipolar spindle form, most cells extending into a straight spindle, with small, unexpanded spike-like lamellipodia. After subculturing, any epidermal cells were lost and only fibroblast cells remained in the cultures. The fibroblasts at an early stage of post-subculturing again displayed a ruffled membrane appearance but in both confluence and post-confluence they were bipolar and spindle-shaped and demonstrated parallel alignment (Fig. 4.4B). In post-confluence stages they formed multiple layers and in some areas they appeared more packed and occasionally showed to some degree an aggregating behaviour.

ii) Dermal papilla (DP)

Dermal papilla (DP) cells were found to grow in a manner similar to that described previously (Jahoda & Oliver, 1981). Cell outgrowths could be observed emerging from intact isolated dermal papillae at 4-5 days incubation (Fig. 4.5A). At this point the original DP structure had collapsed and the cells that had grown had spread out equally in every direction. Cells were relatively closely packed and displayed a fibroblast-like morphology with a stellate appearance. Cells close to the original explants were relatively small in comparison to those at the periphery where they were larger and

more flattened. Sometimes cells were observed isolated from the main body where they displayed a larger size with a more flattened appearance.

During the early stages of growth (5 to 15 days cultivation) the papilla cells appeared to spread rapidly but with continuing growth they divided more slowly and displayed a flatter morphology as the rate of cell outgrowth around the papilla explant decreased. Primary DP cultures were normally passaged at 4-5 weeks cultivation, but with prolonged maintenance of primary cultures for periods over 2 months, the cells sometimes produced organised structures. For example, in several cases cells attached together and produced tube-like structures (Fig. 4.5B). Confluent passaged DP cultures demonstrated multi-layered cell aggregates (Fig. 4.5C) in a manner similar to that described previously (Jahoda & Oliver, 1981). In the space between these aggregations cells remained in a monolayer similar to the pattern of growth seen in skin fibroblasts.

iii) Lower dermal sheath (LDS)

Compared with the DP, it took LDS specimens 2 to 3 days more to display cell outgrowth around the tissue explants which did not break down as completely as those from the DP (Fig. 4.6A). Moreover, unlike the DP cells, the growing LDS cells were more randomly scattered and did not grow out from the explants equally in all directions (Fig. 4.6A). Cells commonly had a spindle-like morphology and despite some variations did not show a significant difference in terms of their size whether at the peripheral or central region of the outgrowth (Fig. 4.6A). At confluence, DS cells were found to be small in size, and had formed dense aggregates, although they never formed as marked clumps as were seen in the papilla cells (Fig. 4.6B). When the DS explants were cultured under glass coverslips the explants produced outgrowths of cells which differed from those seen using normal cultivation methods (without coverslips). Unlike the normal method where the cells showed a regular morphology, the cells in this case displayed multiple phenotypes indicating that different cell types had grown (Fig. 4.6C-D).

iv) Upper dermal sheath (UDS)

These cells displayed early outgrowth behaviour that was similar to that described above for LDS with cells being visible at the edges of the explants at 6 or 7 days after incubation (Fig. 4.7A). These were dispersed and flattened with no obvious

difference in size between those of the peripheral and central regions. At an early stage (6 to 15-days cultivation) the majority of cells possessed multiple cytoplasmic projections but at later stages (after subculturing) they revealed a bipolar fibroblast-like morphology, and were narrower with fewer projections. In contrast to LDS cultures, UDS cells at confluence were narrow, long and less closely packed. At high density UDS cultures formed cell aggregates but again they failed to clump or display the same tight aggregative behaviour of papilla cells (Fig. 4.7B).

To test the approximate growth rate of the LDS and UDS in one case equal numbers of both cell types were plated in culture dishes which were later incubated for 48 hours before examination. When cells were counted using a haemocytometer and the mean was calculated from 3 replicates no substantial difference was found between the two cell types (see appendix, table 3).

4.3.1.2 Immunohistochemical examinations

i) Flow cytometric analysis of MHC I antibody on follicular dermal cells and SF

Spleen lymphocytes are known to be positive for the MHC-class I antigen. Hence, as positive controls and to determine an appropriate working concentration for the antibody, fresh rat spleenocytes were initially isolated and stained with the MHC class I antibody. Data concerning the proportion of these cells which expressed the class I MHC antigen are shown in appendix (Fig. 8).

Cells expressing MHC class I were identified based upon a comparison with their negative controls which had not been incubated with the first antibody. The proportion of cells that stained in the negative controls during this study was never more than 8.0% of the total viable cell population. This and the following results came from experiments that were repeated on three separate occasions. In this study the highest proportion of labelled cells was observed in skin fibroblast cultures. Around 97-98% of these cells expressed MHC-I (Fig. 4.8). On the other hand dermal papilla cells displayed the lowest level of MHC class I expression among all of the follicular and non-follicular cell populations considered (Fig. 4.9). The labelled papilla cells represented only 30-35% of the whole viable cell population. UDS and LDS cells exhibited intermediate levels of expression, with 71-75% of UDS cells (Fig. 4.10) and 63-70% of LDS cells being labelled (Fig. 4.11A). Moreover, to determine whether or not the percentage of MHC-I

positive cells varies among cells with different phenotype, different cell populations within the sample were examined by setting the gate in various positions. It was found that, (as already shown for epidermal cells in section 3.3.3) the percentage of MHC-I-positive cells also increases with size and granularity of dermal cells. For example, figures 4.11B-C show this variation for LDS cells and the histograms concerning the UDS cells can be found in the appendix (Fig. 9).

ii) Labelling of LDS and UDS cells with ASMA antibody

Expression of ASMA in hair follicular dermal sheath cells has already been reported (Reynolds *et al.*, 1993b). In this study, in order to demonstrate that my cultured cells had the same phenotype, follicular UDS and LDS cells were stained with ASMA antibody. The proportions of ASMA positive cells in populations of the LDS and UDS were similar to those reported by Reynolds *et al.* (1993b). In general the number of cells that expressed ASMA was higher in the culture of cells derived from follicular LDS (Fig. 4.12) compared to cells from the UDS (Fig. 4.13). Approximately, 80-85% of LDS cells stained positively for ASMA whereas the proportion of stained cells that originated from follicular UDS was 40-50%. Generally, the large, flat and well-spaced cells tended to stain more intensely for ASMA than the smaller, more tightly packed cells (Fig. 4.13B-C).

iii) Labelling of LDS cells for detection of ECM components

Since it is known that the ECM of dermal papilla cells expresses a number of basement membrane molecules (Katsuoka *et al.*, 1988) and interstitial collagens (Messenger *et al.*, 1991a,b) I investigated the expression of three of the ECM components in cytoplasm and ECM of the papilla's neighbouring cells namely the dermal sheath cells. Laminin and collagen types I and IV were used for this investigation. It was found that dermal sheath cells expressed all of these components in their cytoplasm particularly around the endoplasmic reticulum (Figs. 4.14-16). These elements were also detected in the ECM of dermal sheath cells but with expression of each component differing. Laminin was clearly expressed in the ECM (Fig. 4.14B) but in comparison to this, the ECM was less positive for collagens type IV (Fig. 4.15) and I (Fig. 4.16).

4.3.1.3 Interactive abilities of dermal cells in wound sites

i) Implantation of DiI-stained cells (UDS, LDS and SF) into ear wounds

In order to investigate the effect of DiI labelling on cell viability, in two cases populations of stained dermal/epidermal cells were maintained *in vitro* for extended periods (Fig. 4.17A). Observation of stained cells at 12 days revealed that the cells were healthy while still retaining the vital dye (Fig. 4.17B) and displaying no sign of deterioration.

At 7-10 day post-operation wound sites that had received any of the dermal cell types (UDS, LDS or SF) were clearly visible as a raised and red-coloured area. However, close external examination of these sites did not give any indication as to whether hair follicle induction had occurred. Histological observations of operation sites revealed that the dermis had healed and was continuous with its neighbouring tissues (Fig. 4.18A). Indeed, from the normal histological examination of all wound sites, neither obvious signs of interaction nor the existence of unusual structures were noted. Moreover, there was nothing that distinguished the implanted cells from host-derived cells (Fig. 4.18A). However, examination of the specimens by fluorescence microscopy with a rhodamine filter clearly revealed the distribution of the implanted cells in the wound sites as bright red spots (Fig. 4.18B). Later, detailed examination of the punch biopsy specimens at a higher resolution using a confocal microscope confirmed that these red spots represented cells that still retained the vital dye (see next section). The labelled cells were not only observed within the original wound margins but many of them also could be seen in regions adjacent to the implanted sites suggesting that at least some cell migration had occurred (Fig. 4.18B). Within the implanted sites the labelled cells were seen mainly as dense clumps of cells, but the dye gave no further indications that cells had formed any organised structures. Interestingly though, in the specimens that contained labelled LDS (Fig. 4.19A) cells, some of the stained cells were observed to have been incorporated into hair follicle structures (Fig. 4.19B), a phenomenon that was not observed in the sites which received UDS (Fig. 4.20) or SF (Fig. 4.18B). Because the sections were cut obliquely, the stained LDS cells were seen in rings enveloping small ear follicles (Fig. 4.19B). The location of these labelled cells around the follicle structures suggested that the cells had been incorporated into the hair follicle dermal sheath.

ii) Implantation of the DiI-stained cells (LDS, UDS and SF) into punch biopsy sites

Similar to non-stained cells, DiI-labelled cells introduced into collagen gels contracted the gels. The gels that were employed for these experiments had been inoculated with dermal cells 2 days prior and their volume at the time of operation had contracted to approximately 40-50% of the original.

One week after cell implantation the punch areas had not completely healed and the implanted cells could be still seen within the punch sites (Fig. 4.21A). However, after two weeks, the skin surface at the punch sites had been completely repaired but the sites were still distinguishable as shiny, hairless areas surrounded by darker, hairy regions. When the skin was inverted, the punch biopsy sites were well characterised by their reddish appearance (Fig. 4.21B). Histologically, compared to neighbouring regions the implant sites biopsied two weeks after the operation displayed a thick epidermis, while the dermis was thin with large spaces between the cells (Fig. 4.22A). When specimens were examined with fluorescence (Fig. 4.22B-C) or confocal (Fig. 4.22D) microscopy the implanted cells were again distributed mainly within the healed punch areas, but in some specimens labelled cells had also penetrated into the nearby dermis. On the wounds containing labelled cells, no evidence of induction or formation of organised cellular structures was found (Fig. 4.22B-D). Nevertheless, once again the LDS (Fig. 4.23) cells showed a different behaviour to that observed in the specimens containing UDS (Fig. 4.22) and SF (Fig. 4.24). Although, all three cell types had penetrated into the neighbouring host dermis this migration was most extreme in the LDS cells which were found in areas some distance away from the original sites (Fig. 4.23B). Moreover, again in contrast to the other cell types (UDS and LDS), the stained LDS could be observed in follicle dermal sheath in the same manner as described above for the ear wound sites (Fig. 4.23C-D).

4.3.1.4 Organotypic recombinations of LDS cells and epidermal ORS cells

i) Recombination *in vivo*

On removing the upper domed portion of each chamber, the implanted grafts were observed in the centre of the lower portion (Fig. 4.25). Careful removal and the close examination of this lower portion of the chamber, particularly the filter directly

underneath it was used to confirm whether or not recombinations had remained isolated from the surrounding local tissues. The following description refers to the recombinations that remained isolated.

Histology of the cell associations inside the chambers at 3 days post implantation is shown in Fig. 4.26. At this time the grafts displayed two distinct regions, a broad epithelial region at the top and a thinner dermal layer at the bottom (Fig. 4.26A). The epithelial region was composed of several cell layers and showed variable morphology between the lower and upper level. Superficially, the cells appeared large and scattered but in the lower region of the epidermis they appeared relatively small and more compacted suggesting that they were beginning to organise themselves into a basal layer. However, even in the lowest epidermis the epithelial cells had not yet completely attached to each other to make a composite pattern and still appeared as separated cells. In the underlying component, the dermal sheath cells had partly invaded and populated the collagen gel in which they had established a thin dermis with spindle-like cells (Fig. 4.26B). At the dermal-epidermal junction no indication of basement membrane structure was visible as the epidermal cells lay loosely on the dermal cells.

By day 7, the epithelial cells had produced an epidermis-like structure above the dermis but it was still not possible to make out any specific epidermal cell layers such as a basal layer or stratum corneum. In the epidermis there were also some circular structures which possibly represented an early stage of hair follicle formation. In the dermal component, the dermal cells had penetrated further into the underlying gel but a substantial part of the gel was still free of any cells (Fig. 4.27).

Twenty days after implantation the grafts had formed an organised skin structure (Fig. 4.28A). The epidermis was multi-layered with distinct changes in cell morphology at different levels. Basal cells were small and closely packed (Fig. 4.28B) sending several pronounced finger-like projections into the dermis (Figs. 4.28C). In the middle part of the epidermis, the epithelial cells formed cysts or spheroid structures which appeared to show inward-oriented differentiation, a phenomenon previously described (Bell *et al.*, 1983; Grinnell *et al.*, 1986; Limat *et al.*, 1991 & 1994b,c) in recombination cultures. These spheroid or epithelial cysts were also sometimes observed deep within the dermis (Fig. 4-28A). Nevertheless, the question as to how these structures had formed remains unclear. Ultrastructurally, these epidermal spheroids were surrounded by tightly-joined

epidermal cells (Fig. 4.28D). Detailed examination of the epidermal basal cells showed they had a regular appearance with a small, round morphology and contained a tonifibril network distributed around the nuclei (Fig. 4.28E)). These cells were rich in mitochondria and contained numerous free ribosomes indicating a normal metabolic activity (Fig. 4.28F). Desmosomes between the basal cells were sparse. Small cytoplasmic projections of the basal epidermal cells protruded into the underlying dermal extracellular matrix making direct contact with the amorphous matrix material (Fig. 4.28G).

The underlying dermal sheath cells formed a structure resembling a normal dermis (Figs. 4.28A, H & I). The dermis was thick and dermal cells populated most of the collagen gel, but the density of cells in lower areas was less than those in the upper area close to the epidermis. Cells constituting the dermis displayed various shapes with clear nuclei and their cytoplasm contained numerous ribosomes (Fig. 4.28I). The cells were embedded in a vast extracellular matrix rich in filamentous elements (Fig. 4.28J). Unlike earlier stages, a continuous basement membrane was observed at the dermal-epidermal junction (Fig. 4.28K) and the epidermal cells were connected to this membrane by a large number of hemidesmosomes (Fig. 4.28L). On the dermal side of the membrane, filamentous fibrils particularly collagen fibrils were clearly visible which their proximal ends were attached to the membrane.

In a few cases the dermal sheath-epidermal recombination was placed onto the CAM of a 7-day fertilised egg. Examinations of the grafts after 7 days incubation revealed that the cells had organised to produce a primitive skin-like structure consisting of a discrete epidermis and dermis (Fig. 4.29A). The overlying epidermis constituted of several cell layers with cells in the lower layers being slightly denser than compared to those in the upper layers. The dermis was thin and was composed of 2-3 cell layers (Fig. 4.29B). This structure was relatively similar to its counterparts which were implanted on rat granulation tissue.

ii) Recombination *in vitro*

Histological observations of organotypic recombinations 7 days post cultivation *in vitro*, revealed that in only recombination type 1 (DS cells and the ORS cells seeded onto a collagen gel initially lacking cells) had a primitive structure had been formed. This

structure consisted of two thin dermal and epidermal components covering the underlying collagen gel. The dermis was only 1-2 cell layers thick yet the cells were quite healthy with large nuclei and elongated cytoplasm oriented in a manner so that their long axis was parallel to the underlying gel (Fig. 4.30A). However, the dermal cells had not penetrated into the gel which was virtually free from cells. The epidermis also was comprised of only 2 or 3 cell layers lying directly on top of the dermal cells, and no distinction could be made between cells of the lower and superficial epidermis in terms of cellular differentiation. Such primitive structures were not observed in the other recombination types (type 2 and 3, see materials and methods). In these recombinations epidermal cells did not attach strongly to the dermal substrate, and therefore, during the processing of specimens for histology, epidermal cells detached and were observed floating in the processing solutions. As a result, histologically, no epidermal cells were observed, while dermal cells had often died inside the gel. In three cases, the type 1 recombinations were cultured for 2 weeks. However, when the specimens were harvested and processed for histology it was noted that the epidermal cells were separating from the underlying substrate as seen in type 2 and 3 recombinations after one week in culture. Histological examination showed that only a few epidermal cells were still attached to the dermis which itself had largely disintegrated (Figs. 4.30B-C).

4.3.1.5 Recombination of LDS cells with dissociated embryonic skin on CAM

Histological examination of both experimental (dissociated embryonic skin plus LDS cells) and control grafts (only dissociated embryonic skin) after 7 days on the CAM showed that in both graft types the dissociated cells had developed into bodies containing epidermis, dermis and primitive hair follicles (Figs. 31A & 32A). The epidermis consisted of 4-6 cell layers with each layer showing different cell morphology and densities (Fig. 4.31B). Cells in the basal layer were small and dense, but higher up they became large and more scattered with an acellular layer covering the epidermal surface. The dermis was also distinguishable as an area containing primitive hair follicles beneath the epidermal cells (Fig. 4.31C). These hair follicles were in various stages of morphogenesis (Fig. 31D). Because the majority of the hair follicles were cut transversely they were visible as small structures composed of 3 to 4 circular epidermal cells. Some follicles, which had been sectioned longitudinally, displayed well-developed

structures that had already produced hair shafts. All these follicles were small in size, similar to that of pelage hairs.

In addition to these features which were found in both experimental (Figs. 4.31A-F) and control (Figs. 4.32A-B) implants, in the former a few larger structures were observed at the base of the grafts close to the host CAM (Fig. 4.31E). These structures which were surrounded by dermal cells contained 5-6 epidermal cell layers, and were much larger compared to the other follicle structures (Fig. 4.31F). These structures were never observed in the control specimens suggesting their production was a consequence of the presence of dermal sheath cells.

4.3.1.6 Inductive abilities of rat hair follicle DP in punch biopsy sites

Four days after incorporation of dermal cells into a collagen gel the volume of the gel markedly decreased, to approximately one thirds of its initial diameter. Thus the dermal cells/collagen mixtures put into the punch biopsies *in vivo* had a high density of cells. These mixtures were recombined with gels freshly populated with epidermal ORS cells in punch biopsies in the presence or absence of intact vibrissa papillae. The sites of implantation were recognised by their shiny appearance and lack of pelage hair.

Macroscopic examination 32 days post-implantation revealed that in all 3 sets of experiments the punch sites which had received LDS+ORS + intact DPs produced large external vibrissa-type fibres which were clearly visible by the naked eye. The induced fibres were highly pigmented and much thicker than neighbouring pelage hairs (Fig. 4.33). Unlike the local pelage fibres the operation-site hairs were quite curved and increased in diameter from the tip to their base at the skin surface. The remaining punch sites, including the sites that had received cultured LDS+ORS, SF+ORS and LDS+ DP +ORS cells did not produce any external fibres when observed at a macroscopic level, and the sites remained hairless (Fig. 4.34).

Histological observations of the punch sites demonstrated that, in all cases, a well-defined structure resembling normal skin had been established. The structures were clearly distinguishable from nearby host tissue by different levels of staining in the dermis and on occasions an incomplete hypodermis (Fig. 4.35A). In these sites the dermis was fully reconstructed and populated with a great number of dermal cells surrounded by an extracellular matrix which was rich in fibrillar networks. The dermal cells nearest the

epidermis were denser than the lower regions (Fig. 4.35B). The epidermis was well organised, sometimes thicker than its neighbouring epidermis and composed of basal and supra-basal cell layers comparable to that of nearby regions. The basal cells had a polygonal or oval shape as found in normal epidermis and distally the cells had flattened and keratinized. Downgrowths of epidermal cells were occasionally observed penetrating the dermis, giving the dermo-epidermal junction a highly sculptured profile, a character not observed in neighbouring regions (Fig. 4.35B).

The punch biopsy sites were generally lacking pelage-like follicles, although in a few sections one or two such follicles were also observed (Fig. 4.35A). These follicles like regular pelage follicles were small and demonstrated rounded dermal papillae containing a few cells. In the punch sites where cultured LDS/ORS (Fig. 4.36), SF/ORS (Fig. 4.37) or LDS/DP/ORS (Fig. 4.38) cells were implanted in the absence of intact DPs no vibrissa-like follicles were observed at a histological level. However, the punch sites that had received additional dissected DPs (DS/ORS + DPs) exhibited vibrissa-type follicles aligned through different planes, showing a marked difference in size compared to adjacent pelage follicles (Fig. 4.39A). The vibrissa-like follicles were large and their bulbs were found in various regions. Some were located close to the skin surface and others were seen deep into the dermis. These follicles had vibrissa-sized dermal papillae containing many more cells (Fig. 4.39B) than are usually found in pelage follicle papillae (Fig. 4.39C). Some papillae displayed evidence of having elongated apices as seen in anagen vibrissa papillae in situ. The papilla cells were well spaced and stained positively with Alcian blue, an indication of a glycosaminoglycan rich extracellular matrix. Broad epidermal matrices producing vibrissa-like hair shafts surrounded the papillae. A thick layer of dermal sheath could be seen around the vibrissa follicles (Fig. 4.39D) but there was no indication that these follicles had formed a collagen capsule, characteristic of vibrissa follicles. In some sections sebocytes were also found associated with and attached to the upper half of the induced hair follicles (Fig. 4.39E). Some implanted papillae did not appear to interact with the ORS cells to form follicular structures, but they remained visible as a clump of closely packed dark cell nuclei (Fig. 4.39F) surrounded by a small amount of ECM (Fig. 4.39G).

4.3.2 Characteristics and interactive abilities of dermal cells in other skin appendages

4.3.2.1 Claw unit

i) Morphology

Claw is produced as a result of continuous epidermal-dermal interactions occurring at the posterior tip of the digits. The dermis or papilla that interacts with the epidermal matrix (site of cell production) rests above the dorsum of the distal phalanx. The papilla displayed similar characteristics to the anagen follicle papilla. It was populated with fibroblast-like cells, which were well-spaced in an ECM (Fig. 4.40A). The ECM stained with Alcian blue, an indication that it was rich in GAGs, similar in characteristic to the hair follicle papilla. Above the dermis there was an epithelium which was virtually a continuation of the skin epidermis whose cell layers underwent differentiation towards the surface. As the epidermal cells moved upward they became larger, flattened, elongated and enucleated. They finally made a thick nail plate which was composed of compacted keratinized cells (Fig. 4.40B).

ii) Cell culture

Cultures of claw papilla cells were first observed 3 days after incubation. Cells began to grow from the explant tissues 3-4 days after cultivation and extended from the explants in all directions (Fig. 4.40C). Unlike follicular dermal papillae, the explant tissues of claw papillae did not lose their structure and cells grew from peripheral regions of the tissue. Despite some variations, cells were generally small, less flattened and spindle-like. Initially no difference could be seen in terms of their size or density in areas close to the original tissue explant compared with those at the peripheral regions. Nevertheless, after 2-3 days, cells proximal to the tissue explant were denser than those at periphery (Fig. 4.40D). Cells at the peripheral region of the outgrowth had multiple lamellipodia implying that they were actively migrating. Some cells which were isolated from the main body were longer with extended lamellipodia (Fig. 4.40E). Furthermore, in comparison to follicular dermal cells, the claw papilla cells displayed a greater proliferative activity, and dividing cells were abundant in the outgrowths (Fig. 4.40F). As a result of these high rates of cell division and cell movement, the expansion of claw

cells around the explanted tissue was quick. This in turn resulted in coverage of the whole available substrate in a short time, so that cells from 7-10 tissue explants covered an entire dish within two weeks (Fig. 4.40G). Cells maintained this trait even after several passages, where they reached confluence a few days after subculturing when split one to three. At the confluent stage, claw cells, like hair follicular dermal papilla cells, aggregated and produced clumps, however, these cell clumps were not as marked as those seen in their hair follicular counterparts (Fig. 4.40H).

iii) Immunolabelling of claw dermal cells with ASMA antibody

In order to investigate similarities between claw and hair follicle papilla cells, cultured claw cells at passage 2 or 3 and in a subconfluent state were stained with α -smooth muscle actin antibody. Approximately 85-90% of the cells at passage 2 or 3 stained positively with the antibody, which was expressed throughout the cytoplasmic microfilaments in distinct patterns. Although, staining was visible throughout the entire cytoplasm it appeared that filaments in the lamellipodia stained more intensely than the rest of the cytoplasm (Fig. 4.40I). In agreement with observations by Jahoda *et al.* (1991) on follicular dermal cells, in claw cells, the antibody was also found to be expressed more intensively in flat and well-spaced cells than packed cells.

iv) Interactive abilities of claw dermal cells

In order to evaluate their interactive capabilities, cultured claw papilla were labelled with DiI and later implanted into ear wounds or the upper part of transected vibrissa follicles.

Histological analysis of ears that had received stained claw papilla cells for 7-10 days showed the area of cell implantation was clearly distinguishable because of the persistence of disrupted tissues at the wound sites (Fig. 4.40J). However, no tissue organisation was observed at the site of the implanted cells. To investigate the distribution of claw cells in the tissue, the operation sites were examined under a rhodamine fluorescence filter. Implanted cells were mainly found in the deeper matrix as clumps of cells, though some scattered cells were observed along the edges of the wounds (Fig. 4.40K). However, once again, no sign of follicle formation or any other organisation was visible in association with these cell clumps or single cells. Moreover,

no implanted cells were found to be involved in the structure of host tissues and their appendages.

Early examination of the experimental follicles at 20 days post-operation showed that the base of the follicles had become sealed by the deposition of scar tissue, apparently from the implanted cells. No hair or other structures had emerged from the follicles. Histological observations revealed no sign of hair regeneration or that the initiation of unusual skin appendage formation was taking place in the follicles (Fig. 4.40L). Fluorescence examination confirmed that although some stained cells were found scattered in the upper part of the follicles, the main body of implanted cells had remained at the lower end, around the site of implantation (Fig. 4.40M). As a result, no sign of any new organised structure was found in these follicles.

4.3.2.2. Feather follicles

i) Histology

Growing pigeon feather follicles displayed histological features quite similar to those of anagen hair follicles. A large body of dermal cells was observed in the middle of the follicle (Fig. 4.38A). This dermal structure was comprised of two regions (lower and upper) although their border was not completely distinct in the histological sections. The lower region called the papilla (Welty, 1962) was composed of fibroblastic cells within the extracellular matrix (ECM). Unlike the hair papilla, the ECM of the feather papilla did not stain with Alcian blue (which stains GAGs) and appeared red. Except for its basal stalk the papilla was surrounded by a thick and multi-layered epidermal compartment whose cells were small, densely packed and displayed a strong basophilic reaction, when stained with haematoxylin. This epidermal component, termed the collar, is the region that gives rise to the feather by cell proliferation (Fig. 4.41B). The upper three-quarters of the dermal body named the pulp (Figs. 4.41A & C) which indeed is made of ECM components secreted by the papilla cells. The pulp was enveloped by an epithelium that was thinner than the collar but which contained pigment cells adjacent to the pulp. Large blood vessels entered the follicle through the papilla basal stalk and continued centrally through the papilla and the pulp (Fig. 4.41D).

Most of the feather follicles from adult birds were in a non-growing state and their morphology differed considerably from that of the growing follicles. Non-growing

follicles only had a dermal papilla containing very packed dermal cells at the base of the follicle (Fig. 4.41E). The pulp was not observed in these follicles as during transition from growing to non-growing state it dies and is resorbed. Papillae from non-growing follicles had a wide basal stalk, through which they were joined to the surrounding connective tissue. The papillae was surrounded laterally and at the top by an epidermal compartment which was mainly acellular and keratinized (Fig. 4.41E). This keratinized body (feather shaft), both on the inner side (close to the papilla) and outer side (close to the follicle connective tissue), was covered by thin layers of non-keratinized epithelial cells. These epithelial layers became thicker towards the tip of the collar which is termed papillar ectoderm (Fig. 4.41F). At this point two epithelial layers joined together. The cells of the epithelial layer were very tightly attached to the side of the dermal papilla so that even after dissection of this component the epidermal cells remained attached. The epithelial layer that was lateral to the collar continued upward as a thin layer and finally merged with the skin epidermis (Fig. 4.41G).

For further observation, the dermal papilla of adult sparrow feather follicles (non-growing) were dissected and processed for EM. Examination of semi-thin sections revealed that the papillae were composed of fibroblastic cells distributed more or less in a circular pattern around large central blood vessels (Fig. 4.41H). Ultrastructurally, the dermal cells displayed various morphologies, but were mainly elongated with a few cytoplasmic projections (Fig. 4.41I). The cells possessed large nuclei, which constituted a high proportion of the cell volume, with little cytoplasm. The dermal cells were surrounded by an extracellular matrix rich in fibrils particularly fibrous collagen (Fig. 4.41I). The large blood vessels at the centre of the papillae exhibited a very thick wall consisting of three discrete layers (tunica), the intima, media and adventitia (Fig. 4.41J). The papilla cells were separated from outer epithelial cells by a thick basement membrane rich in fibrous elements (fig. 4.41K). On the epithelial side, the epidermal cells were found to be small with round nuclei and were closely associated with and well attached to the basement membrane. Within the epidermis several dendritic cells were also observed which were closely connected to their neighbouring epidermal cells (Fig. 4.41L-M). They were possibly dormant melanocytes which would serve as producers of pigment for the next feather generation. Similarly, the residual epidermal cells may give rise to the new collar and feather shaft in the next growing phase.

ii) Feather follicle papilla cell culture

As mentioned above, the feather papilla is tightly associated with its surrounding epidermal cells, making separation difficult. For culture purposes to rupture the dermal-epidermal junction, the dermal papillae were isolated along with their attached epidermal cells and placed into dispase solution for a few minutes. Early attempts to grow papilla isolated in this fashion were not successful because the explant tissues did not attach to the substrate. Consequently, to help the tissues stick to the dish, they were subsequently cultured under sterile coverslips. The coverslips remained on the cultures until the cells were subcultured.

Five to six days after cultivation, cell outgrowths were seen in a circle around the tissues. Cells displayed a normal mesenchymal appearance with a spindly morphology and dendritic cytoplasmic processes. Cells in the most peripheral region of the outgrowth were flat and quite dispersed but towards to the centre they gradually became smaller and more closely packed (Fig. 4.42A). Cells attached to the tissue explants were very small in size and dense. These cells grew slowly and it took 30-45 days before cell numbers were high enough for them to be passaged. After passaging, they displayed an unusual behaviour in that only about one third of the cells attached to the substrate while the remaining cells stayed floating in the medium. Moreover, after some time a percentage (25-30%) of the attached cells also started to die while the remaining cells looked healthy and were proliferating (Fig. 4.42B). The dying cells were not confined to specific areas but could be seen in all areas of the culture dish. Areas of cell death were continually observed in these cultures even after multiple passaging of the cells. Nevertheless, cell proliferation always outpaced cell death, which enabled the cell lines to be grown for several generations. Feather papilla cells displayed a degree of aggregative behaviour similar to their hair follicle counterparts, but it was less marked, particularly when compared to vibrissa follicle papilla cells.

iii) *In vivo* and *in vitro* immunolabelling with ASMA antibody

When growing pigeon feather follicles were dissected and labelled with ASMA (Fig. 4.43A) the dermal papilla cells expressed the antibody at a high level but the pulp was negative. The antibody also labelled the wall of the blood vessels, seen as two

distinct lines, rising to the upper part of the follicle in the pulp. Labelling was also detected in the feather connective tissue which surrounded the follicle like a sleeve, but as has been shown for the vibrissa hair follicle only the lower region of this tissue stained with the antibody. In particular the part of the tissue below and around the lower sides of the papilla was highly marked. The expression of the antibody in the connective tissue surrounding the follicle terminated abruptly, so that no staining was observed in the connective cells located around the middle part of the follicle. Apart from the dermal compartment of the follicle, the antibody was strongly expressed within the feather muscle attached to the upper part of the follicle (Fig. 4.43A).

In vitro labelling of cultured pigeon papilla cells revealed that only around 25-30% of these cells were positively labelled by ASMA antibody at passage 2 (Fig. 4.43B). Cells that were larger and flatter stained very intensely with the antibody. These cells, which constituted about 10% of all positive cells, were found individually or in small groups among the unlabelled cells. Subsequent passaging did not appear to have any significant effect on the level of expression, which was similar in cells after the third passage.

iv) Implantation of pigeon feather papilla into the upper part of the hair follicle

Histological analysis revealed that the implanted feather papillae were still evident inside the hair shaft of the amputated vibrissa follicles (Fig. 4.44). Around the implanted tissue the epidermal ORS cells of the hair follicle were also visible attached to feather papilla. However, there was no indication of any interaction between the feather dermal papilla and vibrissa epidermal tissues as no cellular organisation was found in the experimental follicles.

4.3.3. Organ culture of the plucked end bulb of hair follicles

The plucked vibrissa end bulbs were examined at 7 and 15 days after culturing. Of 24 experimental end bulbs which were examined after 7 days, external fibres were observed in none (Fig. 4.45). External examination did not show any significant changes, but in some pigmented follicles a black material inside the bulbs had increased compared to their initial state. Histological examination showed that in nine of them a new epidermal matrix had formed around the dermal papilla (Fig. 4.45A). However, the

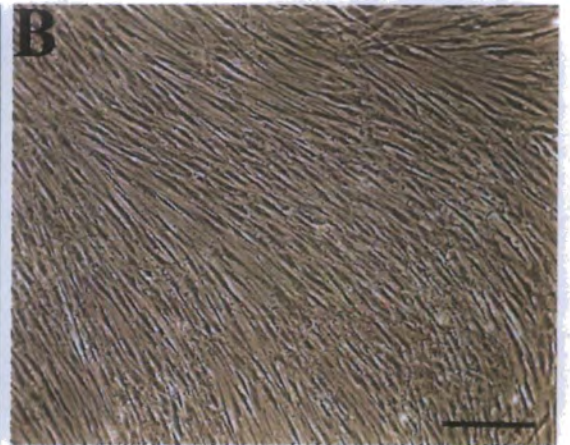
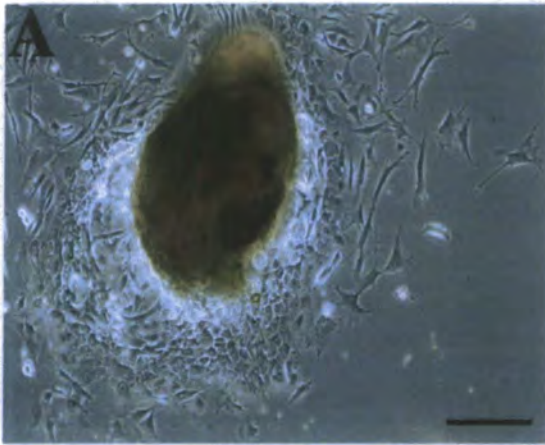
matrix contained dead cells, in a manner that cells close to the papilla had disintegrated and cells close to the dermal sheath had keratinized. The dermal papillae had moved upward slightly and were completely surrounded by the matrix. They had become reduced in size and contained fewer cells in comparison to normal follicles *in vivo*. The remaining 15 follicles demonstrated no regeneration of matrix (Fig. 4.45B). Some of these follicles displayed disorganised structures, the dermal and epidermal cells had been displaced and were hardly distinguishable (Fig. 4.45C).

Of 36 follicles that were examined after 15 days in culture, 28 follicles did not produce fibres or any external projections. However, in 8 follicles small projections could be distinguished emanating from the bulbs. The length of these projections rarely exceeded 0.5 mm and sometimes they were associated with sheaths (Figs. 4.46A-B). Histologically, these follicles displayed a different morphology. Five follicles displayed a cellular architecture which was similar to that seen in telogen follicles *in vivo* (Fig. 4.46C-D). Their dermal papillae were small and compact with reduced intercellular spaces. The matrices were very thin and restricted to a single layer of epidermal cells around the papilla, but in two instances the epidermal cells were indistinguishable. In these follicles the regenerated fibre had moved away from the bulb region. In three other follicles the matrix epidermal cells were found to be dead in a similar manner to that described above for follicles after 7 days (Fig. 4.46E). To determine the nature of the external projections in these follicles, they were examined by electron microscopy. It was revealed that these projections are composed of keratinized epidermal cells similar to that seen in the matrix (Fig. 4.46F and H). Numerous desmosomes were found at the interface of these cells (Fig. 4.46G). Interestingly, although in these follicles the epidermal part had deteriorated, the dermal sheath cells were found to be completely healthy with large nuclei and cytoplasm rich in organelles (Fig. 4.46I).

Fig. 4.4) Phase contrast micrographs of rat skin fibroblasts in culture. **A)** Both dermal (long and spindle-like) and epidermal cells (small and round) have emerged from a skin explant after 10 days in culture. **B)** Swirls of parallel, spindle-shaped rat skin fibroblasts, passage 3. Scale bars 200 μ m.

Fig. 4.5) Phase contrast micrographs of rat vibrissa dermal papilla cells in culture. **A)** Dense papilla cells have emerged from all sides of papilla explants after 6 days in culture. **B)** Papilla cells in a long maintained primary culture (two months) that have produced a tube-like structure. **C)** Dermal papilla cells form multi-layered aggregates at confluence. Scale bars A and C 200 μ m and B 40 μ m.

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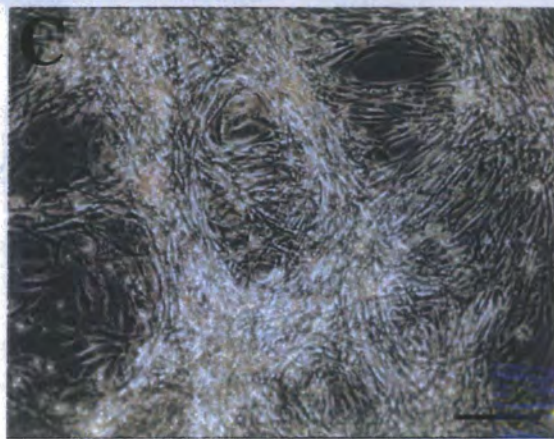
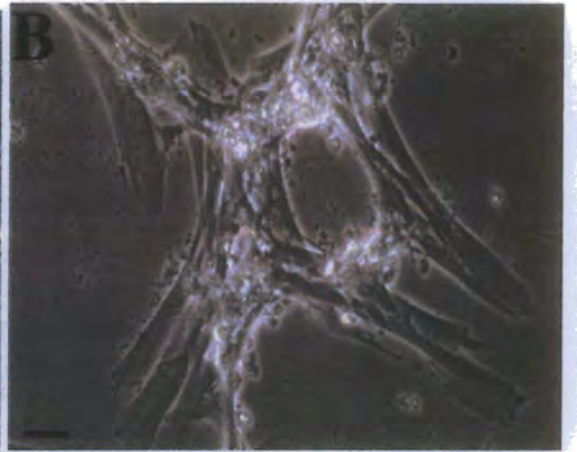
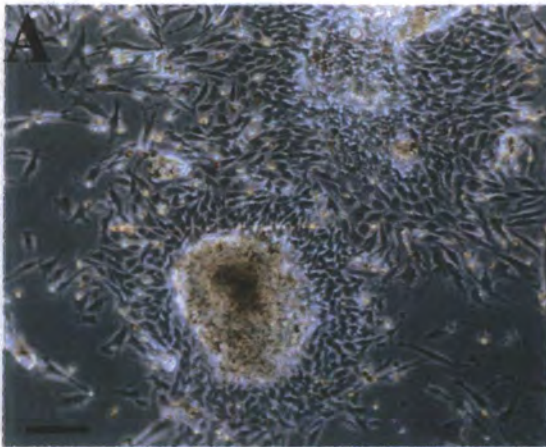
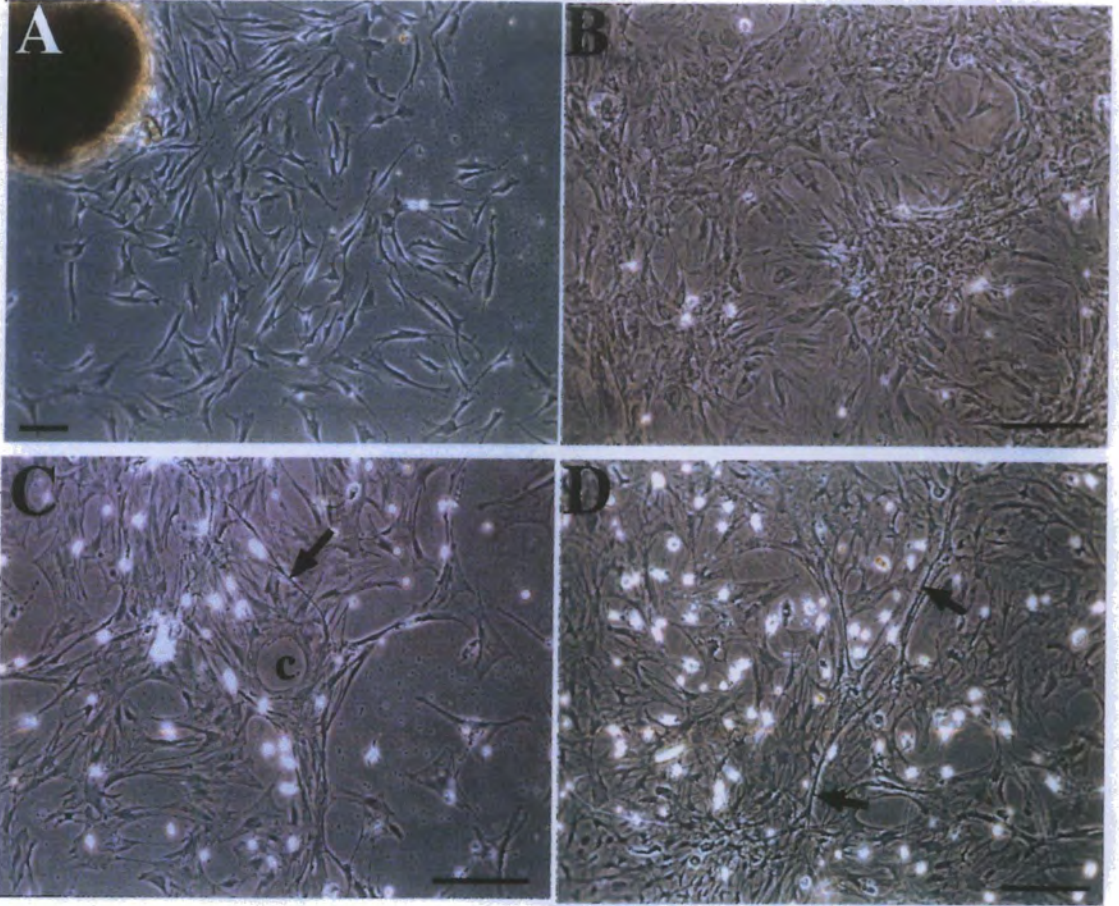


Fig. 4.6) Phase contrast micrographs of rat vibrissa lower dermal sheath cells in culture. **A)** Sparse dermal cells emerging from a tissue explant after 8 days in culture. **B)** Dermal sheath cells showing a degree of aggregation at confluence. **C-D)** Variation in morphology of cells growing from lower dermal sheath explants cultured under glass coverslips. Some cells displayed a morphology somewhere between that of dendritic and muscle type cells (arrows). Cells also often produced organised structures (c). Scale bar A 50 μ m and B-D 100 μ m.

Fig. 4.7) Phase contrast micrographs of rat vibrissa upper dermal sheath cells in culture. **A)** Dermal cells emerging from a tissue explant after 7 days in culture. **B)** The upper dermal sheath cells show less marked aggregative behaviour at confluence than the lower dermal sheath cells (Fig. 4.6B) above. Scale bars 100 μ m.

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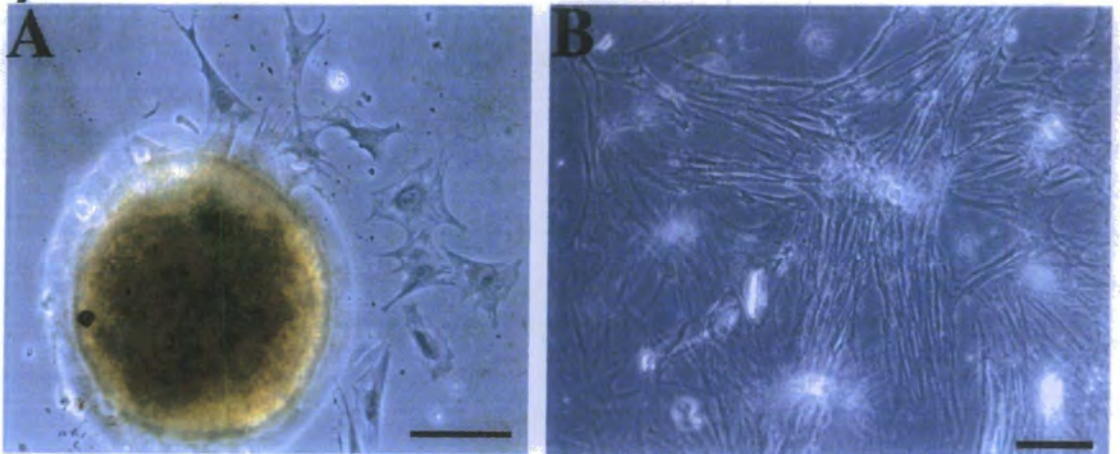


Fig. 4.8) Flow cytometric histograms displaying the distribution (left) and the percentage expression of MHC-I (right) in rat skin fibroblasts. 97% of the cells expressed MHC-I. Passage four.

Fig. 4.9) Flow cytometric histograms displaying the distribution (left) and the percentage expression of MHC-I (right) in rat dermal papilla cells. Passage four.

Fig. 4.10) Flow cytometric histograms displaying the distribution (left) and the percentage expression of MHC-I (right) in upper dermal sheath cells. Passage four

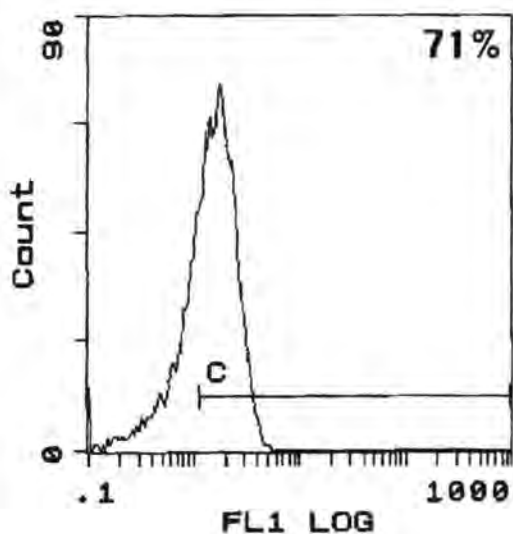
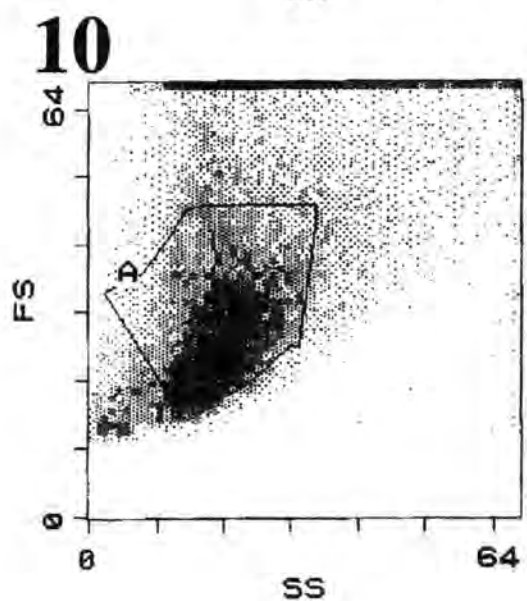
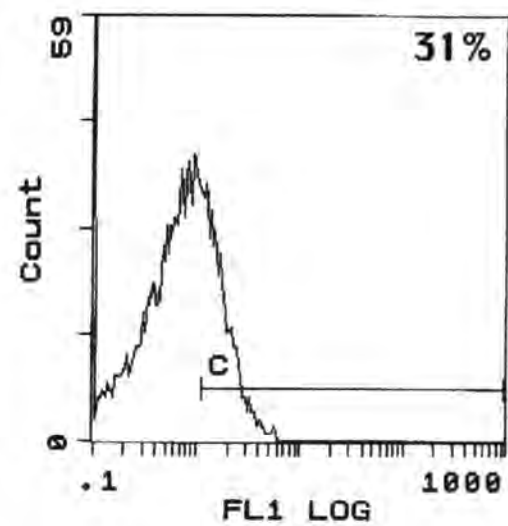
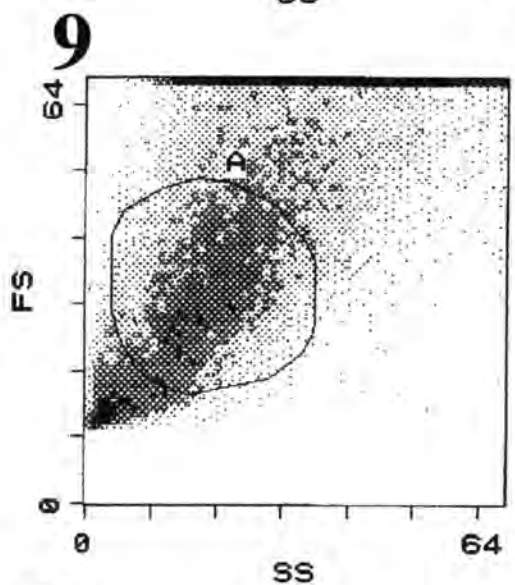
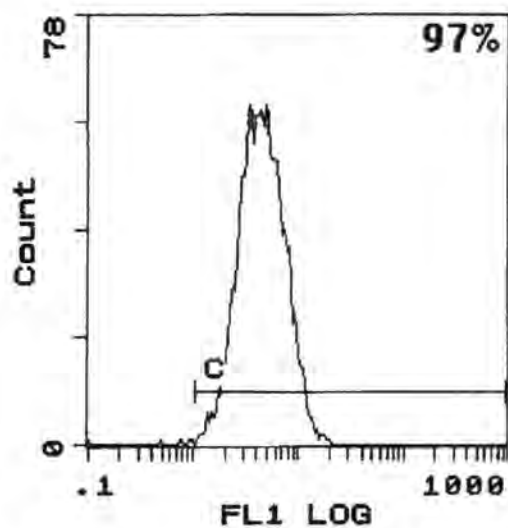
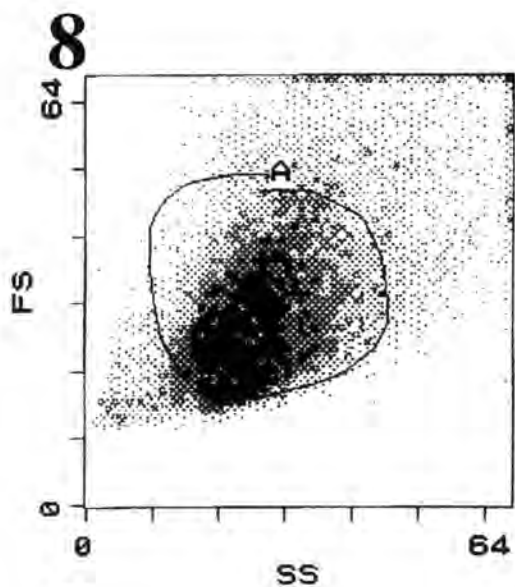


Fig. 4.11) Flow cytometric histograms displaying the distribution (left) and the percentage expression of MHC-I (right) in lower dermal sheath cells. Passage four. A) When the gating was set up to include the majority of cells 63% of cells expressed MHC-1. The smaller and less granular cells (B) expressed MHC-I at a lower level (42%) compared with the larger and highly granular cells (C) in which 71% of cells were positive for MHC-I.

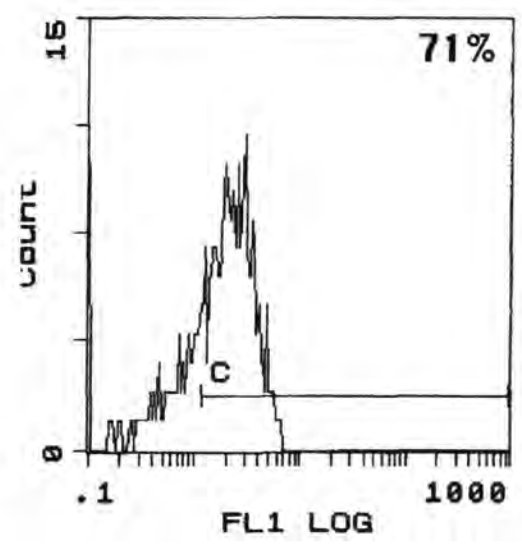
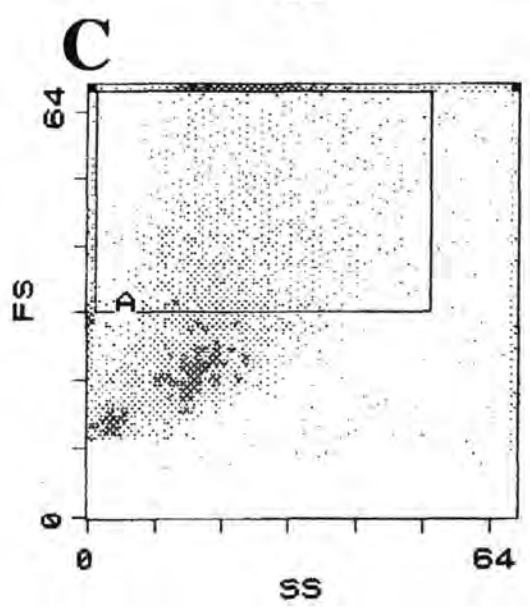
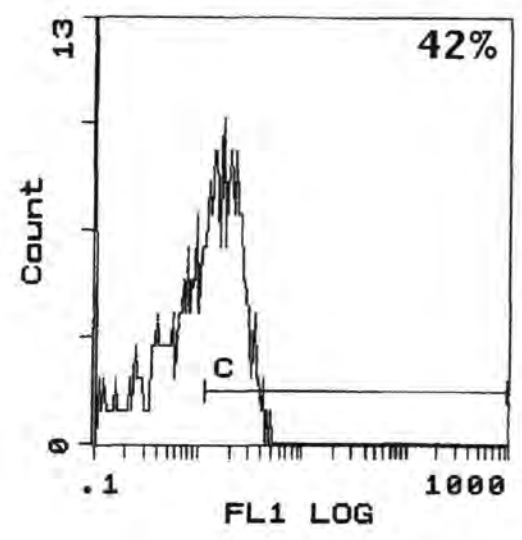
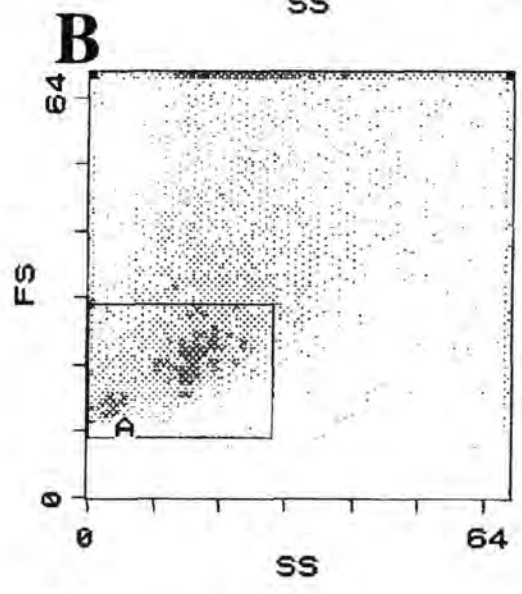
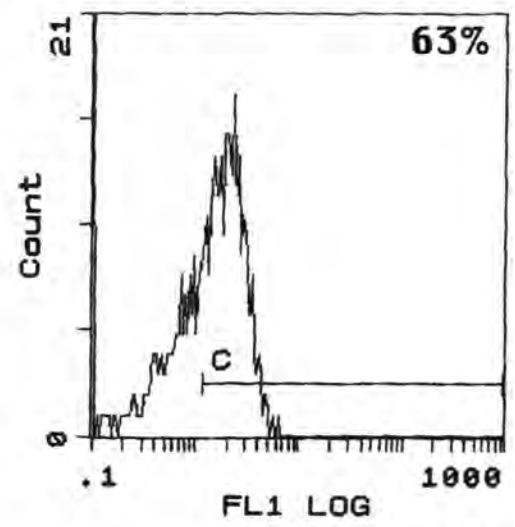
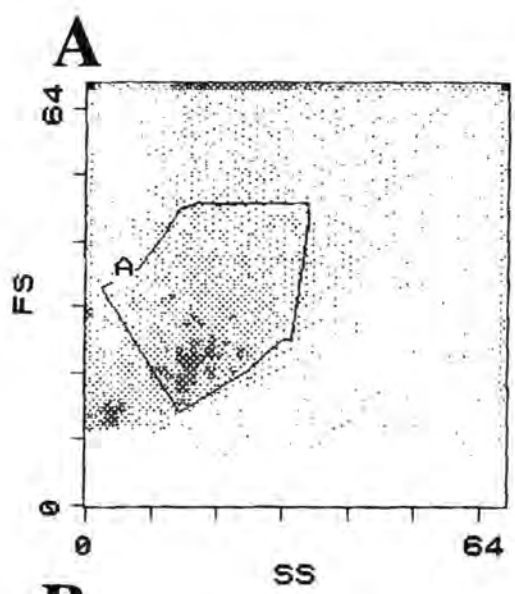


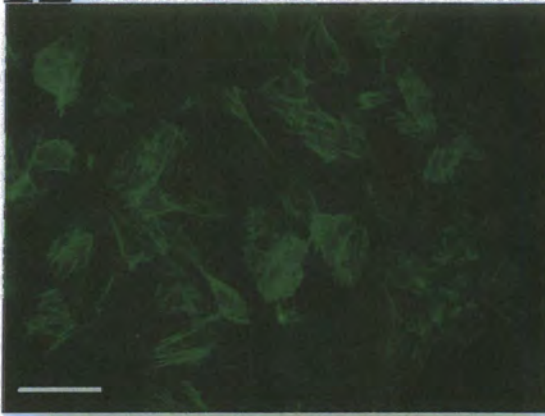
Fig. 4.12) Immunofluorescence staining of rat vibrissa lower dermal sheath cells (passage two) with ASMA antibody. The majority of cells were ASMA positive. Scale bar 50µm.

Fig. 4.13) Immunofluorescence staining of rat vibrissa upper dermal sheath cells (passage two) with ASMA antibody. A) Compared with LDS cells fewer of these stained positively with ASMA. B) ASMA was normally expressed more strongly in larger and flatter cells (B). A flattened cell showing stronger staining at its periphery than closer to the nucleus (C). Scale bars A 50µm, B and C 25µm.

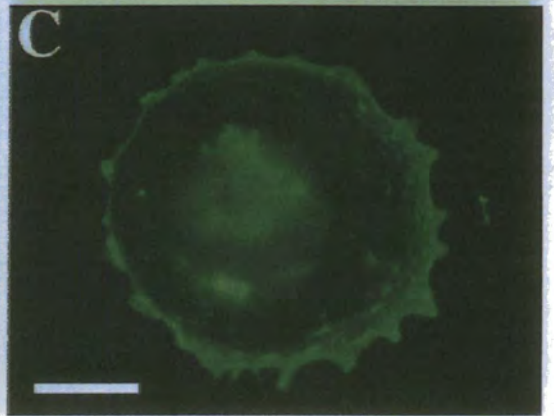
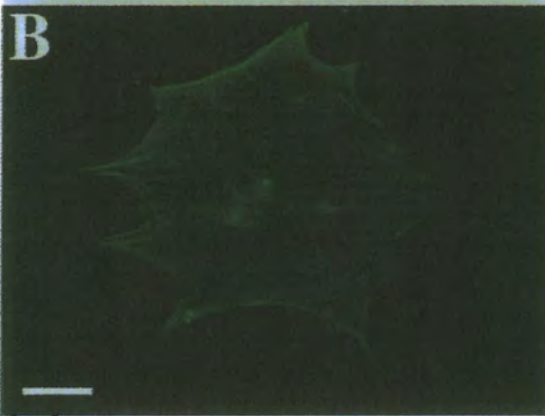
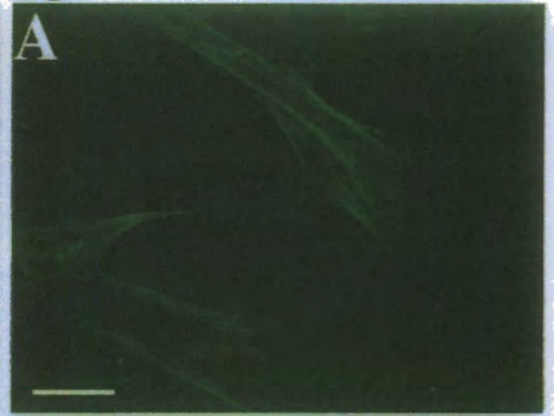
Fig. 4.14) Immunofluorescence labelling of lower dermal sheath cells with laminin. Lower (A) and higher (B) magnification. Laminin is expressed intensively intracellularly (arrow). Note that there are patches of positive extracellular material. Scale bars A 100µm and B 50µm.

Fig. 4.15) Immunofluorescence labelling of lower dermal sheath cells with collagen type IV. Lower (A) and higher (B) magnification. Cells are predominantly positive for this basement membrane component which is also expressed in extracellular matrix. Scale bars 100µm and B 50µm.

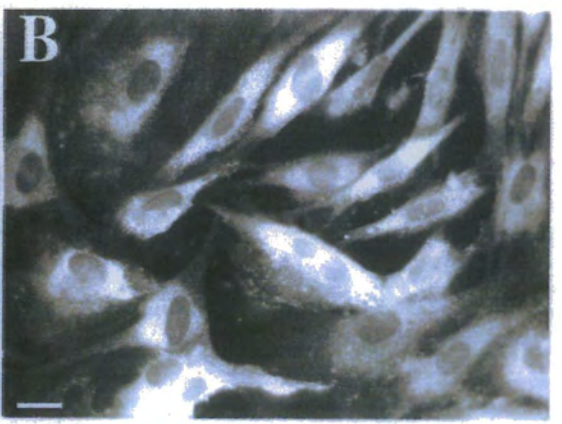
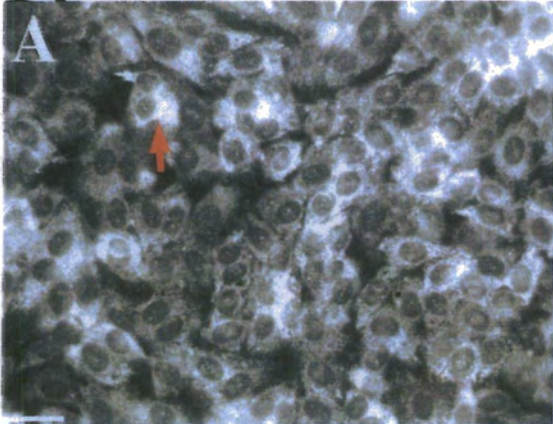
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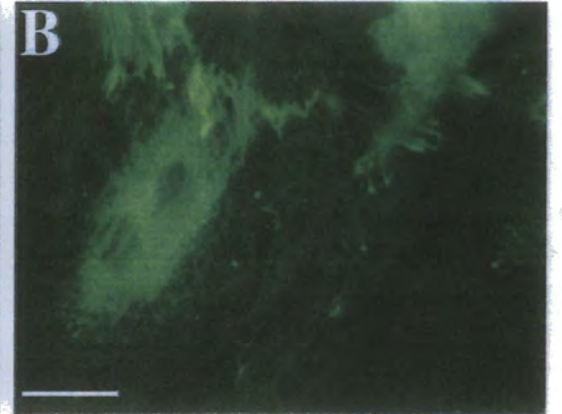
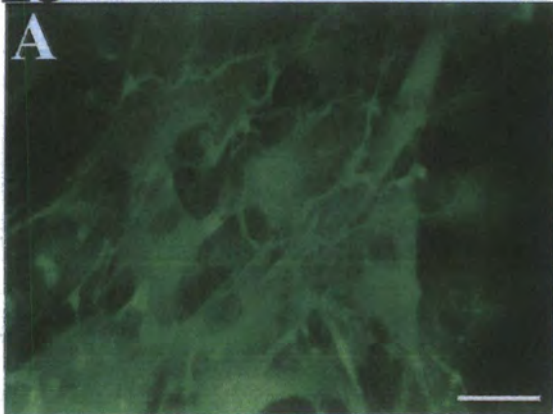
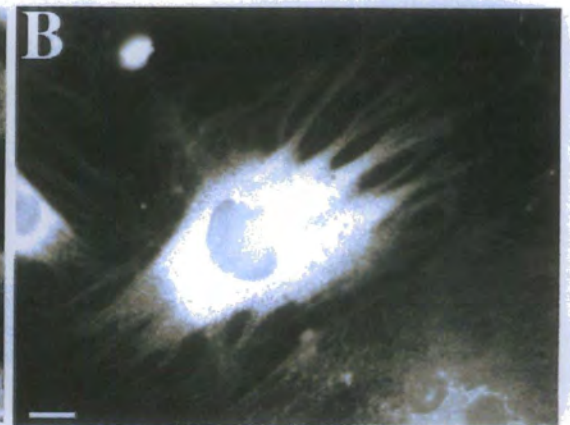
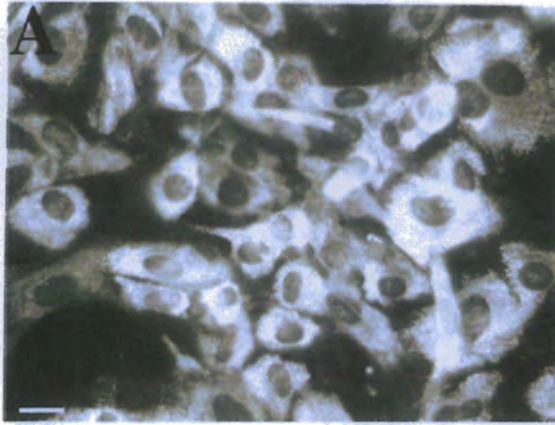


Fig. 4.16) Collagen type I-expressing cells derived from the lower dermal sheath. Low (A) and higher (B) magnification. Scale bars A 50 μ m and B 25 μ m.

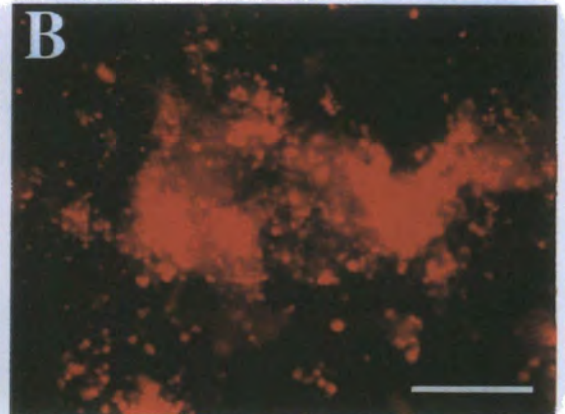
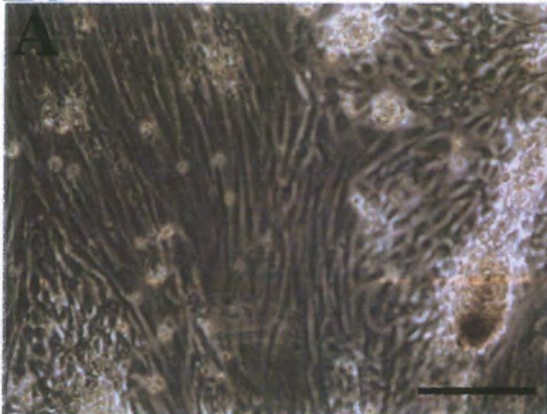
Fig. 4.17) Phase contrast (A) and fluorescence (B) micrographs of a combination of DiI-stained follicle-derived dermal/epidermal cells after 12 days in culture. Cells are healthy and display no sign of deterioration. Scale bars 200 μ m.

Fig. 4.18) Phase contrast (A) and fluorescence (B) micrographs of a region of an ear wound that had received DiI-stained fibroblasts. Implanted fibroblasts are seen as scattered red spots within the dermis (der). A few patches of stained cells are also seen at the epidermal (arrows) surface. These most likely represent implanted cells that have remained outside of the wound and dried. Scale bars 200 μ m.

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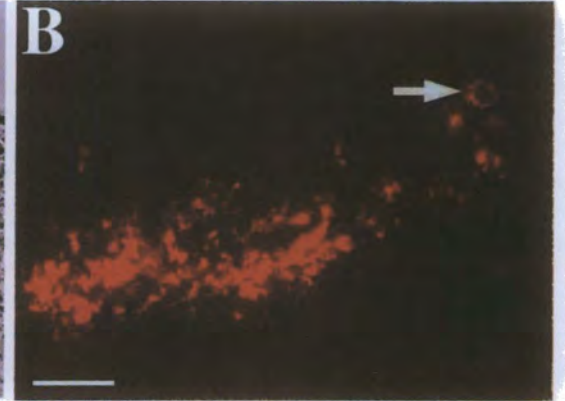
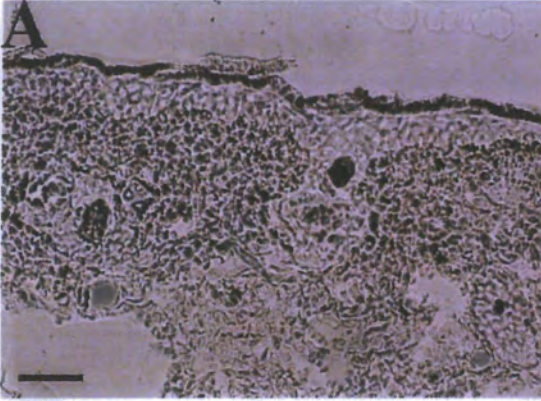
Fig. 4.19) Phase contrast (A) and fluorescence (B) micrographs of a region of an ear wound that had received DiI-stained lower dermal sheath cells. Implanted cells are seen within the structure of a local hair follicle (arrow). Scale bars A 200 μ m, and B 100 μ m

Fig. 4.20) Phase contrast (A) and fluorescence (B) micrographs of a region of an ear wound that had received DiI-stained upper dermal sheath cells. In contrast to the lower dermal sheath cells (Fig. 4.19) no implanted cells are seen contributing to hair follicles. Scale bars A 200 μ m and B 100 μ m.

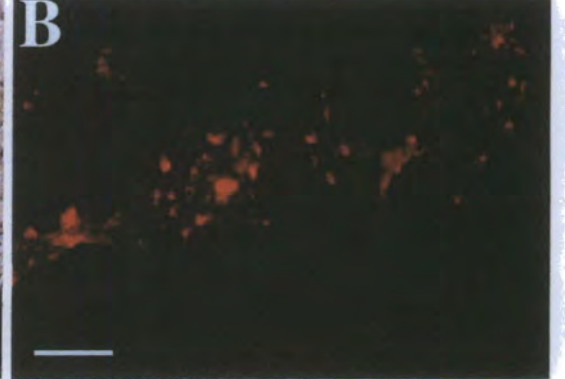
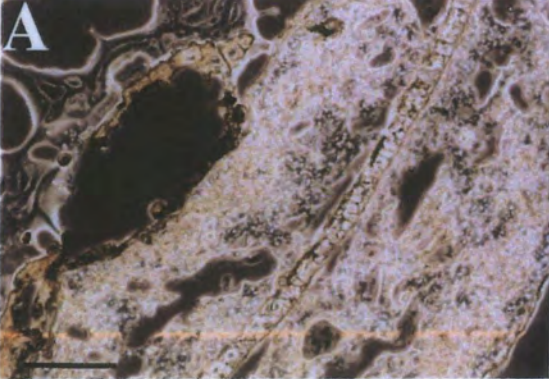
Fig. 4.21) Micrographs show the external (A) and internal (B) surfaces of punch biopsy implantation sites (arrows) that had received DiI-stained cells. Punch sites one week after operation have not repaired yet and DiI-stained cells can be seen within them (A). Internally the punch areas were clearly distinguishable from their surrounding tissues by their red-coloured appearance even after two weeks. Scale bars A 12mm and B 4mm

Fig. 4.22) Phase contrast (A) and fluorescence (B) micrographs of punch biopsy sites that had received DiI-stained upper dermal sheath cells. Cells were seen as clumps (B) or scattered within the dermis (der), but they were not found in hair follicles. Scale bars 200 μ m.

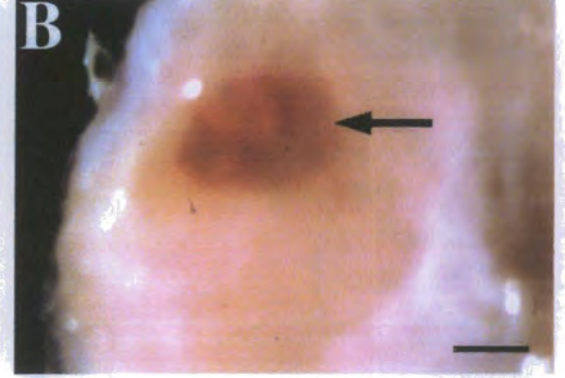
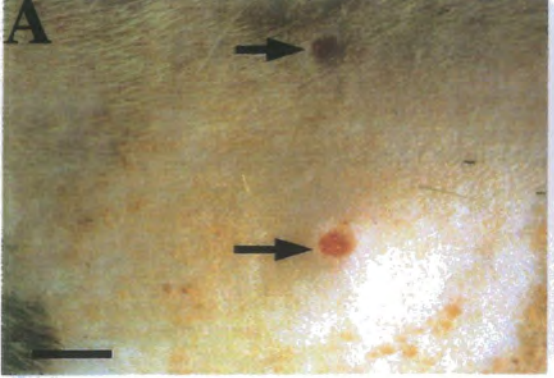
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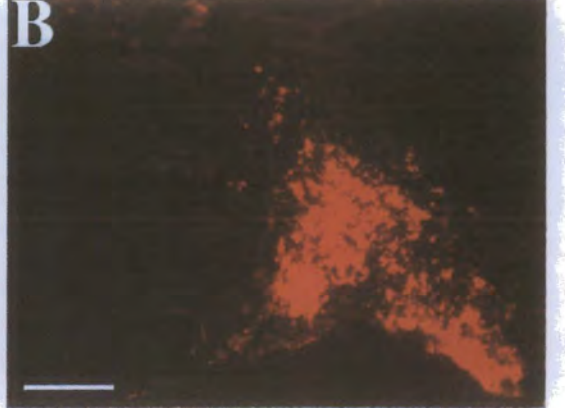
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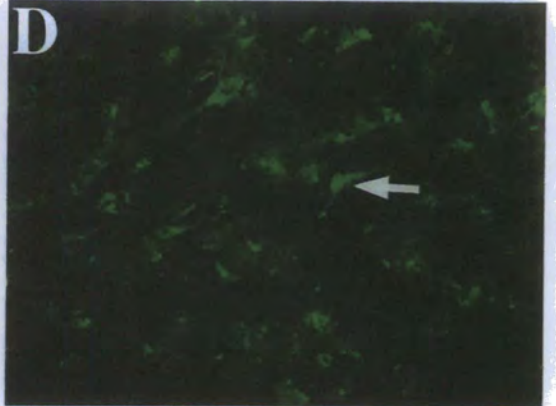
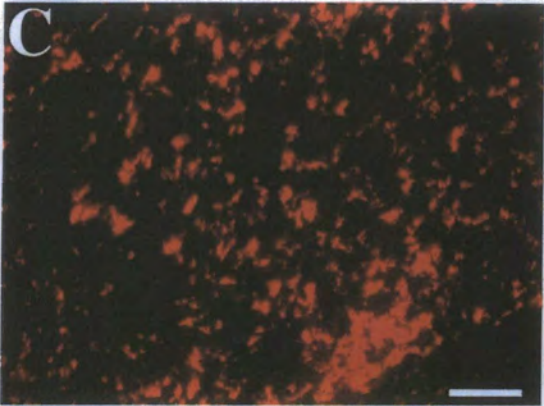
Figs. 4.22C-D)

C) Higher magnification micrograph of a punch site that had received DiI-labelled upper dermal sheath cells. Implanted cells are seen scattered in the dermis. D) Confocal laser microscopy revealed that the red spots corresponded with cellular structures (arrow). C 75 μ m.

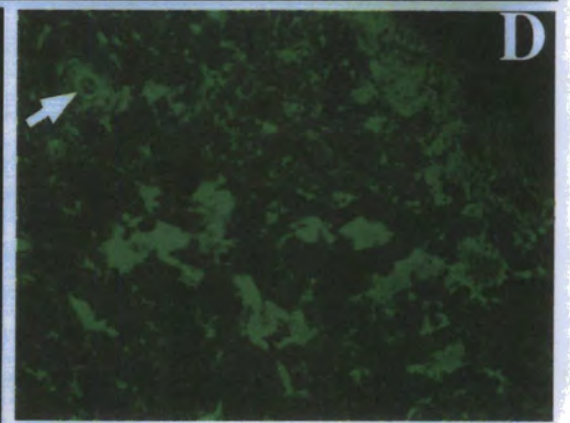
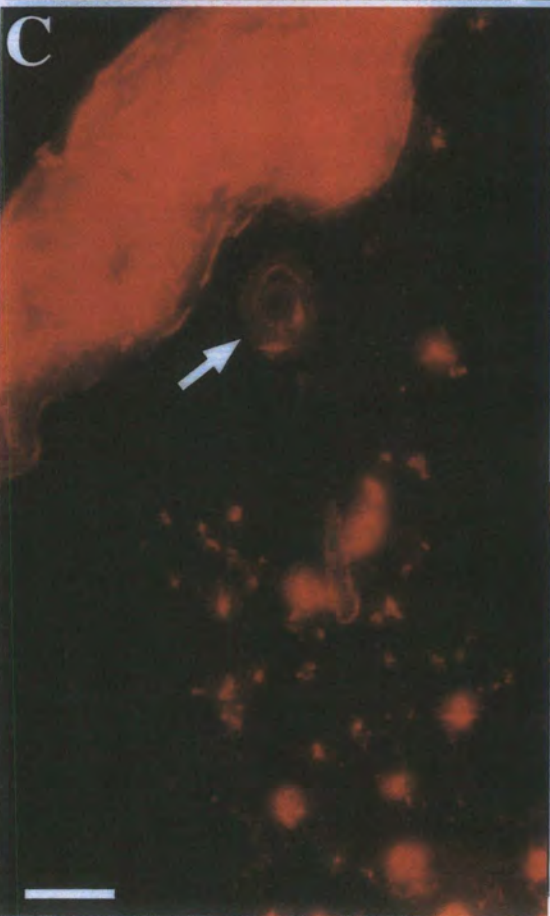
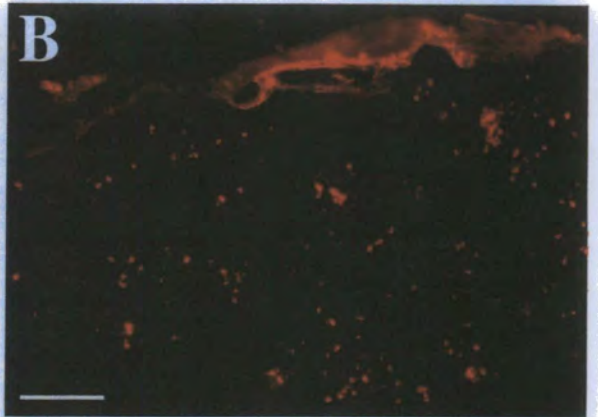
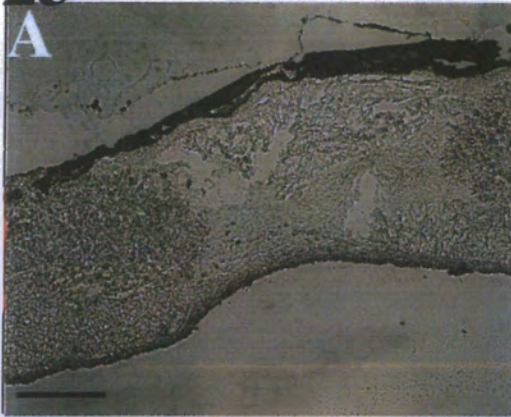
Fig. 4.23) Phase contrast (A), fluorescence (B-C) and confocal (D) micrographs showing a punch biopsy site that had received DiI-stained lower dermal sheath cells. Dermal cells are widely distributed (B) and labelled cells are seen within the connective tissue of local pelage hair follicles (arrow in C), the incorporation of sheath cells into local hair follicles is also seen (arrow) on the confocal micrograph (D). Scale bars A 300 μ m, B 150 μ m and C 50 μ m.

Fig. 4.24) Fluorescence micrograph of a punch site that had received labelled skin fibroblasts. As with the upper dermal sheath cells no staining is seen in the hair follicles. Scale bar 100 μ m.

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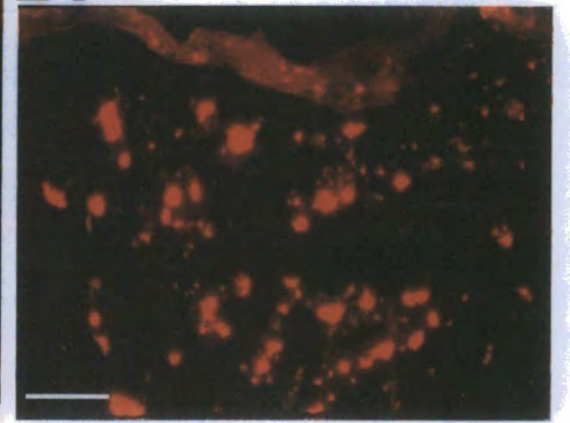


Fig. 4.25) A silicon chamber with its recombinant tissue 20 days after implantation into a host rat. Scale bar 1.5mm.

Fig. 4.26) Semi-thin sections through a lower dermal sheath-epidermal ORS cells recombination which was implanted for 3 days onto the dorsum of a host rat. **A)** At lower magnification distinct dermal (arrow) and epidermal (epi) cell layers are clearly visible. Epidermal cells at the surface are very scattered but closer to the dermis they become dense. **B)** At higher magnification the dermis (der) is clearly multi-layered but the epidermis does not display a normal stratified arrangement. The lower part of the collagen gel (g) is free from any cells at this stage. Resin embedded sections stained with toluidine blue. Scale bars A 100 μ m and B 25 μ m.

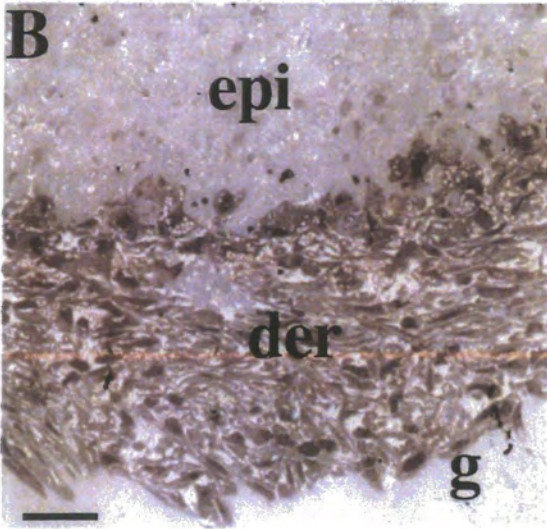
Fig. 4.27) Morphology of a tissue reconstructed from recombination of dermal sheath cells with epidermal ORS cells after 7 days in vivo. Both epidermis (epi) and dermis (der) are evident. At this stage the epidermal cells are more organised but have a fewer cell layers. Resin embedded section stained with toluidine blue. Scale bar 25 μ m

Fig. 4.28) Morphology and ultrastructure of a skin-like structure formed by recombination of lower dermal sheath cells with epidermal ORS cells after 20 days in vivo. **A)** A low power micrograph displaying the general structure of the reconstructed tissue. The collagen gel has become densely populated with dermal sheath cells and produced a normal-looking dermis (der). The epidermis (epi) is thick and contains multiple cell-layers. Within the epidermis and dermis a few epidermal spheroids (arrows) are seen. Resin embedded section stained with toluidine blue. Scale bar 100 μ m.

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B

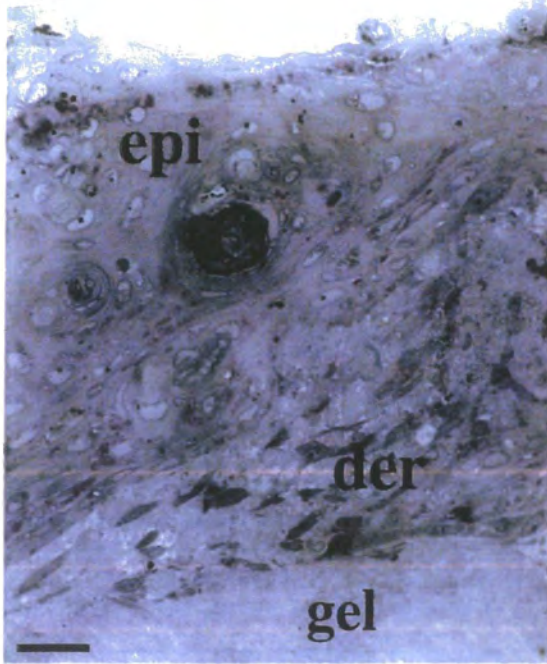


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A



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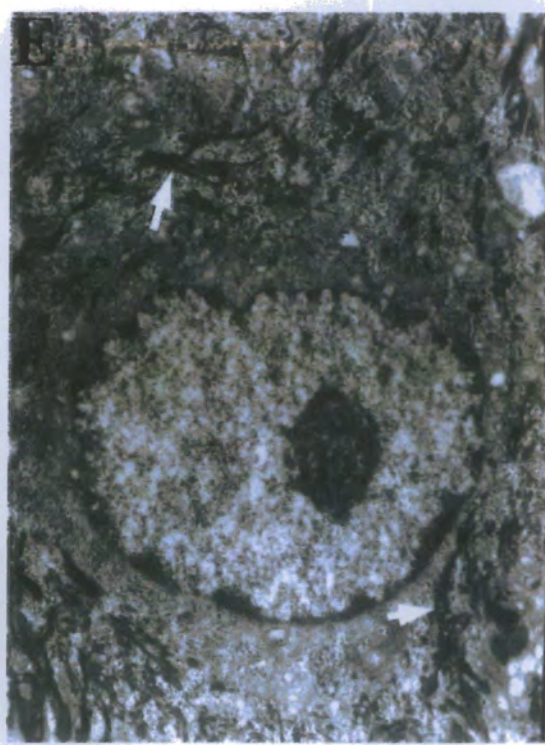
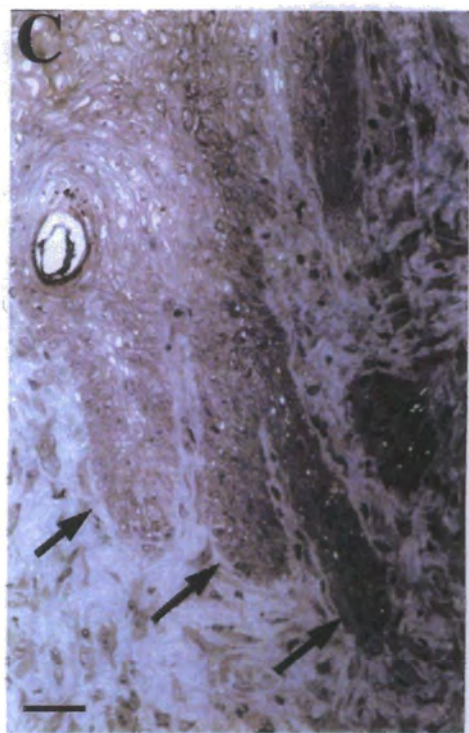
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A



Figs. 4.28B-E) Histology and ultrastructure of LDS/ORS cell recombinations 20 days post-implantation.

B) A higher power micrograph of the previous specimen. Epidermal cells (epi) in areas close to the dermis (der) are small and tightly packed but towards the surface they become larger and elongated. A clear stratum corneum is clearly evident at the surface of the tissue. **C)** The epidermis has produced finger-like projections (arrows) that have penetrated deep into the underlying dermis. **D)** Ultrastructure of an epidermal spheroid seen in Fig. 28A. It appears that epidermal cells around the central cyst are differentiating towards the centre since unlike the outer cells, the cells adjacent to the cyst do not have nuclei. **E)** Ultrastructure of basal epidermal cells. Cells contain a network of tonofibrils (arrows) around the nucleus. B and C resin embedded and stained with toluidine blue. Scale bars B and C 25 μ m, Dx2800, Fx10000.



Figs. 4.28F-K)

F) Basal epidermal cell (epi) from a 20 days LDS/ORS cell recombination showing cytoplasm rich in mitochondria (arrow) and ribosomes. (der) dermis. **G)** Basal cells display some small cytoplasmic projections jutting towards the dermis. **H)** Morphology of tissue in the lower dermal areas close to the filter (see material and methods). Unlike the upper dermis, cells found in this area very scattered. F and G x28000, H resin embedded and stained with toluidine blue, scale bar 40 μ m.

I-J) Ultrastructure of dermal cells. **I)** Dermal cells are fibroblast-like and display distinct nuclei with large cytoplasmic volume. The cytoplasm is rich in endoplasmic reticulum and associated ribosomes (**J**). Dermal cells were located in an extracellular matrix rich in collagen fibrils. **K)** A continuous basement membrane (arrows) is seen at the dermal-epidermal junction (see also F and G). Ix6000, Jx46000, Kx17000.

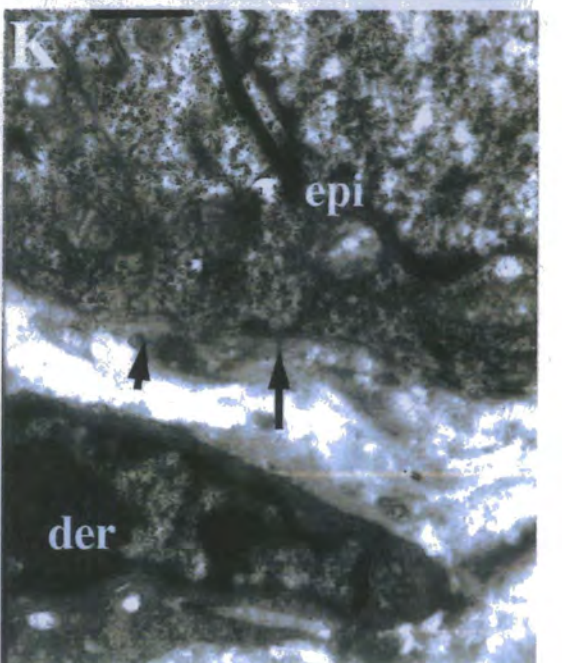
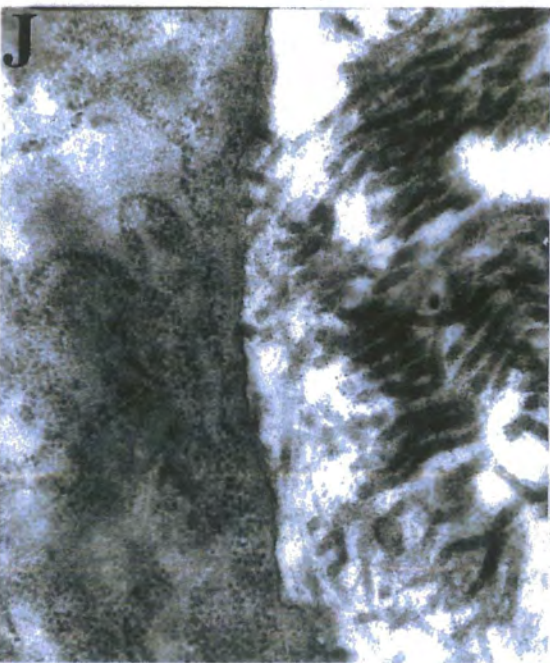
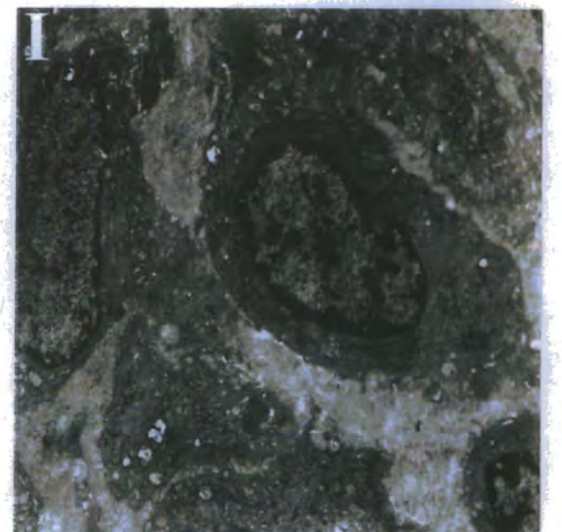
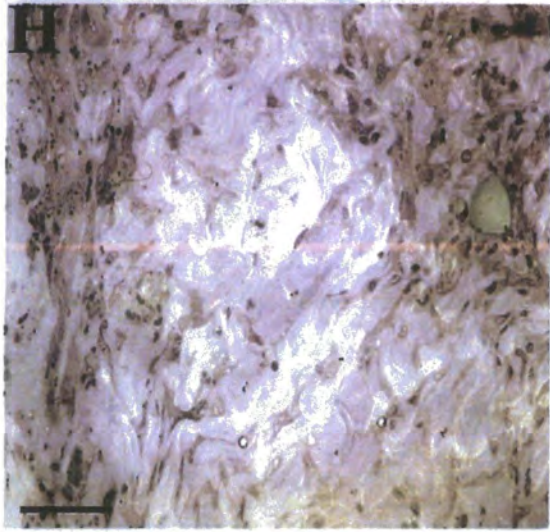
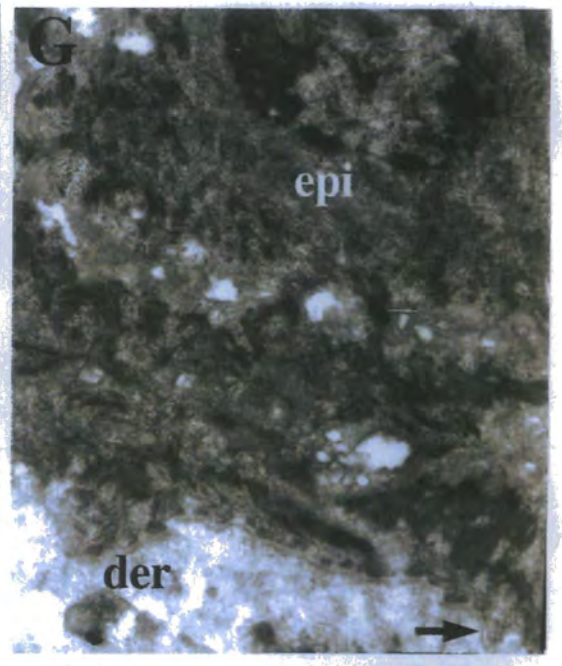
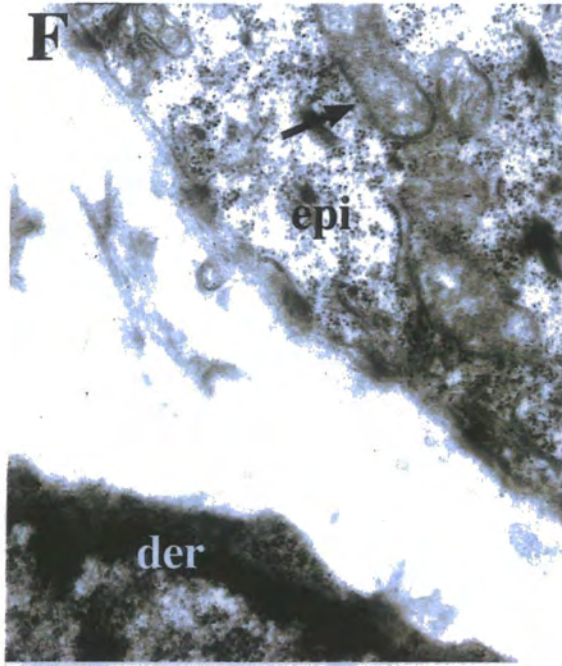


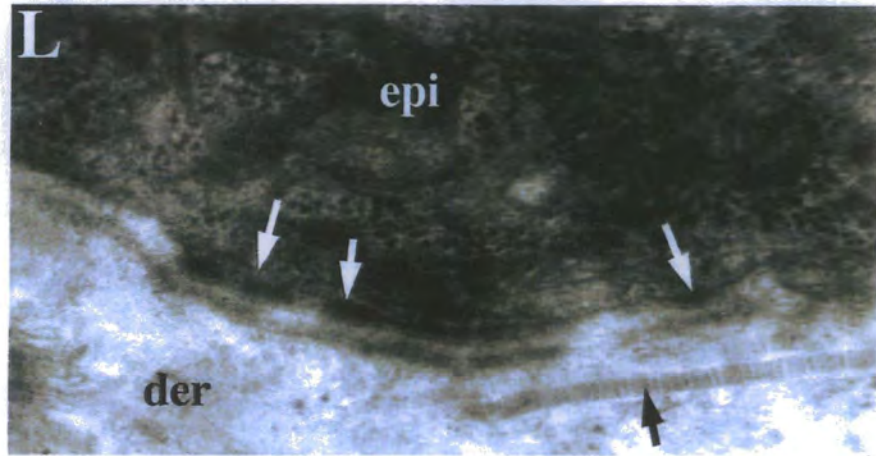
Fig. 4.28L) Dermal sheath/ORS recombination 20 days post-implantation. The epidermal basal cells attached to the basement membrane by numerous hemidesmosomes (white arrows). A collagen fibril (black arrow) is seen in the proximity of the basement membrane. $\times 60000$.

Fig. 4.29) Lower (A) and higher (B) power morphology of a tissue reconstructed by recombination of dermal sheath cells with epidermal ORS cells on the chick CAM after 7 days. The epidermis (e) contains approximately 6-8 cell layers situated above a thin layer of dermal cells (arrows). Resin embedded sections stained with toluidine blue. Scale bars A 50 μm , B 25 μm .

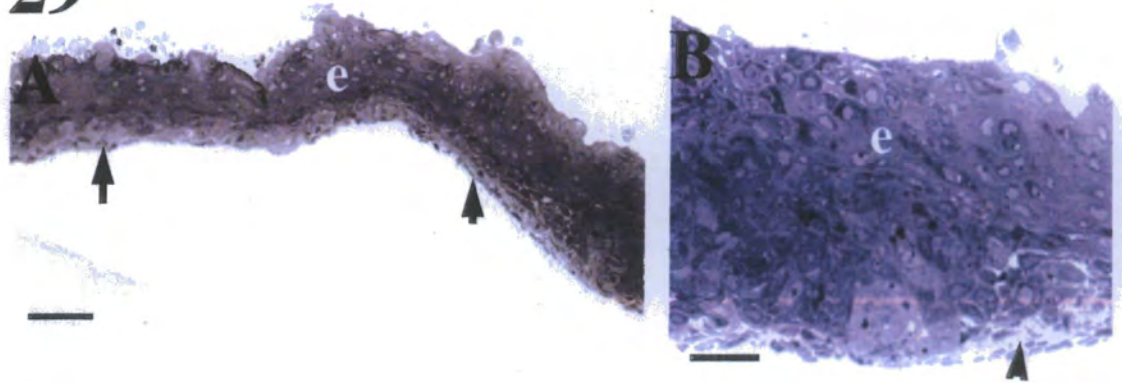
Fig. 4.30) Lower dermal sheath-ORS recombinations *in vitro*. Recombinations were prepared as method 1 (see materials and methods). A) Morphology of a reconstructed structure grown for 7 days in culture. Both epidermis (e) and dermis (arrow) are present but they are very thin. (g) collagen gel. B-C) Lower (B) and higher (C) power micrographs of recombinant cells after 14 days in culture. The dermal-epidermal tissues have been segregated and cells appear dead. Resin embedded sections stained with toluidine blue. Scale bars A and C 25 μm , B 50 μm .

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L



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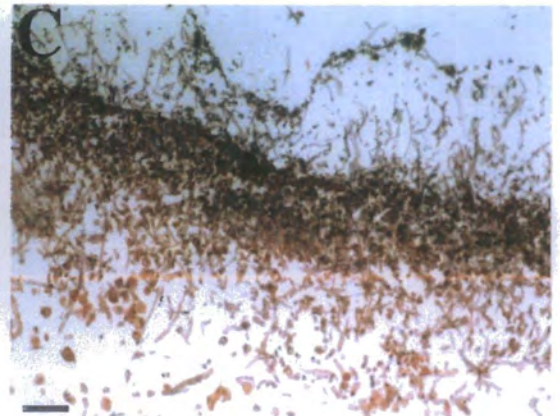
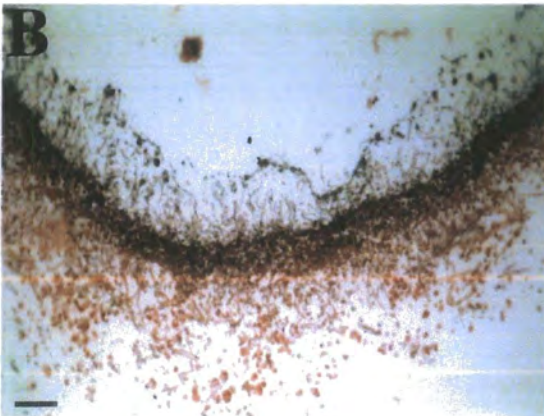
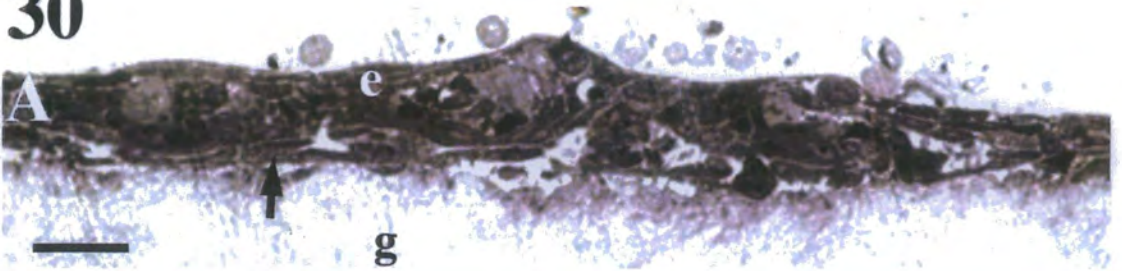


Fig. 4.31) Paraffin embedded sections through a tissue reconstructed from the recombination of dissociated embryonic skin cells with lower dermal sheath cells derived from the adult rat vibrissa follicle. **A)** A general morphology displaying the reconstructed epidermis (epi) and dermis (der) along with primitive hair follicles (arrow). **B)** A higher power micrograph through the epidermis in Fig. A showing that it is composed of several cell-layers. The basal cells are small and well packed but towards the upper regions cells become larger and sparser. An acellular layer is evident at the epidermal surface. **C)** A higher power micrograph through the dermis in Fig. A showing that it is populated with dermal cells. Hair follicles (arrow) which appear circular, because they have been cut transversally, are seen within the dermis. **D)** A micrograph from an area whose follicles (arrow) have been cut semi-vertically. It shows that some follicles have developed well and formed hair shafts. **E)** In areas close to the host tissue some large epithelial structures (arrows) are also observed. These structures are much larger than hair follicles seen in other parts of the tissue and contain more cell layers (arrow in F). Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bars A and E 100 μm , B, C and 25 μm , D 50 μm .

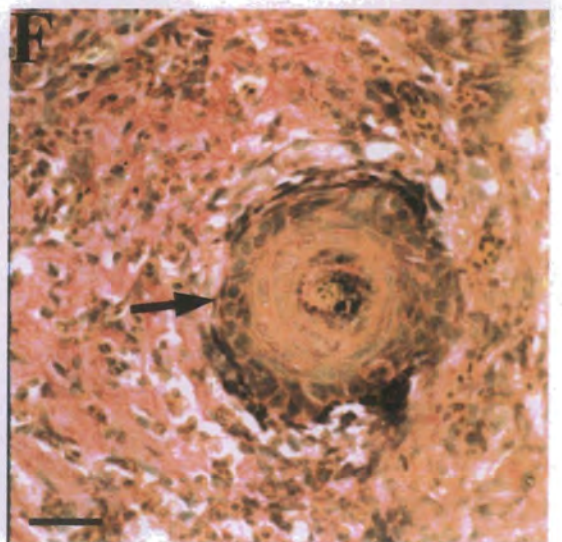
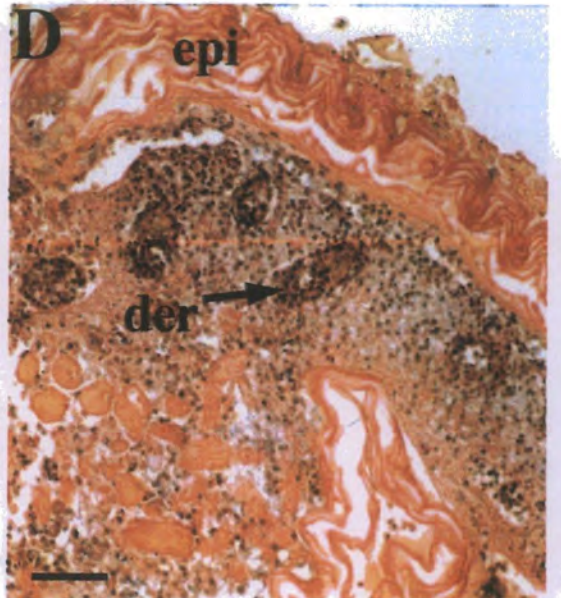
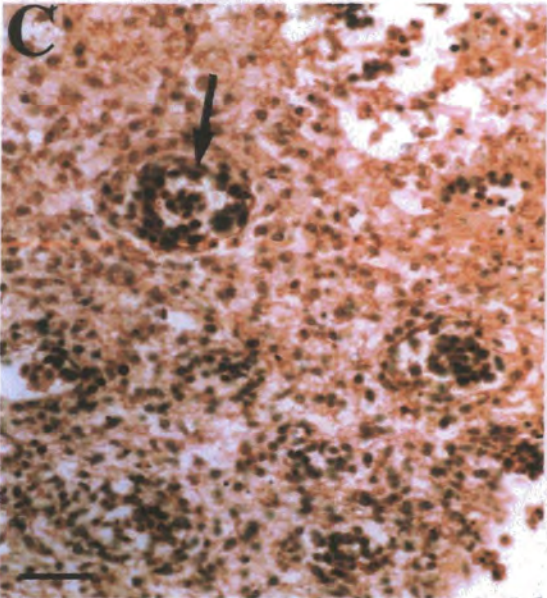
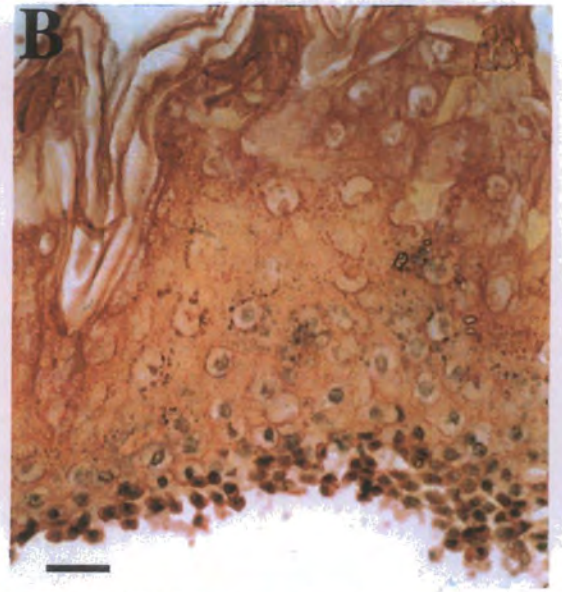


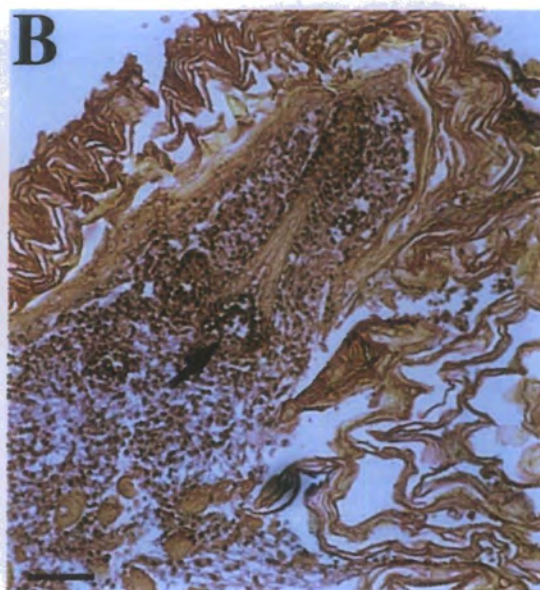
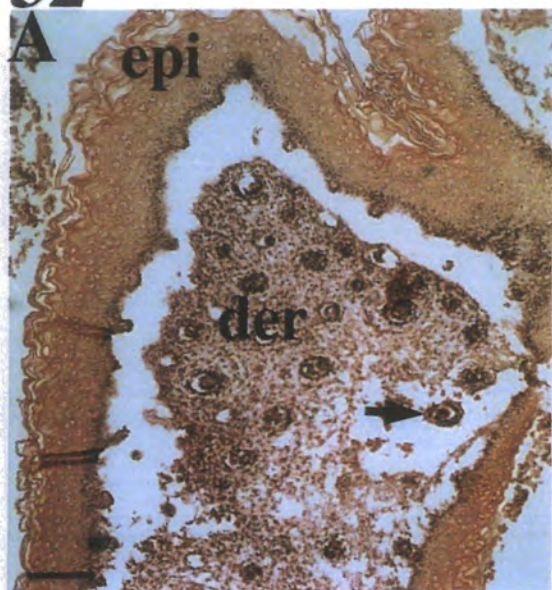
Fig. 4.32) Low (A) and higher (B) power micrographs displaying the morphology of a tissue reconstructed from dissociated cells derived from embryonic rat skin after 7 days on the chick CAM. A) Dermis (der) and epidermis (epi) has been reconstituted and pelage-like hair follicles (arrow) are seen within the dermis. B) Longitudinal section through a pelage-like follicle (arrow) showing a full-developed structure with bulb and hair fibre. However, no larger epithelial structures (as seen in Fig. 31E-F) were found in this tissue. Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar A 100µm and B 50µm.

Fig. 4.33) A punch biopsy site that had received LDS + ORS + intact DPs has produced thick and long vibrissa-type fibres (arrow). Scale bar 3mm.

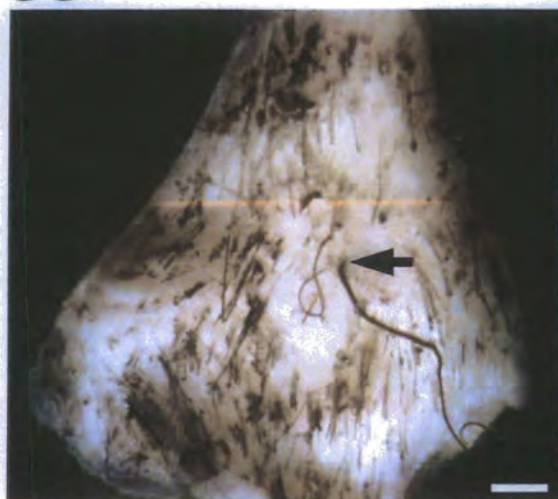
Fig. 4.34) A punch biopsy site (arrow) that had received LDS + ORS. This site did not produce vibrissa-type fibres. Scale bar 2mm.

Fig. 4.35) Lower (A) and higher (B) power micrographs displaying the histology of punch sites that had received LDS + ORS+ DP cells, 30 days earlier. A) The punch site (p) has completely repaired in terms of the dermis and epidermis, but the hypodermis is still impaired. Epidermis is thicker than adjacent areas. Unlike the neighbouring regions the punch site is relatively free of pelage follicles. B) The dermis is well populated with dermal cells. In the region just below the overlying epidermis the dermal cells become denser (arrow). The epidermis contains several layers and unlike neighbouring regions sends finger-like projections into the dermis. Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar A 200µm and B 25µm.

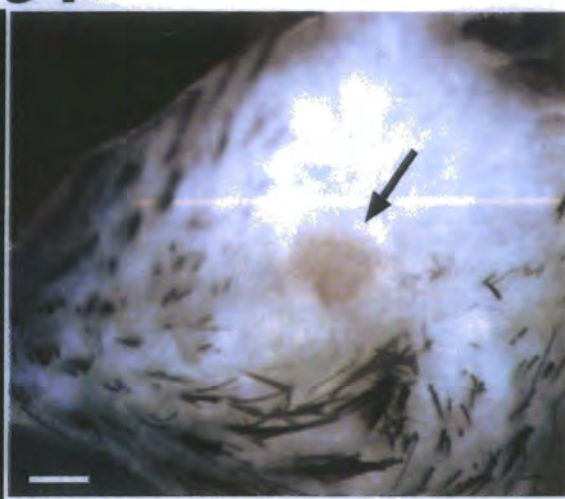
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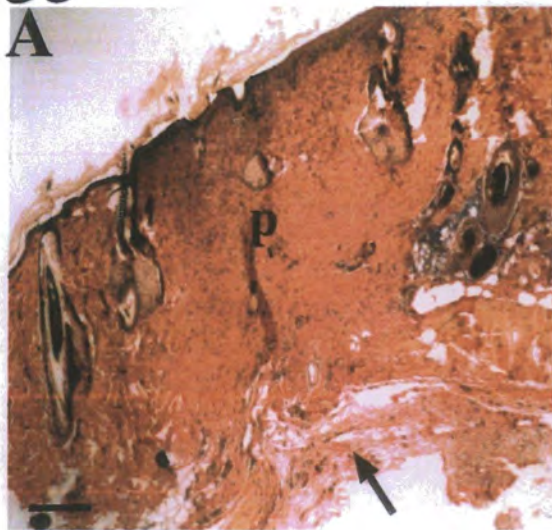


Fig. 4.36) Morphology of a punch site that received LDS + ORS cells. No vibrissa-type or pelage-type follicles are seen at the operation site. Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar 200 μm

Fig. 4.37) Histology of a punch site that had received SF + ORS cells. Again no hair follicles are observed at the punch site. Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar 200 μm .

Fig. 4.38) Section through another punch biopsy that had received LDS + ORS + DP cells. As with Fig. 4.36 and 37 no follicles were found at the punch sites. Staining Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar 100 μm .

Fig. 4.39) Histology of punch sites that had received LDS + ORS + whole DPs. **A)** General morphology displaying vibrissa-type hair follicles (white arrows) produced by implanted whole DPs. A follicle in mid-dermis that has been cut vertically shows a pear shaped and Alcian blue stained dermal papilla. Another two large follicle, which have been cut transversally are visible within the dermis. A large and abnormal structure is also seen at the surface of the punch. Smaller pelage-like follicles are also seen deeper in the dermis (black arrow). **B)** Higher magnification of a large follicle displaying a big dermal papilla (p) and a broad epidermal matrix (m). **C)** A vertical section through local pelage follicles which are positioned in regions neighbouring the punch site. They are much smaller than vibrissa-type follicle and have small papillae (arrow) with far fewer cells. **D)** A vibrissa follicle which has been sectioned transversally. A thick layer of dermal sheath (arrow) is seen surrounding the epithelial core.

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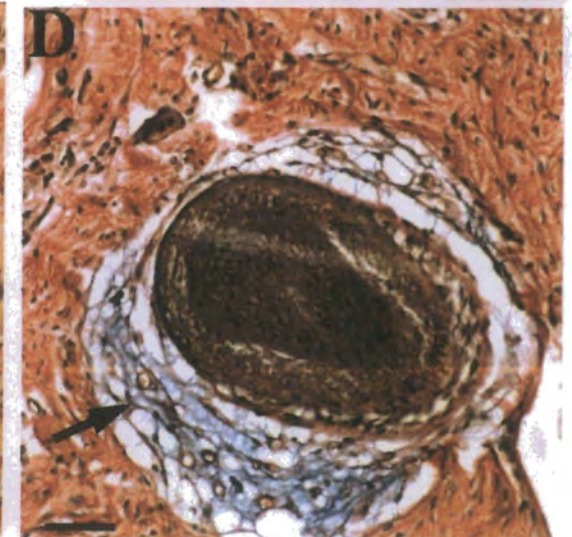
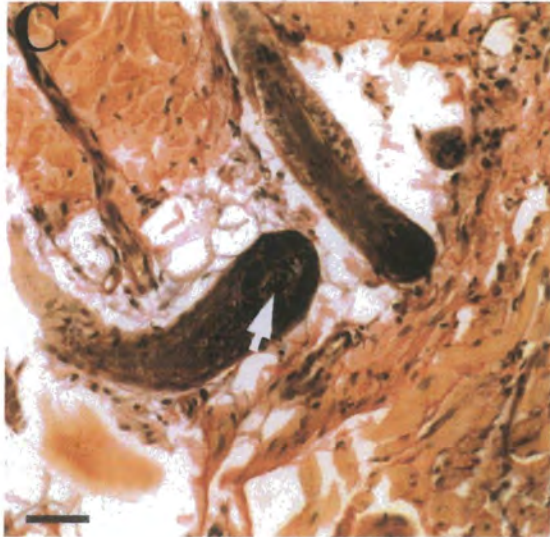
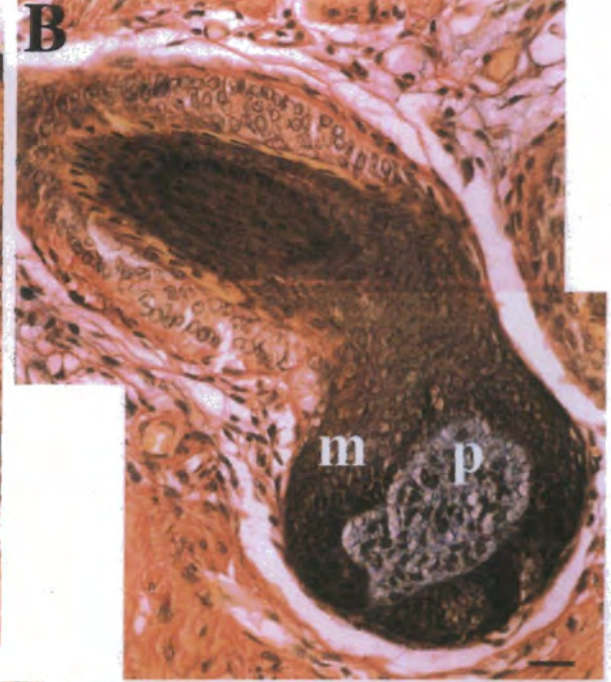
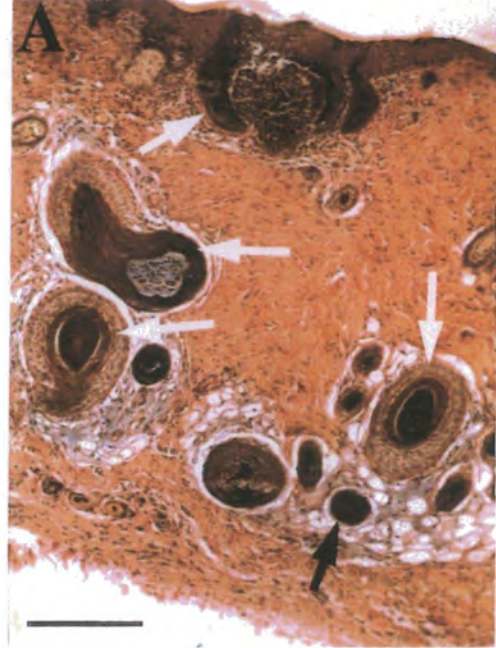
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Figs. 4.39E-G) Histology of a punch site that had received LDS + ORS + whole DPs. **E)** An induced vibrissa-type follicle showing a sebaceous gland (arrow) on both sides of its neck. **F)** Two isolated dermal papilla cell clumps seen within the dermis (arrow). **G)** Higher magnification of one of the papilla cell clumps seen in Fig. F. The papilla cells are densely packed, as seen during telogen in vivo but a reduced amount of extracellular matrix is still present in intercellular spaces. Scale bar A 200 μm , B and G 40 μm , C-F 100 μm .

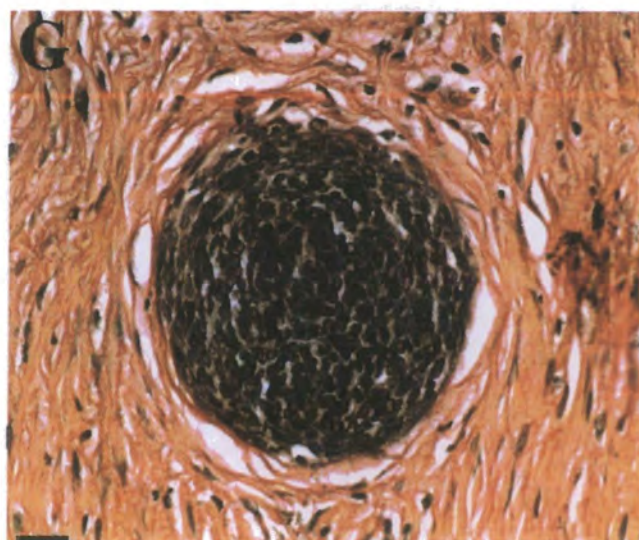
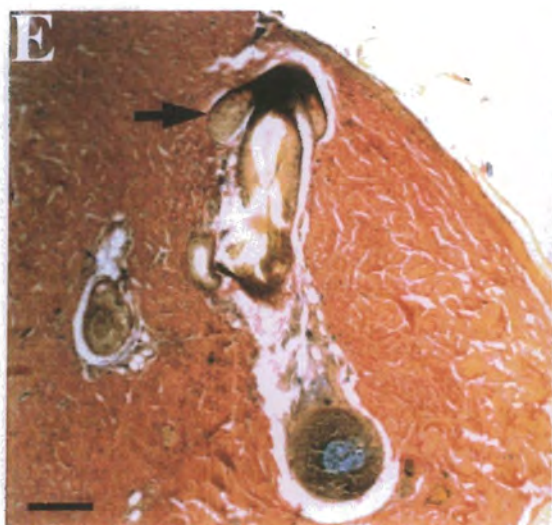
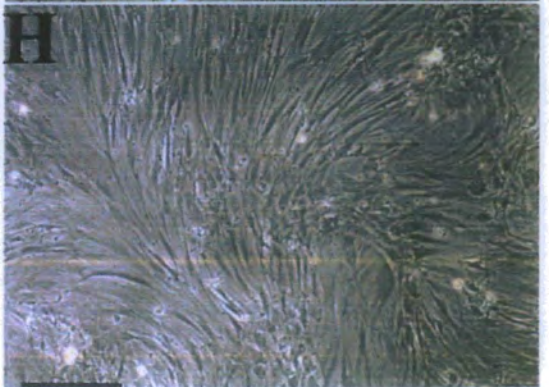
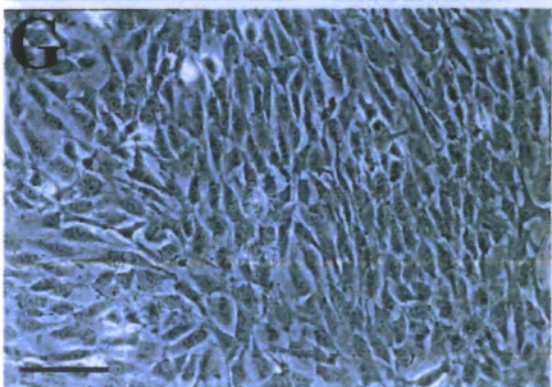
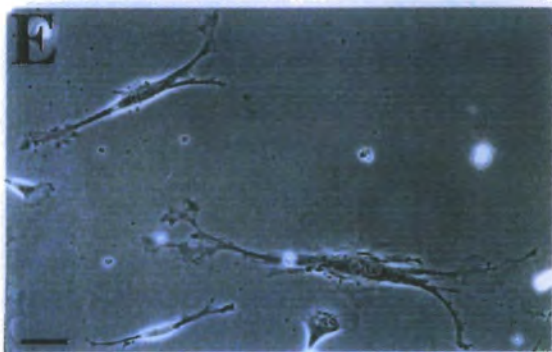
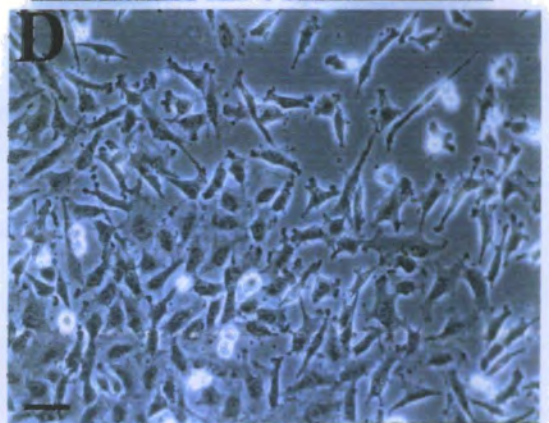
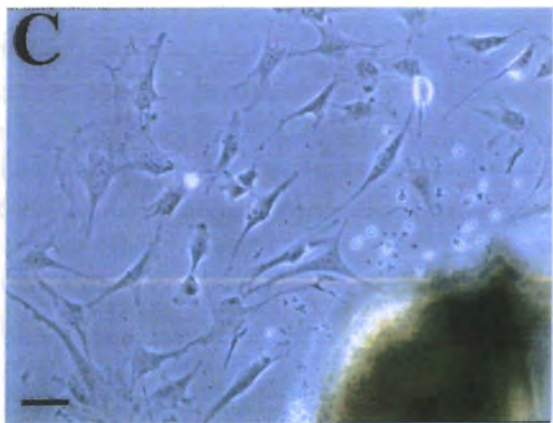
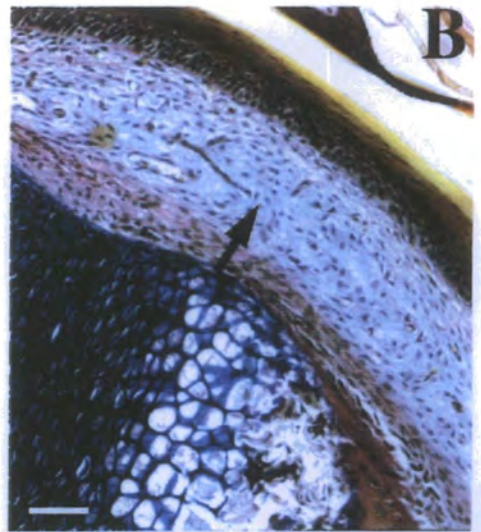


Fig. 4.40) Claw unit dermal cells.

A-B) Lower (**A**) and higher (**B**) power longitudinal sections of a claw unit and accompanying structures in a 4-day-old rat. **A**) The claw unit is essentially composed of two parts, epidermal (arrow) and dermal (p). As a result of dermal-epidermal interactions epidermal cells are produced by claw matrix which is positioned at the proximal end of the claw plate. After production cells move forward and upward and during this movement they undergo a special process of keratinization. The keratinized cells later form claw plate (arrow): (b) bone. **B**) Claw dermal tissue (arrow) is populated with dermal cells which are well-spaced in a GAGs rich ECM. This dermal tissue was used for cell culture.

C-H) Phase contrast micrographs of rat claw dermal cells in culture. **C**) Dermal cells have emerged from a tissue explant after 4 days in culture. Cells mainly show spindle shaped morphology and are closely not packed either in areas near to the tissue explant or in peripheral regions. **D**) After 7 days in culture, cells closer to the tissue explant are denser than in peripheral regions. **E**) Some claw dermal cells display a long morphology with multiple lamellipodia. **F**) There are numerous dividing cells (arrow) among the claw dermal cells in culture. **G**) After two weeks in culture cells from 7 tissue explants cover all of the available substrate. **H**) At confluence the claw dermal cells show a low level of aggregation. **A** and **B** wax embedded and stained with Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bars **A** 100 μ m, **B** 50 μ m, **C-E** 35 μ m, **G** 70 μ m, **H** 150 μ m.



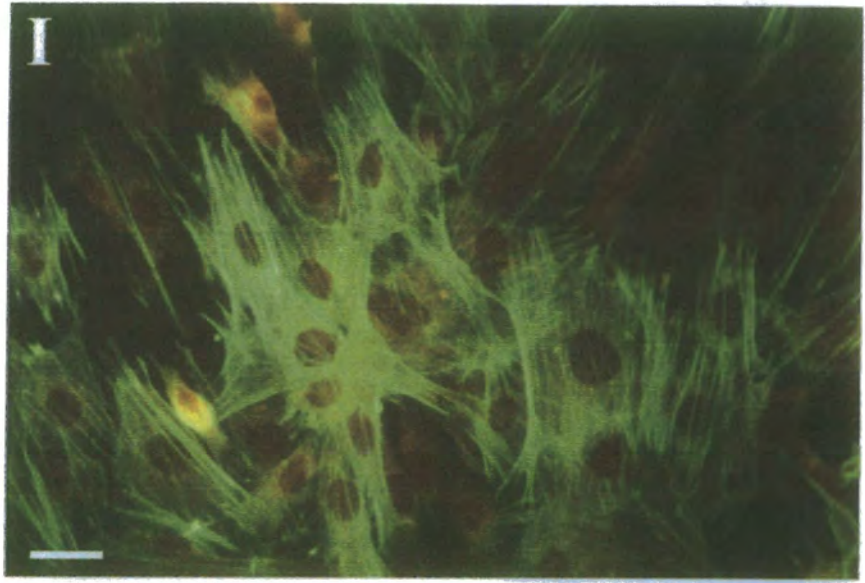
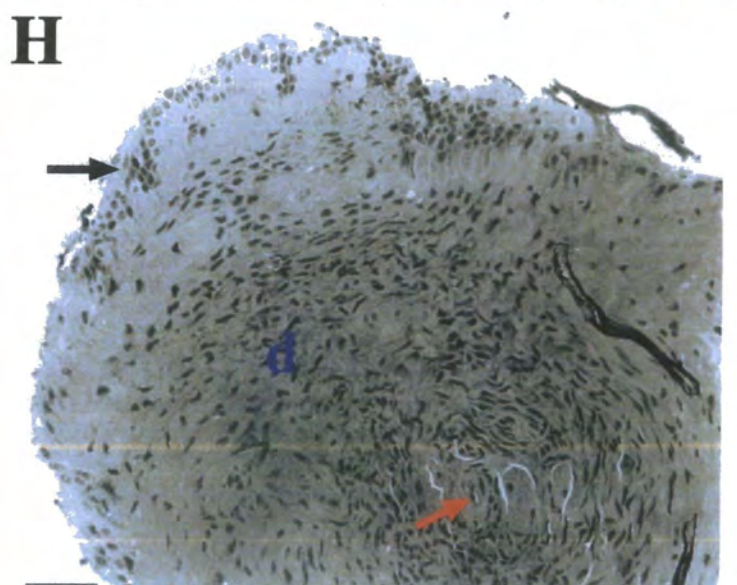
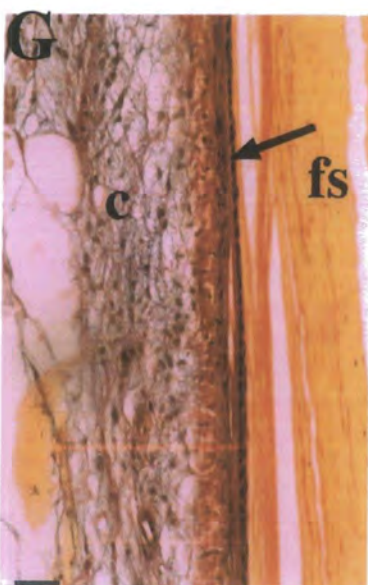


Fig. 4.41) Morphology and ultrastructure of feather follicles. **A)** A longitudinal section through a growing pigeon follicle. The follicle has a large dermal component which is divided into two regions, the lower is termed the dermal papilla (d) and the upper which is named the pulp (p). **B)** Higher magnification of the lower region of the same specimen showing the papilla (d) which is surrounded by a thick epidermal tissue or collar (c). **C)** Higher magnification of the upper region of the same specimen displaying the pulp (p) and its surrounding epidermal tissue (e). At the interface between the pulp and epidermal tissue numerous black pigments (arrows) are visible. **D)** A vertical section through the lower region of another growing follicle showing a large blood vessel along with its endothelial cells (arrow) which runs through the length of the papilla (d) and pulp (p). The epidermal collar (c) is also visible. **E)** A longitudinal section through a non-growing pigeon follicle. Compared to the growing follicle, the dermal papilla (arrow) is small and confined only to the base of the follicle. The epidermal feather shaft (fs) is mainly keratinized and acellular. **F)** Higher magnification of the lower region of the same specimen (Fig. E). The feather shaft (fs) is surrounded by a thin layer of epidermal cells at both sides (black arrows) and bottom, papillary ectoderm (white arrow). In some areas of the papilla (d) interface this layer of epidermal cells is relatively thick. **G)** Higher power micrograph of the upper region of the same section displaying a thin layer of epidermal cells between the inner feather shaft and outer connective tissue (c). **H)** A semi-thin section through a feather papilla dissected from a non-growing sparrow follicle. Note that there is a layer of epidermal cells still attached to the papilla (d). A blood vessel (arrow) is also visible at the centre of the papilla. A-G Paraffin embedded and stained with Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. H) Resin embedded section stained with toluidine blue. Scale bars A and E 200 μ m, B and C 50 μ m, D 30 μ m, and F-H 25 μ m.



Figs. 4.41I-M) Ultrastructure of a dissected sparrow feather papilla.

I) Dermal cells displaying a fibroblastic appearance are well-spaced within a fibril-rich (arrow) ECM. **J)** A cross section through the central blood vessel showing three distinct layers of intima (black arrowhead), media (m) and adventitia (white arrow). **K)** The dermal cells (der) were separated from epidermal cells (epi) by a thick basement membrane (arrow). **L)** The epidermal cells had round nuclei and some dendritic cells (m) were also observed within them. The dendritic cells were most likely inactive melanocytes. **M)** Higher magnification of a dendritic cell displaying multiple cytoplasmic projections. Ix17000, Jx6000, Kx2800, Lx13000, Mx36000.

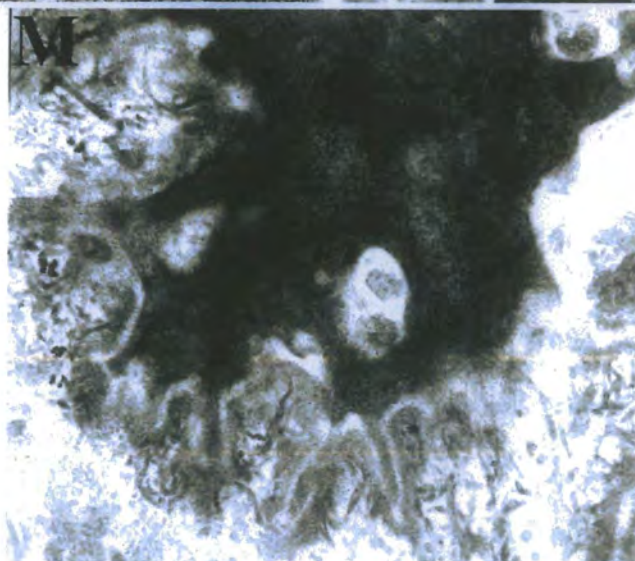
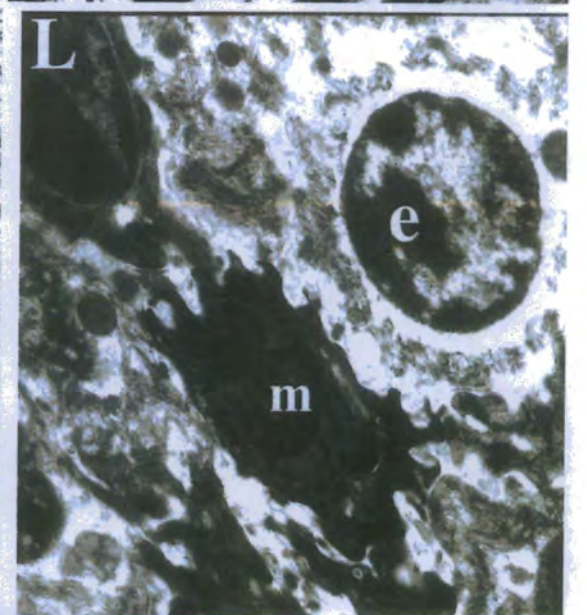
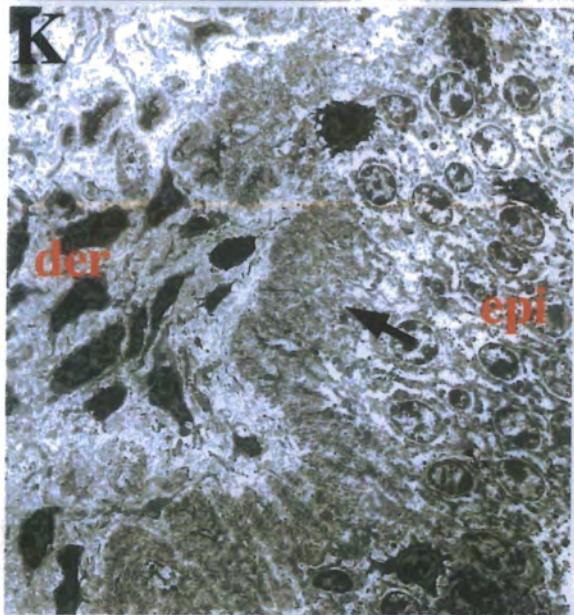
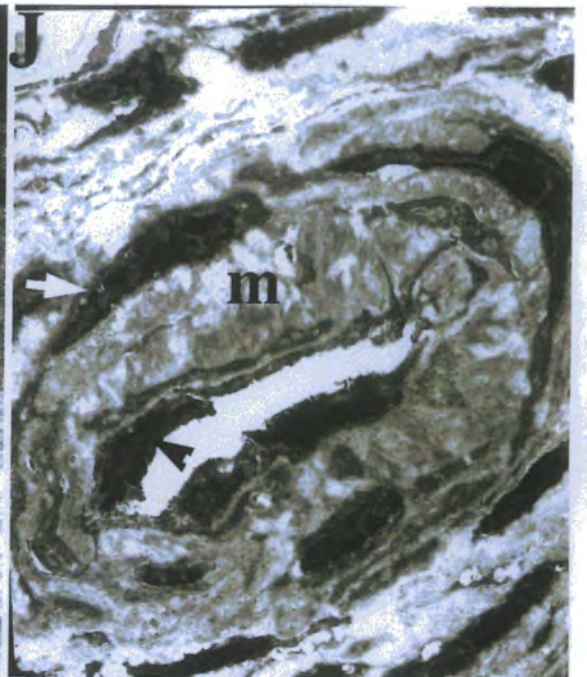
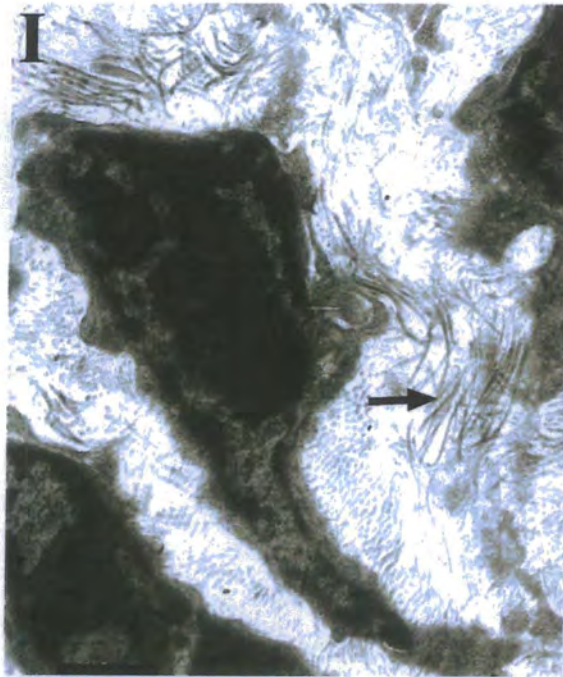
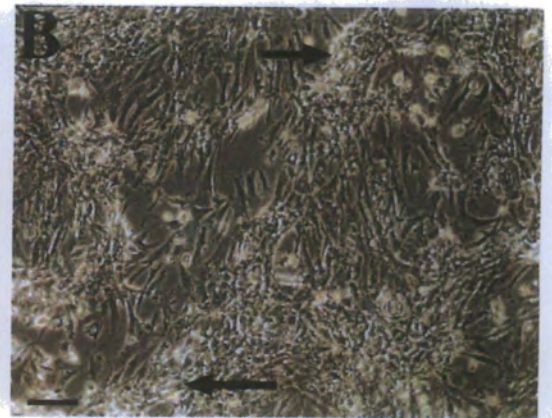
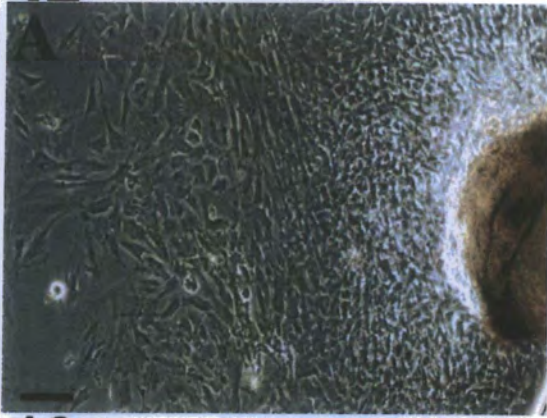


Fig. 4.42) Phase contrast micrographs of pigeon feather papilla cells in culture. **A)** Cells have emerged from a tissue explant after 2 weeks in culture. Cells in the areas close to the tissue are smaller and tightly packed whereas at the peripheral regions cells are larger and sparser. **B)** Cells at confluence showed a degree of aggregation. Arrows point to two areas of culture in which cells were dying. Scale bars 100 μ m.

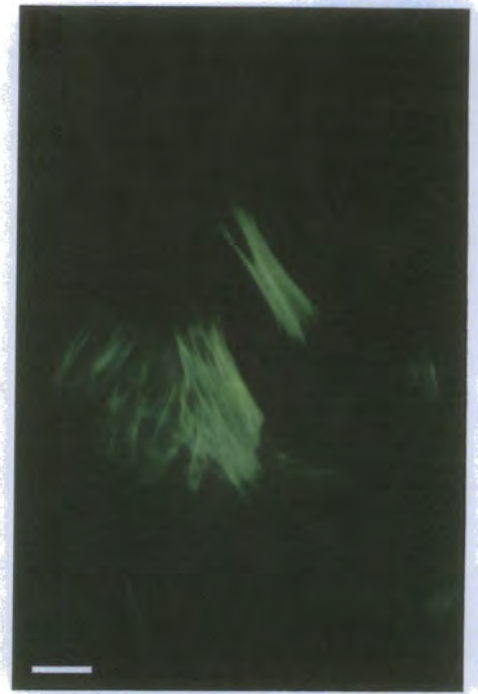
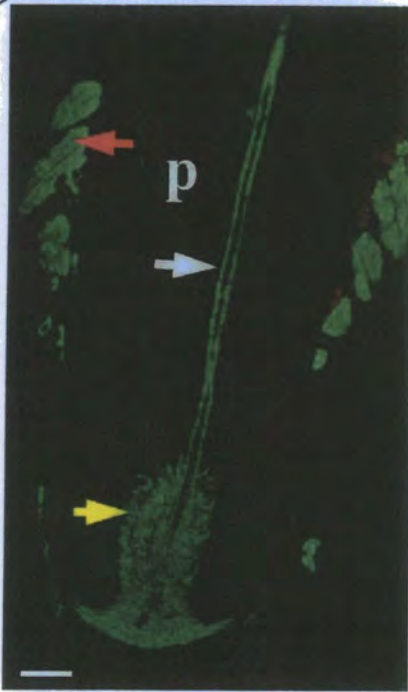
Fig. 4.43) Immunofluorescence micrographs displaying the expression of ASMA in feather components in situ (**A**) and in vitro (**B**). **A)** A longitudinal section through a growing pigeon feather follicle stained with ASMA. Dermal papilla (yellow arrow), blood vessel (white arrow), pulp (p), arrector muscle (red arrow). **B)** Only around 25-30% of the cultured papilla cells expressed the ASMA. Scale bars A 100 μ m and B 25 μ m.

Fig. 4.44) Morphology an amputated vibrissa follicle which had received pieces of a pigeon feather papilla implant 4 weeks previously. The feather papilla (arrow) is still present in follicle but no structure has been formed. Paraffin embedded and stained with Alcian blue, Weigert's haematoxylin and Curtis's Ponceau S. Scale bar 75 μ m.

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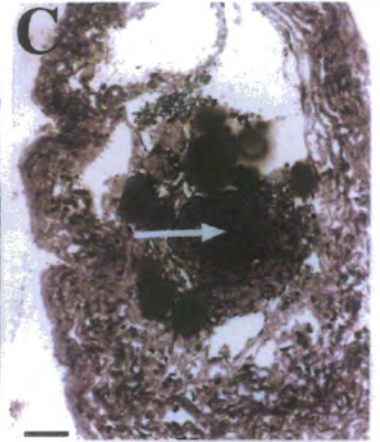
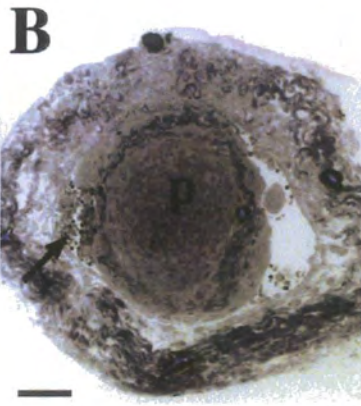
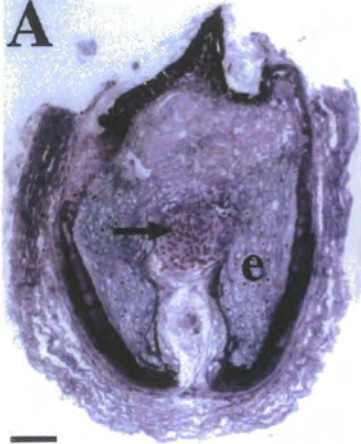
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Fig. 4.45) Plucked follicle end bulbs after 7 days in culture. **A)** The dermal papilla (arrow) has moved up. It is more compact than that of a normal anagen follicle *in vivo* and contains fewer cells. An epidermal matrix (e) has regenerated around the papilla but most epidermal cells appear to be dead. **B)** The papilla (p) and epidermal cells (arrow) in this follicle are both healthy, but no epidermal regeneration has taken place. **C)** This follicle is disorganised and what appears to be the remnant of the dermal papilla is seen in the middle of the micrograph as a ball of tightly packed cells (arrow). Resin embedded and stained with toluidine blue. Scale bars 50µm.

Fig. 4.46) Morphology and ultrastructure of plucked follicle end bulbs after 14 days in culture. **A and B)** Two follicles that have produced small external projections which are associated with a sheath (arrows). **C)** A longitudinal section through a follicle end bulb that has grown a fibre-like projection. The bulb displays a telogen-like morphology and contains a compact papilla (p) with packed cells. The fibre has lifted up and its base is now seen above the papilla. **D)** Higher magnification of the previous specimen showing the structure of the papilla (p) in more detail. The papilla cells after 14 days in culture are still healthy. **E)** Another follicle end bulb that has produced a matrix with two external projections. As with Fig. 45A in this follicle the matrix cells appear to have keratinized. Resin embedded and stained with toluidine blue. Scale bars A-C 50µm, D and E 25µm.

45



46

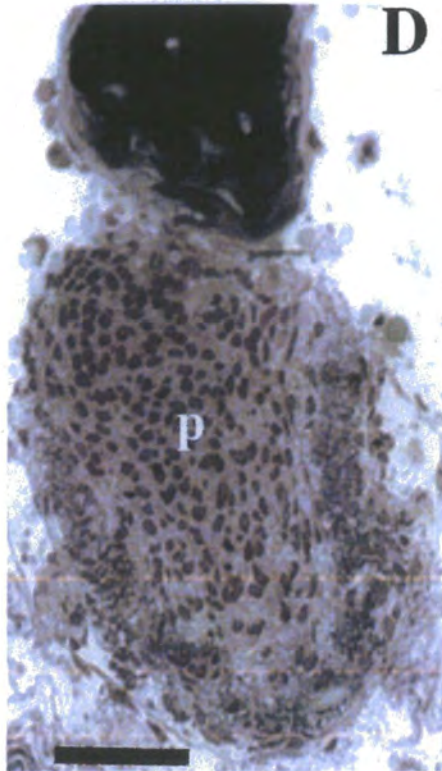
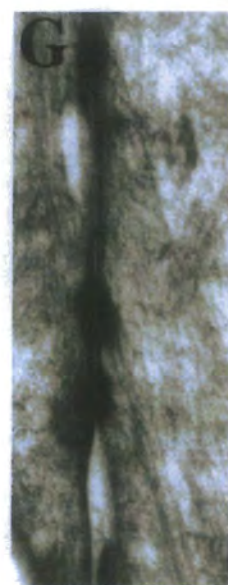


Fig. 4.46F-I) Electron micrographs of the specimen shown in Fig. 4.46E.

F) The cells within the external projections are epidermal and have already keratinized.

G) Many desmosomes are present at the interface between these cells. **H)** Within the bulb region, the epidermal cells (e) have also keratinized but in a few cells the nuclei are still visible. Lateral to the epidermis dermal sheath cells (ds) are also observed. **I)** The dermal sheath cells are healthy with a large nucleus and a cytoplasm rich in organelles. F and Hx2800, Gx46000, Ix8000



4.4 Discussion

4.4.1 Hair follicle dermal sheath

In contrast to other hair follicular-derived cells, most of which are now well characterised, less is known about the dermal sheath cells, which surround the follicle all along its length like a sleeve. According to what is known these cells generally display an intermediary nature between follicular dermal papilla cells and interfollicular skin fibroblasts in terms of their morphology, behaviour and phenotype *in vivo* and *in vitro*. Moreover, there is documentation to suggest that, unlike the papilla, which apparently contains a homogenous fibroblast-like cell population, dermal sheath cells are heterogeneous, to the extent that depending on their position in the hair follicle, they display different characteristics (Horne *et al.*, 1986). This change in behaviour is seen both horizontally and vertically. It has been reported that in human follicle only the internal horizontally aligned layer of the dermal sheath express ASMA (Urabe *et al.*, 1991) implying that this structure is composed of two layers as first was suggested by Kligman (1988). He proposed that each of these two layers possibly have a separate function in the follicle. In agreement with this, in the present study I have shown that culture of the dermal sheath under coverslips resulted in the growth several of cell types, emphasising that this dermal tissue contains cells of different origin. However, the most interesting evidence of dermal sheath heterogeneity derives from the experiments that revealed that these cells also exhibit different characteristics depending on their position along the longitudinal axis of the hair follicle. Oliver (1966a,b & 1967a) demonstrated that a new dermal papilla and hair bulb was formed in the rat vibrissa follicle after experimental removal of the lower third, but when more than a third of the follicle was removed no regeneration occurred. This indicated that the dermal sheath attached to the lower third portion of the follicle has the capacity to reform the papilla whereas the dermal sheath cells above this region do not. This capacity in the lower dermal sheath has also been shown in human hair follicles (Jahoda *et al.*, 1989 & 1996) where amputated follicles regenerated a new bulb when implanted into nude mice. In line with these experiments, it has been documented that deactivated upper follicles can regain the ability to produce a bulb and a hair fibre when implanted with intact dermal papillae

(Oliver, 1967b), cultured dermal papilla cells of early passage (Jahoda *et al.*, 1984a,b; Horne *et al.*, 1986) or dermal sheath cells derived from the lower third of the vibrissa follicle (Horne & Jahoda, 1992). Furthermore, Oliver (1967a) reported that when lengths of the lower third of the vibrissa follicle wall (composed of ORS and dermal sheath) were transplanted into ear skin, hair bulbs were formed, but this was not the case with equivalent specimens from the upper half of the follicle. Hence, this body of evidence indicates that cells from the upper and lower regions of follicular dermal sheath have different abilities when it comes to altering their phenotype and accepting different roles.

The current study has generated further evidence of differences between upper and lower dermal sheath cells *in vitro*. Firstly, in agreement with previous studies (Horne, 1987) it was demonstrated that cells derived from the upper dermal sheath displayed a morphology similar to fibroblasts at confluence and post-confluence, and had a lower level of aggregation than cells from lower dermal sheath. Secondly, as previously reported (Skalli *et al.*, 1986; Jahoda *et al.*, 1991; Reynolds *et al.*, 1993b), cells from the lower dermal sheath were found to be highly positive for ASMA whereas this cytoskeletal element was expressed at a lower level in dermal sheath cells from the upper follicle. Thirdly, when cells were stained with an antibody to MHC class I molecules it was revealed that while the papilla cells and skin fibroblasts expressed the lowest and highest level of this antigen respectively, the mean level for LDS cells was close to that of the papilla whereas for UDS it was close to that of skin fibroblasts. Moreover, in my implantation work when stained cells were implanted into ear pinnae the subsequent observations demonstrated that cells from LDS contributed to local hair follicle structures whereas UDS and SF cells failed to display this activity. These results, along with evidence provided from dermal-epidermal recombinations not only show that the dermal sheath represents a heterogeneous cell population in the upper and lower follicle but in agreement with previous studies, they also demonstrate that LDS cells have distinct characteristics which emphasise the important roles they play in the hair follicle and skin biology in general. To further clarify these roles, in the following sections I discuss various properties of dermal sheath cells separately, but before this duo to the fact that some properties of follicle-derived dermal cells have been shown to relate to their aggregation behaviour I would first like to consider this matter.

4.4.1.1 Possible importance of cell aggregation

Mesenchymal cell condensations are a vital stage in the morphogenesis of many organ systems, and they are associated with the formation of all integumental appendages including feather and hair follicles (Wessells, 1965; Kaplan & Holbrook, 1994). During embryonic development of the hair follicle, dermal condensations precede the papilla formation, and the papilla cells retain a propensity to aggregate not only through later embryonic stages but also throughout their entire post-natal life (Oliver & Jahoda, 1989). Since the hair follicle is continuously involved in cyclic processes of growth and regression it has been proposed that this aggregative property of papilla cells protects the follicle from destruction, so that when in telogen while the papilla is not enveloped by epidermal matrix, it prevents the loss of papilla cells and assures the retention of a stable structure for renewal of follicle morphology (Jahoda & Oliver, 1984c). On the other hand there is a good deal of evidence to show that papilla cells (possessing a high capacity to aggregate) also have high inductive capabilities (see Jahoda & Oliver, 1990; Ferraris *et al.*, 1997b) a property that is not observed in skin fibroblasts which do not have the capacity to aggregate. Similarly, as was shown in the current study LDS cells at confluence show higher level of aggregation compared to that was seen in UDS cells and there are reports that show LDS cells display greater potential for hair induction than UDS cells (Oliver, 1966a, 1967b; Horne & Jahoda, 1992). An obvious question to follow on from this is, whether or not there is a relationship between the inductive ability of cells and their aggregative behaviour and if so, what role does this process play in interactions? Providing definitive answers to these questions would require a huge amount of investigation but there is circumstantial evidence that suggests that there is a possible connection between these two phenomena (Jahoda, 1982). For example, it has been shown that several factors, particularly cell adhesion molecules, are involved in the process of cell aggregation (Chuong & Edelman, 1985; Chiquet *et al.*, 1986; Lyons *et al.*, 1990; Chuong *et al.*, 1991; Kaplan & Holbrook, 1994). In this context Horne *et al.* (1986) showed that after implantation of low passage number papilla cells into amputated vibrissa follicles, the implanted cells remained in their sites and produced cellular aggregates which later became the new dermal papillae, whereas when late passage cells were employed for the same experiment

they did not form recognisable cellular aggregates and they migrated from the implantation sites. These observations showed that the loss of inductive potential by papilla cells after extended culture may be associated with loss of aggregative behaviour while *in vivo*. However, if this is true and an aggregative property provides cells with greater potential in inductive processes *in vivo*, we would also expect low and high passaged papilla cells to show different degrees of aggregation *in vitro*. There is documented data that shows that papilla cells after multiple passages are still capable of forming clumps of aggregative cells similar to those from low passaged cells (see Horne *et al.*, 1986). Therefore, this suggests that the aggregative potential is not the only factor responsible for the high inductive abilities of the papilla cells and there are others which remain to be discovered.

4.4.1.2 Dermal sheath and expression of ASMA

In agreement with previous observations of rat vibrissa follicles (Reynolds *et al.*, 1993) this study showed that dermal sheath cells from the lower portion of the follicle expressed ASMA at high levels, while the same cell types in the upper part of the follicle displayed a lower level of expression. Similarly, it has been reported that an epidermal-specific actin (F-actin) has a similar gradient of expression along the length of the follicle so that it is more prominent in the lower ORS compared with the upper follicle ORS both in the growing and resting stages (Furumura & Ishikwa, 1996). These observations point to the possible role of actin in the follicle as a functional element in follicle contraction, a process which is vital for the follicle to serve its function. The hair follicle during its periodic cycle undergoes profound morphological changes. During hair growth, the newly formed hair shaft is pushed continuously toward the skin surface thereby passing through the dermal and epithelial sheaths of the follicle. Thus, the cells in these sheaths may play an important role in pushing the produced fibre upwards. Additionally, at catagen in most follicle types, the lower portion of the follicle contracts and moves upwards which again implies that the cells in these regions are like to be capable of contraction. The high level of ASMA in dermal cells in this area is probably associated with this process. Furthermore, the presence of contractile actins in these cells may influence the arrangement of cells in the hair shaft. Electron microscopy has revealed the actin bundles in the ORS cells of the lower follicle form continuous

circumferential bands around inner root sheath (Furumura & Ishikawa, 1996). This region corresponds to the keratogenous zone which is a crucial site for fibre production. During hair growth, cells proliferate in the hair matrix of the bulb and as they move upwards they increase in volume before keratinization. Above the follicle bulb, the enlarged cells are funnelled through a narrow keratogenous zone, where they may be subjected to a constraining force resulting in their condensation into the slim and elongated bodies of hard keratin that move up the hair shaft and inner root sheath (Kassenbeck, 1981). There is a possibility that the funnelling process is dependent on the force exerted from continuous circumferential actin bundles in the ORS cells (Furumura & Ishikawa, 1996). In respect to this, regarding the cessation of hair production at the end of anagen, it appears that during catagen and telogen all of the contractive elements of the follicle are directed towards pushing the club fibre upwards. Hence, it would be interesting to investigate whether there is a change in alignment of the cytoskeleton within the lower follicle cells at these stages. In brief, based on this body of evidence it appears that follicular actins serve as contractile fibres whose presence and distribution within the follicle are crucial for fibre structure and the integrity of the follicle.

In addition to the role of ASMA within the hair follicle there is evidence to support the idea that this protein is also important for maintaining the integrity of skin, particularly in the process of wound healing. It has been established that in normal skin only a low number of skin fibroblasts express ASMA but Darby *et al.* (1990) demonstrated that during cutaneous wound healing, ASMA, at a certain and restricted time, is highly expressed in the fibroblasts. This high level of ASMA expression in wounded tissues was later shown by other authors (Germain *et al.*, 1994; Jester *et al.*, 1995; Ghassemifar *et al.*, 1997; Cass *et al.*, 1997) and corresponds to the time when cells are needed for contraction to occur (see Ronnov-Jessen & Petersen, 1996). Moreover, it has been shown that during wound healing, myofibroblasts (characterised by ASMA expression) appear in granulation tissue and it is thought that these cells are responsible for increased contractility of the affected tissues (see Zhang *et al.*, 1996). Interestingly, it is believed that the myofibroblastic cells derive mainly from local fibroblasts, as well as other cells including pericytes and smooth muscle cells (see McCluskey & Martin, 1995; Gabbiani, 1996). The conversion of fibroblasts into myofibroblasts is triggered by growth factors such as TGF- β 1 (Desmouliere *et al.*,

1993). After wound healing myofibroblasts disappear (probably by apoptosis) and granulation tissue transforms into scar tissue (see Desmouliere, 1995; Gabbiani, 1996). These findings suggest that ASMA plays an important contractive role in wound healing (Cintorino *et al.*, 1991). On the other hand the presence of ASMA in the hair follicle dermal sheath raises the possibility that the dermal sheath is also involved in wound healing. In the treatment of burn wounds with split-thickness skin there is evidence that the use of scalp skin as a donor site provides better results than skin from less hairy areas such as the thigh (see Martinot *et al.*, 1994). There are also indications that wounds in hair-bearing sites heal earlier than those in hairless sites (Muller, 1971). This can be attributed in part to the involvement of follicular ORS cells in the regeneration of skin epidermis as it has been shown that in wounds where stumps of hair follicle are left, a large contribution to the healed epidermis is derived from these hair follicle remnants (see Martin, 1997). However, at the same time, given the similarity between follicular dermal cells and myofibroblast properties in terms of the expression of ASMA it has been suggested that the dermal sheath cells might be a specific source of myofibroblasts in cutaneous wounds (see Jahoda *et al.*, 1991). While follicular ORS cells provide the means for covering the wounds, it appears that in a parallel fashion dermal sheath cells could help to close wound gaps and act in dermal repair. In our laboratory there are experiments underway to clarify the role of follicle dermal sheath in wound healing.

4.4.1.3 Dermal cells and expression of MHC-I

Along with many other factors believed to be involved in the regulation of hair cycling, the immune system has also been suggested to play a part (Paus *et al.*, 1989, 1994 & 1996). This concept comes from the fact that some immunomodulatory agents such as cyclosporin and UV light have the potential to stimulate hair growth and because some hair diseases are associated with immunopathological phenomena (see Paus *et al.*, 1994). However, the exact role of the immune system in hair follicle regulation remains unclear.

During this study it was found that among the follicle and non-follicle-derived dermal cells examined, dermal papilla cells showed the lowest level of expression of the MHC-I antigen, while the highest level was observed in skin fibroblasts. Dermal sheath cells showed an intermediate expression, yet in UDS cells expression was higher than

that of the LDS cells. To my knowledge, such comparative experiments have not been performed on follicular dermal cells, although the current results are generally compatible with previous *in situ* and *in vitro* studies on rodents and humans where MHC class I molecules were found to be expressed at a lower level in the lower part of the hair follicle compared to the cells of the upper follicle or interfollicular compartments (Harrist *et al.*, 1983; Westgate *et al.*, 1991; Limat *et al.*, 1994a; Paus *et al.*, 1994). Nevertheless, while MHC-I immunoreactivity in hair follicles has been shown to be cycle-dependent *in situ*, cells at the proximal end of the follicle tended to be completely negative. Using mouse pelage follicles Paus *et al.* (1994) demonstrated that during anagen the entire hair bulb was MHC negative, as opposed to the distal portion of the follicle including the bulge area that was MHC positive. In earlier studies similar data has been provided where human and rat anagen hair follicles appeared to lack MHC-I expression in their proximal regions (Harrist *et al.*, 1983; Westgate *et al.*, 1991). However, as was previously discussed there is contradictory data concerning the possible role of MHC-class I in the immuno-privilege processes. Westgate *et al.* (1991) suggested that the absence of MHC-I expression in the proximal portion of follicles leads to the recognition of this part by activated macrophages which play a role in regression of the follicle during catagen. In other words, according to this explanation, missing MHC-I expression evokes immune-recognition. On the other hand, there are several reports that show that a low or complete lack of MHC-I expression results in escape from immune-recognition. Based on this, Paus *et al.* (1994) suggested that the lack of MHC-I in the lower portion of the follicle might serve to prevent recognition of this portion by the immune system. If this is true it may explain why dermal papilla cells, with a low level or lack of MHC-I expression show great integrity when grafted onto non-privileged sites (e.g. ear) where macrophages have been activated as a result of wounding (Jahoda, 1992).

Another point of interest in the current study was the comparative expression of MHC-I in the lower and upper DS cells. While the lower expression of the antigen by LDS cells, compared to UDS cells, was generally in accordance with previous experiments, it again confirmed that the follicular dermal sheath has different cells with distinct characteristics in the lower and upper regions of the follicle. It also suggested that unlike the UDS cells that are characteristically close to skin fibroblasts, the LDS

cells possess properties more closely related to the DP. If the above idea concerning the relationship between MHC and immuno-privilege, is correct LDS should be capable of prolonged survival in wound sites where activated macrophages are present.

4.4.1.4 Dermal sheath and interactive abilities in wound sites

There is accumulative data which has not only established the properties of follicle-derived dermal and epidermal cells and those from interfollicular skin but has also documented their differences in terms of morphology, behaviour and biochemistry (Messenger, *et al.*, 1986; Reynolds & Jahoda, 1996; Hibberts *et al.*, 1996, 1998). Dermal and epidermal cells of the hair follicle, like other skin appendages have a widespread experimental use and powerful properties, and in this respect the follicular dermal cells have been outstanding. The dermal papilla (either as isolated intact tissue or cultured cells) from adult whisker follicles can be associated with skin epidermis from various sites and initiate follicle morphogenesis (e.g. Oliver, 1966a,b; Reynolds & Jahoda, 1992; Ferraris *et al.*, 1997b). Following isolation and implantation of follicular lower dermal sheath into amputated follicles it has been shown that dermal sheath cells have the ability to initiate bulb formation and hair growth (Horne & Jahoda, 1992). However, in all similar experiments to date cultured dermal sheath cells by themselves have not been capable of inducing hair growth or follicle formation in follicular or non-follicular sites (Horne *et al.*, 1986; Horne & Jahoda, 1992). Nevertheless, the combination of cultured LDS cells with GE cells implanted into ear wounds formed large new follicle structures (Reynolds & Jahoda, 1996). This showed that non-inductive cultured LDS cells could have follicle-inducing capabilities conferred on them by the influences of GE cells. In the present study it was shown that although the LDS cells cannot induce or form a type-specific follicle in implanted sites, by staining and tracking the cells in implanted sites I demonstrated that these cells did have the capacity to be incorporated into local follicle structures, a property that UDS and SF cells lacked. This ability was not only demonstrated in ear wounds but was also observed with cells introduced into punch biopsies. This suggests that although UDS and LDS have a common developmental origin and can be structurally and functionally substituted for each other *in vivo*, LDS cells possess a greater capacity for inductive interactions and are closer to papilla cells in terms of their intrinsic characteristics.

Vital dyes have been widely employed for monitoring cells and have proven to be a reliable tool in this respect (e.g. Fukiishi & Morrisskay, 1992; Brandsaberi *et al.*, 1995; Oyesiku & Wigston, 1996; Chuong *et al.*, 1996). However, for this experiment, a couple of safeguards were included in an attempt to avoid misinterpretation. Firstly, by keeping stained cells in culture for a long period it was shown not only that the dye did not damage cell viability but also that the labelled cells could grow healthily for a long period while bearing the vital dye. Secondly, by taking advantage of confocal laser microscopy which presents a high magnification 3-dimensional image, it was confirmed that the labelling observed with conventional fluorescence microscopy was of cells rather than extracellular matrix. Two different implantation sites were used in this experiment, each providing appropriate conditions for cell interactions. Ear wound sites created a space that was relatively free of fibrous extracellular collagen and hence the extracellular matrix was loose, which provided a suitable medium for cell movement and morphogenetic activities. Similarly, in punch biopsy sites, cells were in a loose artificial tissue which, by supporting them and releasing them from physical constraints allowed them to migrate and interact with host cells.

The observations that neither DS nor SF cultured cells were capable of forming cell aggregates and initiation of follicle development in implanted sites have also been reported previously (Horne *et al.*, 1986; Reynolds & Jahoda, 1992; Jahoda *et al.*, 1993). However, the advantage of this study was that the implanted cells could be precisely localised in the host sites, a practice which was not carried out previously. This allowed LDS cells to be identified within the local hair follicles. Unfortunately, because in all of the histological sections these follicles were cut transversally it was impossible to ascertain the distribution of LDS cell along the length of the follicles, but based on their position within the follicle they were clearly situated in the follicular dermal sheath. There are various possibilities concerning the origin of these follicles which this experiment did not clarify; i) they were newly developed follicles which formed entirely after wounding and LDS became engaged in their structure during the morphogenesis process or, ii) they were the follicles which had been damaged due to wounding and LDS cells were involved in the regeneration process. Or, as labelled cells were also observed in local follicles located outside of the wound margins the third possibility would be that these cells migrated and were recruited by local undamaged follicles.

Whatever the answer, it demonstrated a significant point which had not previously reported, that these cells are capable of being incorporated in an appendage (pelage-type follicle) which morphologically and possibly developmentally differs from their original appendage (vibrissa follicle). This is interesting because there is also evidence showing that in addition to the induction of type-specific follicles, dermal papilla cells also can direct the growth of hair follicles with different features. It has been shown that vibrissa follicle papilla cells implanted into wounded rat ears yielded hair follicles corresponding in size to the vibrissal origin of the papilla (Reynolds & Jahoda, 1991). On the other hand, in a heterospecific experiment in which vibrissa follicle papilla cells were implanted into embryonic mouse skin, the implanted papillae yielded follicles of an intermediate size between those from where the papilla cells originated and those of the host skin (Pisansarakit & Moore, 1986). Moreover, recently, Weinberg *et al.* (1993) showed that vibrissa follicle papilla cells in combination with developing mouse pelage hair follicles directed the growth of hairs that were narrower than those supported by papilla cells from pelage follicles. These observations suggest that the DP cells have the versatility to accept different phenotypes and functions in various conditions. At the same time, based on the observations presented here (vibrissa LDS cells incorporated into pelage-type follicles), and those from Reynolds & Jahoda (1996) who showed that vibrissa LDS cells could make large vibrissa-type hair follicles in ear wounds when associated with GE cells, it is logical to consider that LDS cells also are capable of changing and accepting different roles. However, this question as to what factor(s) regulate the change in behaviour of these cells remains unanswered.

The results discussed up to now support the idea that the cells of the LDS are different from cells originating from the UDS, so that UDS are more like SF, but LDS behave more or less like DP. However, it does not appear logical to consider that there is a distinct border between these two cell populations. It was shown that the LDS is a heterogeneous tissue in terms of its constituent cell types and the change in behaviour of DS cells along the length of the follicle could be due to the selective loss of a specific cell type or molecular element from the DS tissue in the higher parts of the follicle. Further study of this interesting tissue should yield important information about the cellular and molecular differences between cells residing in the lower and upper parts of the hair follicle

4.4.1.5 Dermal sheath in recombination with ORS cells *in vivo*

The use of hair follicles as a source of keratinocytes for dermal/epidermal organotypic recombinations has previously been shown by others (Noser & Limat, 1987; Lenoir *et al.*, 1988 & 1993, Lenior & Bernard, 1990; Limat *et al.*, 1991, 1995 & 1996; Verma & Shaw, 1994). However, the use of follicular dermal sheath cells in these experiments has been less fashionable. After establishing some of the *in vitro* and *in vivo* properties of LDS cells, my main goal for this part of the study was to investigate whether LDS cells in combination with the ORS were capable of interacting to produce a skin phenotype. Over the past decade skin substitutes produced from cultured cells have proven not only to be valuable tools in the treatment of burn wounds, but also helpful in the study of skin biology and development (Cooper & Hansbrough, 1991; Smola *et al.*, 1993 & 1994; Nolte *et al.*, 1994; Harriger *et al.*, 1995; Auger *et al.*, 1995; Hansbrough, 1995; Breitzkreutz *et al.*, 1997). Moreover, these artificial structures have been shown to be suitable for use in pharmacological and immunological studies (Rouabhia, 1996; Boyce *et al.*, 1994). The skin substitutes are composed of two compartments- dermis and epidermis. The dermis, historically considered as a passive support system, plays a key role in controlling growth, differentiation, and attachment of the epidermis. Using *in vitro* studies it has been well established that dermal cells enhance epidermal cell growth (Rheinwald & Green, 1975; Limat *et al.*, 1989; Reynolds *et al.*, 1991; Navsaria *et al.*, 1994). The critical role of the dermal component in the growth and proliferation of epidermal cells in studies aimed at making skin equivalents is also well documented (Hansbrough, 1995). One of the common methods used in the production of the dermal substitutes has been to seed the dermal cells on or into a solid collagen substrate. In these dermal equivalents interfollicular skin fibroblasts have commonly been used as the dermal constituent (Bell *et al.*, 1979, 1981 & 1983; Graeter & Hull, 1996). However, this study has demonstrated the usefulness of cultured follicular dermal sheath cells as a convenient source of the dermal component within these experiments. It was shown for the first time that these cells are also capable of direct interactions with epidermal ORS cells *in vitro* and later *in vivo*. Dermal sheath/ORS recombinations after 20 days growth on granulation tissues produced structures resembling normal skin with distinct dermal and epidermal compartments and

with a continuous basement membrane separating them.

The observation that dermal sheath cells penetrated well into the underlying collagen gel suggested that these cells could have collagenolytic activity. Since the collagen gel is composed of type I collagen, the enzyme released by dermal sheath cells is likely to be a type I collagenase. This observation is consistent with previous experiments where it was shown that hair follicles from new-born mice exhibit the collagenolytic activity when cultured in collagen matrices (Roger *et al.*, 1987; Scandurro *et al.*, 1995). This lytic activity which facilitates the invasion of the substrate by cells is likely to be one of the mechanisms involved in hair cycling, when early anagen hair follicles invade the entire depth of the dermis to form enlarged follicles. In this experiment the collagenolytic activity of the DS cells resulted in formation of a suitable 3-dimensional dermal substrate that facilitated their interactions with epidermal cells. Additionally, for the first time it was shown here that cultured dermal sheath cells by themselves and without the involvement of other follicular or non-follicular dermal cells can support the attachment and growth of epidermal cells as previously seen in fibroblasts (Limat *et al.*, 1989). In previous experiments it has been revealed that fibroblasts in skin equivalents secrete many macromolecules, including collagens (Demarchez *et al.*, 1992) fibronectin, chondroitin-4-sulphate, chondroitin-6-sulphate and type III laminin (Bouvard *et al.*, 1992) which are possibly responsible for the effect of dermal on epidermal cells. For example, it is known that fibronectin induces keratinocyte spreading and growth, while type III collagen promotes stratification and differentiation. Regarding the DS cells, it has been shown that *in vitro* they provide a good supportive substrate for the growth and proliferation of epidermal cells (Reynolds, 1989) and here using immunohistochemistry I have demonstrated that these cells produce various macromolecules in culture including type IV collagen as well as laminin, both of which are important constituents of the basement membrane.

Histological and ultrastructural observations demonstrated that the reconstructed tissue 20 days post-implantation had an organisation similar to that of the normal integument. This was clear from the fact the reconstructed tissue was composed of both dermal and epidermal components, which revealed normal characteristics as found *in vivo*. Dermal cells had a fibroblastic morphology and were distributed in a rich extracellular matrix. The epidermal cells revealed a normal phenotype in both the lower

and upper layers in terms of their internal structure and connections with neighbouring cells. Morphological changes observed in different layers of the epidermis confirmed that, in addition to the stimulation of epidermal growth, the presence of living LDS cells in the dermal equivalent could also contribute to quite normal differentiation of the epidermis. In addition to the important direct role of the dermis on epidermal growth and differentiation, it has also been suggested that the dermis influences these processes through its role in the formation of the basement membrane. It has been suggested that a normal epidermal structure and differentiation depends on the presence of an intact basement membrane since abnormalities in the growth and function of skin are often associated with disturbances of the basement membrane zone (see Fusenig, 1994). There is an increasing amount of data to suggest that the constituents of the basement membrane are produced mainly by the epithelial cells (see Marinkovich *et al.*, 1993; Yamane *et al.*, 1996). However, *in vitro* studies have demonstrated that keratinocytes grown under conventional culture conditions show insufficient basement membrane components, a lack of polar deposition and no formation of a structured basement membrane unless contact with a dermal tissue is provided (see Fusenig, 1994). It has been speculated that this deficiency in the production of a basement membrane is the main reason for a disappointing take of grafts composed of keratinocyte sheets when alone implanted on full-thickness wounds (Woodley *et al.*, 1988). The dependency of a basement membrane formation on dermal tissue is also shown using mesenchymal cells, where human keratinocytes could not produce basement membrane components unless under mesenchymal influence (Limat *et al.*, 1991). A dermal contribution is also required for formation of the basement membrane constituents in organotypic cultures and skin composites *in vivo*.

Subsequent to the use of composite grafts (containing both epidermal and dermal components) implanted directly onto wound sites, several groups have shown the formation of basement membrane components at the dermal-epidermal junction (Compton *et al.*, 1989; Krejci *et al.*, 1991; Mommaas *et al.*, 1992; Cooper *et al.*, 1993). This formation is attributed to the presence of a dermal component. In fact, synthesis and linear deposition of the basement membrane constituents, which increases with improved epidermal differentiation has been considered to be another differentiation product of keratinocytes, but regulated by mesenchymal cells (Smola *et al.*, 1993).

Nevertheless, the reciprocal induction of mRNA of some basement membrane components has also been observed in co-cultured fibroblasts in collagen gels when combined with epidermal cells (see Fusenig, 1994). Despite reports of the establishment of a basement membrane in composite grafts, formation of a continuous lamina in an environment isolated from host tissues has not yet been reported. Here, using conventional transmission electron microscopy, it was shown that a continuous basal lamina along with scattered anchoring fibrils could be detected at the dermal-epidermal junction 20 days after grafting. Although in this experiment the exact source of these components was not clarified, this success may be attributed to the replacement of SF cells by dermal sheath which appears to provide a better substrate for the establishment of a basement membrane. In support of this idea, as shown in chapter 2, the junction between the epidermal component and dermal sheath in the hair follicle is much thicker than that seen between fibroblasts and interfollicular epidermis (see Mommaas *et al.*, 1992) as well as between the epidermal matrix and dermal papilla. Dermal sheath cells are likely to secrete more dermal extracellular matrix components which encourage the production of the basement membrane constituents by keratinocytes. If this is the case, it suggests that, in addition to the presence of a dermal component, the type of dermal constituent is very important to the establishment of a basement membrane and the use of dermal sheath can be considered as a better alternative to fibroblasts in the design of dermal equivalents.

The strength of epidermal attachment is critical for the long-term maintenance of the graft integrity (Nolte *et al.*, 1994) and the presence of rete ridges between the dermis and epidermis is believed to strengthen the attachment between these two tissues. Mommass *et al.* (1992) reported the appearance of a normal rete ridge pattern in epithelial grafts transplanted into patients suffering from chronic leg ulcers. Later, Cooper *et al.* (1993) demonstrated the formation of rete ridges in the epithelium of composite grafts. However, the regeneration of epithelial projections in organotypic grafts inside an isolated chamber has not been reported to date. In the current study epithelial tongues which penetrated deep into the dermis were clearly evident in histological sections of grafts 20 days post implantation. Interestingly, these epithelial projections are not seen in the normal structure of rat skin. These results led me to the conclusion that the formation of epithelial projections enhanced the surface contact area

between dermis and epidermis. This along with the establishment of basement membrane components at the epithelial/dermal junction, which were attached to epidermis by numerous hemidesmosomes, improved the attachment of the epidermis to the underlying dermis. This physical connection in turn led to more dynamic interactions between the two neighbouring tissues and these interactions were responsible for the development of an organised and mature epithelium.

Maintaining material in chambers over well-vascularised granulation tissue, has previously been shown to be effective in allowing the formation of organised epidermal and skin-like structures from skin-derived cells (Reynolds & Jahoda, 1992; Rouabhia, 1996; Xu *et al.*, 1996), and the present study has also shown the usefulness of this methodology for the formation of organised structures from follicular cells. In previous studies the recombinations have been placed directly onto host tissues which not only provided extra support for the graft but clearly made a cellular contribution to it. The difference in this experiment was that the recombinations were separated from the host tissues by a filter which restricted any cellular contribution from the host tissues in interactions occurring inside the chambers. Therefore, in the present study I was certain that except for nutrients which were provided by the host, all developmental processes were as the result of activities between experimental cells only. This fact was confirmed by two observations made on examination of the specimens: i) a thin acellular layer or sparsely populated collagen gel was still present in the lowest part of the chamber even 20 days post implantation, ii) no blood vessels were found within the dermis of the organised tissues.

Although, as mentioned earlier, the main objective of this study was to investigate the ability of cultured DS cells to direct the reconstruction of epidermis from cultured ORS cells, the formation of epithelial-spheroids which were possibly primitive follicular structures is a point worth noting here. The formation of skin appendages in graft systems has previously been reported in skin equivalents, either *in vivo* or *in vitro*. English *et al* (1992) observed sebaceous glands in one skin recombination, but these structures were considered to have resulted from stem cell contamination of dissociated epidermal cells used to make an epidermis. In addition, Reynolds & Jahoda (1992) reported that hair follicles were induced in footpad skin recombinations but they used freshly dissociated epidermis and dermis as the source of the recombinant cells.

Similarly, Watson *et al.* (1994) demonstrated follicle formation in sheep skin equivalents containing SFs with epidermal cells derived from non-follicular or follicular epidermis. However, follicle formation was only observed in recombinations that had received extra whole dermal papillae implants. Those that did not contain whole papillae or received cultured papilla cells did not display any features that could be related to the development of hair follicles. In the context of using only hair follicle-derived epidermal and dermal cells as the source of recombinant cells, there are also several reports that show that follicle development and hair growth have occurred (Yuspa *et al.*, 1970; Worst *et al.*, 1982; Weinberg *et al.*, 1993; Lichti *et al.*, 1993; Kamimura *et al.*, 1997). However, in these experiments the recombinant cells were all or partly derived from embryonic or immature hair follicles. It has also been well-established that dermal/epidermal recombinations containing adult follicle-derived cells are capable of producing a normal dermis and epidermis, but so far the formation of hair follicles has not been observed in these recombinations. In the present study, it was shown that cultured DS cells in association with ORS cells (both from adult animals) produced structures that appeared to be components of primitive hair follicles. To confirm whether or not these structures really were hair follicles is difficult especially, as they did not show a bulb, and further examination is required particularly utilising appropriate immunohistochemical markers which recognise follicular tissues.

Grafting of ORS/DS recombinations onto the chick CAM reinforced the value of this site as a suitable place for cell/tissue implantation, and confirmed that, in those recombinations that were implanted onto host rats, the host tissues had made no cellular contribution to the organisation of developed skin-like structures. When specimens on the CAM were compared with their chronological equivalents grafted on rat tissue, the structures that had developed displayed similar configurations in terms of their dermal and epidermal morphologies.

In summary, these results highlighted the interactive character of adult hair follicle dermal sheath cells in combination with ORS cells. In addition to providing a convenient environment for the growth and proliferation of epidermal cells the results showed that dermal sheath cells are also able to induce the production of basement membrane constituents in epidermal cells. This supports the concept that dermal sheath cells (like dermal papilla cells) retain abilities similar to those seen in embryonic

mesenchymal cells in that they can direct the normal morphogenetic processes in epidermis. Furthermore, as it has been shown that during follicle morphogenesis, mesenchymal cells express numerous signalling molecules (Holbrook *et al.*, 1993) which conduct the epithelium toward the formation of the hair follicle. I would therefore predict that dermal sheath cells make these molecules.

4.4.1.6 Dermal sheath in recombination with ORS cells *in vitro*

The use of an artificial three-dimensional culture system of skin comprising a dermal substitute and cultured epidermal cells has been shown to be advantageous for investigating biological interactions between epidermis and dermis *in vitro* compared to single-layered cell culture systems on plates (see Horiguchi *et al.*, 1994). The main objective of this study, was to examine whether such an artificial system containing cultured epidermal ORS cells and a dermal substitute made of collagen gel with cultured LDS cells, was able to acquire the same dermal and epidermal phenotype *in vitro* that was shown *in vivo*. By preparing three different types of dermal equivalents and subsequently seeding ORS cells on top at different time intervals I tried to optimise the conditions for growing the cells in culture. Moreover, as the beneficial effects of an air interface on keratinocyte growth and differentiation have been previously established (Asselineau *et al.*, 1985 & 1986; Coulomb *et al.*, 1986; Lenoir *et al.*, 1993; Limat *et al.*, 1994c) this system was applied to the cultures to enhance the possibility of success. Histological observations demonstrated that none of the attempted combinations were able to produce skin-like structures *in vitro*, in the way that they did when implanted *in vivo*. Only primitive skin-like structures comprising a 2 to 3 cell-layered dermis and epidermis were observed in combinations which were prepared exactly like their *in vivo* counterparts. However, even in these combinations; cells were not able to maintain their growth for an extended time.

There are many reports that a normal epidermis can be reconstructed *in vitro* with combinations of dermal and epidermal cells using various methods (e.g. Lenior & Bernard, 1990; Limat *et al.*, 1991; Regnier & Darmon, 1989). It has also been documented that, under these conditions, dermal and epidermal cells can even express the specific markers which are normally expressed by epidermal cells *in vivo* (Horiguchi *et al.*, 1994; see also Fusenig, 1994). However, as already mentioned to the best of my

knowledge, there are no reports of the use of follicular DS cells as a dermal substrate. It is difficult to explain why my experiments did not work, but this deficiency can be attributed to a number of reasons. Firstly, it is possible that the DS cells were not able to provide the suitable conditions for the maintenance and growth of ORS cells.

Consequently, it could be argued that LDS cells are not able to secrete diffusible factors such as KGF, members of the FGF family, EGF as well as other cytokines that have been demonstrated to promote epidermal cell growth (see Smola *et al.*, 1993). However, as discussed earlier I have shown that they are able to secrete ECM components and that they were competent to support the growth of keratinocytes *in vivo* as efficiently, or better than skin fibroblasts. Therefore, the failure to produce a skin-like organisation in this experiment cannot be attributed to the abilities of LDS cells to support epidermal survival but is more likely to be due to technical problems *in vitro*, other properties of the DS cells, or species differences.

Although, in organotypic cocultures dermal and epidermal cells from both mouse and humans have been shown to have the ability to produce a nearly normal skin architecture, this ability has not been observed with cells from rat (see Fusenig, 1994). Hence, this is one possible reason why these experiments were not successful. I do not believe that this was the case, since Reynolds & Jahoda (1994) using a different model showed that the combination of 4 follicle cell types from rat hair follicles (ORS, GE, DS and DP) were able to interact and reconstruct hair follicles *in vitro* within the collagenous shells of vibrissa follicles. On the other hand, according to a very recent report (Almond-Roesler *et al.*, 1997), when human DP and SF cells were introduced into collagen gels, in contrast to SF, the DP cells showed a low capacity to reorganise a 3-dimensional collagen matrix and they lysed the collagen gel. Although this feature was not noted in the present experiments with LDS cells, since there are similarities between DS and DP cells, the LDS cells may have upregulated this activity during recombination with ORS cells. Therefore, a second reason for the results of the present experiment could be due to the lytic property of LDS cells preventing the maintenance of a 3-dimensional organisation of the collagen for a sufficient length of time. Nevertheless, it was shown that LDS cells still produced well-organised architectures *in vivo*. The third possibility, which I consider the main reason for this deficiency is related to technical problems. Despite the fact that culture systems will generate a degree of organisation

approaching that of the normal skin architecture *in vivo*, the *in vitro* systems still require considerable improvement before the reconstruction of skin with all its specifications is obtained. For example, it has been shown that many of the biochemical changes characteristic of terminal differentiation, such as K1, K10 and filaggrin expression, do not occur (see Fuchs, 1990) or are delayed (Smola *et al.*, 1993) in human reconstructed epidermis *in vitro*. Moreover, as mentioned earlier, to date, hair follicle and other skin appendage formation has not been reported using adult-derived dermal-epidermal cell recombinations *in vitro*, whereas this event has been demonstrated in a few cases *in vivo*. Another possible reason could be due to the poor quality of the collagen gel which did not provide an adequate substrate for cell growth and proliferation, but again the collagen produced good results when employed for *in vivo* recombinations. In brief, although at the present time we do not know exactly why the ORS-LDS cell recombination *in vitro* were less successful, I attribute this failure to technical problems in the culture system, rather than the properties and capabilities of the LDS cells. Indeed overcoming this problem presents a main task for future work in this area.

4.4.1.7 Dermal sheath and interactive abilities in an embryonic environment

In agreement with previous experiments on chick and mouse (Weiss & Taylor, 1960; Garber & Moscona, 1964; Garber *et al.*, 1968), in this study I have shown that dispersed embryonic rat skin cells when implanted on chick CAM could reorganise themselves and produce a skin-like structure. This demonstrates that at the time of their isolation the skin cells must contain specific properties or information which could be translated into a message causing morphogenesis to be repeated. The observation that several large epithelial structures were seen only in grafts which had received extra dermal sheath cells suggested that the presence of DS cells was responsible for some morphogenetic activities that did not take place in their absence. Although due to time limitations for grafts on chick CAM it was not possible to follow the fate of these large structures, yet morphologically they appeared to be vibrissa-like follicle structures that had been cut transversely. However, to confirm this more immunohistochemical evidence is required and experiments are underway to achieve this.

In conclusion, these observations reinforce the idea that follicular DS cells even in maturity possess the same repertoire of information as their embryonic precursors. In

particular, in this specific case it was confirmed that dermal sheath cells are able to participate in morphogenetic activities with epidermal cells to some extent, if not to the same degree as DP cells.

4.4.2 Inductive abilities of the hair follicle DP

As a permanent and stable population of specialised fibroblasts in the hair follicle the dermal papilla possesses unique properties and plays a profound role in controlling hair follicle activities (see Jahoda & Oliver, 1990). As was mentioned earlier, the inductive influences that the dermal papilla exerts on epidermal cells have been clearly shown using several experimental approaches (see Jahoda & Oliver, 1990). In the present study it was shown that when cultured dermal and epidermal cells were recombined in punch biopsies, vibrissa-like follicles were induced only in punches that had received intact papillae. Other variations, including the combination of DS/ORS and cultured DP cells (passage 2) did not produce type specific follicles in the punch biopsy sites.

Vibrissa follicles have specialised features, including a high level of innervation related to their important function as mechanoreceptors (see Jahoda & Oliver, 1984b). They also produce hair fibres which despite their similarity to pelage hair in terms of keratin composition, have some specific characteristics in terms of size, thickness and medulla type (Jahoda, 1992). In comparison to the pelage hair, the hair fibre of the vibrissa follicle is longer, thicker and it has an open medulla. It also grows as an elongated cone shape, which represents a continuous increase in fibre productivity until just before growth ceases. Although in this study the type and structure of externally induced fibres were not examined ultrastructurally or biochemically, their size and thickness was typical of vibrissa follicles and not pelage. On the other hand the fact that the whisker-like fibres were found to be surrounded by bare skin within the punch sites, suggested that the implanted material must have produced the new follicles. The existence of large follicles with Alcian blue stained dermal papillae within the punch sites that had received intact papillae not only suggested that they had originated from vibrissa-derived implanted components, but that the DPs had stimulated the growth of the large hair follicles. This finding is consistent with previous work which has shown

that the dermal papilla is capable of inducing follicle formation in non-follicular sites (Jahoda, 1992) and also confirms the idea that the size of the follicle is determined by the papilla size (Van Scot & Ekel, 1958).

It has already been reported that vibrissa dermal papilla cells alone (without vibrissa epidermal cells) induce vibrissa-like follicle formation in heterotopic epithelium from wounded ear pinnae (Jahoda *et al.*, 1993; Filsell *et al.*, 1994) and oral epithelium (Oliver, 1973). In these experiments it was clear that the epidermal component of the new large follicles originated from host epidermis. However, in this study because it was not possible to differentiate between the implanted epidermal cells and those from the host site the question of how much of the new follicle epithelium derived from the implanted ORS cells remains uncertain? Nevertheless, the observation of a thick epidermis with multiple deep downgrowths, which were not found in the neighbouring host skin, indicated that the implanted ORS cells were involved in morphogenesis at the operational sites. Moreover, the fact that in some areas these epidermal downgrowths looked to be in the process of forming large follicles in the vicinity of the epidermis (Fig. 4.39A) reinforced the likelihood that the cells in these large follicle-like structures were derived from the implanted ORS. Nevertheless, as shown by Reynolds & Jahoda (1996) the role of ORS cells in this experiment was principally as a responding tissue rather than an inductive one, otherwise they should have stimulated follicle formation in other punch sites without the presence of intact DPs. While the finding that skin fibroblasts were not able to stimulate follicle formation is consistent with previous experiments (Reynolds & Jahoda, 1996), the lack of follicle formation in sites that received LDS cells was intriguing. Nevertheless, these results do not contradict my previous results obtained from LDS/ORS recombinations within chambers *in vivo*, as the latter did not show complete follicle formation either. However, since LDS cells displayed good interactive capabilities in chambers it was expected that they might induce the formation of follicles in punch sites. Nevertheless, there are possible explanations for this, and one of these could relate to the process of cell aggregation. As I discussed earlier, cell aggregation plays a crucial role in the morphogenesis of skin appendages including hair follicles, where cell aggregation and an increase of cell intensity precede organogenesis. In this experiment punch areas were very small, hence shortly (2-3 days) after each operation they closed as a result of wound contraction and the occupation of wound space by

surrounding tissues. Therefore, there was possibly not enough time for the cultured follicle dermal cells to find each other amongst the different cell types present and form the aggregations which are prerequisites for follicle formation. Another explanation which is more likely relates to the role of dermal sheath cells in wound healing. To clarify this, it is worthwhile recalling my other experiments where it was shown that DS cells express ASMA. The significance here is that in mammalian skin ASMA containing fibroblasts are vital for contraction and wound healing, therefore in the punch biopsy experiments the implanted ASMA-positive DS cells most likely participated in wound healing rather than follicle formation and even though they may have follicle forming capabilities, these would be subjugated in an environment where the DS cells were required for a wound healing role. Moreover, since cultured DP cells were not able to induce follicle formation in the same protocol it would be too much to expect DS cells to do so.

On the other hand, this observation that cultured DP cells failed to induce follicle morphogenesis in operational sites is not consistent with previous studies where it has been shown they are capable of doing so (Reynolds & Jahoda, 1992). However, there are again explanations for this result. Since cultured DP cells are ASMA-positive a similar explanation to the one put forward for DS cells can be used to account for the failure of follicle formation in punch sites containing cultured DP cells. In other words it is possible that DP cells can take part in wound healing activities if the conditions appropriate. The second explanation, as was just discussed for the DS cells, would be due to the fact that the DP cells did not have sufficient time to come together and provide an aggregation as the basis for follicle formation. There is a report that supports this idea. After implanting sheep skin equivalents onto nude mice, Watson *et al.*, (1994) found that follicle formation only took place in grafts that contained intact papillae and was not observed in grafts containing first passage cultured papilla cells. However, in the Reynolds and Jahoda (1992) work where it was shown that hair follicles were induced in rat footpad skin recombinants by early passaged pelage papilla cells, the recombinations were implanted in isolated chambers. This allowed the cultured DP cells enough time to aggregate and induce follicle development. This was not provided in my experiment due to wound contraction and the quick influx of the neighbouring host cells. Moreover, my experiment differs from the foot pad work, as Reynolds & Jahoda (1992) used isolated

epidermis and dermis for recombination whereas here only cultured cells were employed. Furthermore, in the footpad work, materials were implanted into chambers in which the cells were isolated from the host tissue and this reduced the possibility that the implanted cells would be recruited into the wound healing processes. In the present study the ability of discrete papillae to induce follicle formation can most probably be attributed to the fact that the papilla cells were already aggregated within the structure, and therefore the conditions were appropriate for rapid follicle development. In addition, since dermal papilla cells do not express ASMA *in situ* (Jahoda *et al.*, 1991) they probably did not become involved in wound healing and their abilities were directed solely towards follicle formation.

In conclusion this experiment reconfirmed the exclusive inductive powers of the follicular DP in follicle formation and emphasised the important role of cell aggregation in the initiation of hair follicle development. Moreover, it implies that sometimes a property of a cell may not be recognised or apparent because of its other capabilities.

4.4.3 Dermal cells in other skin appendages

Extrapolating the idea that dermal and epidermal cells from skin appendages might retain their embryonic flexibility throughout maturity, I have examined the properties of adult rat claw and feather follicle dermal cells, and then investigated the results of associating populations of cells from these two skin appendages with hair follicles.

4.4.3.1 Claw unit

Although the histology of the claw was described a very long time ago (see Nelson, 1953) in comparison to the hair follicle there is very little literature concerning its precise structure and the properties of its different cell types. Mammalian claws have evolved into nails in humans which are structurally very similar. Because of the importance of the nail for humans the majority of available literature concerns the nail. In this study, in agreement with others (Nelson, 1953) I found that like the hair follicle, the claw unit consisted of two principal components dermal and epidermal which lie directly on the dorsal side of the last phalanx bone. The dermal component was comprised of

cells found within an alcian blue-positive ECM showing that it is rich in GAGs. These molecules which are also found at high levels in the anagen hair papilla have been shown to stimulate hair growth in the rabbit (Mayer *et al.*, 1961). Although, the role of these macromolecules even in hair follicles is not yet fully understood. With respect to the fact that it seems they have diverse roles in cell-matrix adhesion, transmembrane signalling, growth factor binding and activation, macromolecular traffic, thrombogenesis and the immune system (Couchman, 1993) their expression in the claw dermis possibly indicates that as for the hair dermal papilla, the claw dermis via these and other molecules was supporting claw formation. The epidermis displayed several cell layers but the observation that the basal cells were only found in the proximal half of the claw (claw matrix) implied that this region is the site of claw formation, corresponding to the germinative region of the hair follicle matrix. Immunohistochemical studies of the matrix of the human nail have demonstrated that compared with the hair follicle epidermis the pattern of integrin expression is similar, although some differences in the distribution of $\alpha 2$, $\alpha 3$ and $\beta 1$ subunits are detectable (Cameli *et al.*, 1994). At the dermal-epidermal junction there is a basement membrane which in the human nail has been shown to display a very similar antigen composition to that of normal non-appendageal skin (Sinclair *et al.*, 1994). Collagen VII, fibronectin, chondroitin sulphate and tenascin were among the antigens detected using antibody probes (see Dawber *et al.*, 1994).

In the context of cell culture, although primary cultures of claw dermal cells behaved in a typically fibroblastic manner in terms of their growth characteristics, the observation that they showed some degree of aggregative behaviour at confluence suggested that the claw dermal cells might have similar properties to hair follicle papilla cells. Moreover, the high level of ASMA expression by the claw cells *in vitro*, which was reported in this study, again reinforced the idea that these cells have a papilla-like phenotype. The role of ASMA in the hair follicle was discussed previously but due to the lack of literature the function of ASMA in the claw unit remains to be clarified.

I have also shown that cultured dermal cells from the rat claw, irrespective of their passage number, were not able to induce the formation of skin appendages either in rat ear wounds or vibrissa follicles although they could not have been expected to induce well-developed appendages in the relatively short period that they were implanted for. However, the fact that they showed no sign of the initiation of any appendage formation

implies that they probably lack the inductive capacity to do so. Moreover, the observation of a mass of implanted cells confirmed that the cells had remained at the operational site until biopsied. Therefore, in comparison with the inductive properties of hair follicle papilla cells which have been demonstrated in previous work (Jahoda *et al.*, 1984 & 1993) the present study has shown that this property is not universal to all skin appendages, at least in this case it is not seen in the claw dermis.

4.4.3.2 Feather follicle

I have found that the feather follicle (during growing and non-growing stages) generally displayed a morphology which was comparable to that seen in the hair follicle but some differences between these were also apparent. One of these, noticed in this study, was that in addition to small blood vessels (capillary-like) seen scattered in both the feather and hair papilla, the feather papilla contained large central blood vessels which ran through the papilla and pulp. The existence of these large, ASMA-rich vessels in the centre of feather follicles reinforces the vital role of these appendages in the survival of flying birds, a role which is probably less important for mammals and the function of hair. I did not examine the structure of feather follicles in a non-flying bird such as the domestic chicken, but it would be interesting to investigate whether there is a difference between flying and non-flying birds with respect to this?

ASMA was highly expressed by feather follicles and their arrector muscles. This cytoskeletal protein was expressed in the connective tissue of the feather follicle in a similar pattern to that observed in the hair follicle (Jahoda *et al.*, 1991) where only the lower connective tissue expressed the antibody highly. However, unlike the hair follicle in which the papilla cells do not express ASMA *in situ*, this cytoskeletal element was seen in feather dermal papillae *in situ*. The high level of expression of this contractive protein in feather follicles and their arrector muscle emphasises a possible role of ASMA in the feather, however, again due to the lack of literature this role remains unclear.

The presence of a population of epidermal cells attached to the papilla of the non-growing feather follicle (not seen in hair follicle) was an interesting observation which may provide a clue to the location of epidermal stem cells in the feather follicle. These cells could be easily separated from the acellular and keratinized part of the feather matrix but they were tightly attached to the papilla by a very thick basement

membrane. As a result these cells are possibly left intact during molting or plucking of the feather. The primitive characteristics of these cells including their round-shape, and small-size with a thin cytoplasm suggests that they are a likely source of epidermal cells for the formation of the next feather shaft generation. This idea is supported by the fact that, in contrast to hair follicles which display a substantial population of epidermal cells in the upper part of follicle (see chapter 2), in the feather follicle no such population was observed (Fig. 4.41G).

In this study, feather dermal papilla cells initially grew very well in primary culture but after subculturing some cells started to die. In spite of this, they displayed aggregations at confluence, though not as distinct as vibrissa papilla cells, suggesting that they have comparable properties with hair follicle papilla cells. The observation of cell death which increased progressively in cultured cells could possibly be due to the fact that the medium was specific for the growth requirements of mammalian cells and was not modified for avian cells. Nonetheless, the rate of cell loss did not decline even when FCS was replaced with chick serum. This excluded the possibility that the bovine serum was responsible for the avian cell death in culture, the main reason therefore remains to be established.

In previous experiments it has been demonstrated that embryonic dermal and epidermal cells of birds and mammals have the capacity to interact with each other and induce the initiation of hair or feather formation (see Sengel, 1976). However, in this experiment it was shown that feather papillae from an adult bird were not able to interact with the hair follicle epidermis in any significant manner when introduced into adult rat vibrissa follicles. This failure could be related to the solid structure of the feather papilla. As I have shown, feather papillae have a fibril-rich ECM which could have possibly prevented the cells from migrating and interacting with the rat follicle epidermis. Moreover, although prior to implantation I tried to remove the thick basement membrane surrounding the papilla, from cell culture observations, parts of this membrane were always found to be left attached to the papilla. Therefore, perhaps this membrane did not allow direct contact between the papilla cells and the host epidermis. On the other hand, a deficiency in my experiment could be due to the fact that unlike in embryonic recombinations where messages sent by cells (e.g. bird dermal cells) can be understood by the other cells (e.g. mammals epidermal cells), the adult cells from birds

and mammals are not capable of recognising or responding to signals from each other. However, finding a clear reason for lack of success in this experiment requires further investigation.

4.4 4 Organ culture of plucked hair follicle end bulbs *in vitro*

In this experiment I employed a method, which was developed in our laboratory (Robinson *et al.*, 1997) from the original work of Philpott *et al.* (1989) for studying the growth of hair follicles in culture. I have shown that plucked end bulbs from rat vibrissa follicles can be isolated and maintained in a viable state in organ culture. I have also demonstrated that in culture the plucked end bulbs not only regenerated a new matrix around the dermal papilla but were also able to grow short fibres. Afterwards a number of follicles ceased growth and entered a telogen-like stage while in others the follicle components had disintegrated.

A number of authors have reported on the successful growth of adult and embryonic hair follicles *in vitro* using specimens from human or other species (see Philpott *et al.*, 1996). In some reports it has been shown that follicles can be maintained *in vitro* in a viable state for 40 days (Li *et al.*, 1992). However, in all of these studies where follicles were cultured and demonstrated hair growth, a preformed fibre was already present and to the best of my knowledge this ability has not been previously reported in plucked follicles. Although the percentage of success was low in this study I have for the first time demonstrated that the epidermal germinative cells left behind after plucking are able to proliferate and produce a new fibre. This result is in agreement with my previous studies that showed the GE cells have ability to regenerate a new fibre *in vivo* after plucking. However, the fibre growth *in vitro* was less than that observed *in vivo* and the time of growth was limited. This limitation is not only restricted to this experiment but has also been reported by several other workers using non-plucked follicles (see Table. 4.1). In all of these investigations the follicles terminated their growth in culture earlier than their counterparts *in vivo*. It has been suggested that this earlier cessation might result from the absence of a factor or factors normally available *in vivo* or from the possible build-up of one or a number of inhibitors in the medium or within the follicles (Bates *et al.*, 1997). The fact that the general conditions in the

Follicle type	Duration of growth <i>in vitro</i>	References
Mouse pelage, (NB, 3-5d)	5 days	Frater & Whitmore, 1973
Mouse pelage	10 hours	Uzuka <i>et al.</i> , 1977
Rat pelage (NB, 12-14d)	48 hours	Frater, 1980
Mouse pelage	7 days	Roger <i>et al.</i> , 1987
Mouse vibrissa (NB, 3-4d)	3 days	Buhl <i>et al.</i> , 1989
Rat pelage (NB, 8-12d)	7 days	Philpott <i>et al.</i> , 1989
Human scalp	9- 10 days	Philpott <i>et al.</i> , 1990
Human (various body sites)	8 days	Kondo <i>et al.</i> , 1990
Human scalp	10 days	Li <i>et al.</i> , 1992
Rat pelage	48 hours	Philpott <i>et al.</i> , 1992
Human scalp	96 hours	Imai <i>et al.</i> , 1993
Mouse vibrissa	5 days	Waldon <i>et al.</i> , 1993
Angora goat pelage	96 hours	Ibraheem <i>et al.</i> , 1993
Human scalp	12 days	Tobin <i>et al.</i> , 1993
Mouse vibrissa	3 days	Jindo <i>et al.</i> , 1994
Human scalp	7 days	Sanders <i>et al.</i> , 1994
Merino sheep wool	7 days	Bond <i>et al.</i> , 1994
Human facial	7 days	Harmon & Nevins, 1994
Sheep wool	8 days	Williams & Stenn, 1994
Mouse pelage	4 days	Kamiya <i>et al.</i> , 1995
Mouse vibrissa	22 days	Robinson <i>et al.</i> , 1997
Sheep wool	8-10 days	Bates <i>et al.</i> , 1997

growth medium are not as ideal as *in vivo* has also to be considered. On the other hand, there is an idea that proposes that cessation of hair growth *in vitro* results from the depletion of the pool of differentiated follicular keratinocytes which in turn results in the loss of matrix cell proliferative activity (Harmon & Nevins, 1994). In other words, because GE cells are TA cells, the cessation of hair growth is due to the fact that these cells do not have the ability to proliferate any more. However, if this is the case we would expect to observe a longer growth period in the intact follicles or in the follicles that possess the upper part (the region which is believed to contain stem cells), but this does not happen. Moreover, I have shown that plucked follicles, in the absence of the upper epidermal cells, were able to grow for lengthy periods *in vivo* without any limitations (see section 2.3).

Williams & Stenn (1994) showed that the transection level dictated the pattern of hair growth in cultured sheep follicles. They stated that if a follicle is cut below the sebaceous gland a sheath grows up along with the fibre but when a follicle was cultured either after transection above the sebaceous gland or with an intact attached epidermis, the hair shaft grew without sheath. Although, in the current study similar sheaths attached to fibres were observed in cultured follicles, Robinson *et al.* (1997) showed that in mice vibrissa follicles, the growth of this sheath was related to the stage of the follicle cycle rather than the transection level.

In conclusion, using an improved *in vitro* system, I have shown that the GE cells are able to regenerate a new matrix and hair fibre in rat vibrissa follicles maintained in culture. With this development I have not only confirmed the results of my earlier data concerning the proliferative ability of GE cells, but I have developed a useful model for studying the interactions between dermal papilla and GE cells- the two principle cell populations which determine all follicular characteristics including follicle cycling.

Chapter 5

Concluding discussion

In the previous chapters I have addressed, a variety of questions concerning the general properties and the proliferative and interactive capabilities of particular dermal and epidermal cells from adult skin appendages. In chapter 2 and 3 in connection with the question of follicle cycling, I investigated the proliferative ability of rat vibrissa follicle epidermal cells *in vivo* and *in vitro* respectively. In chapter 4 the characteristics and interactive abilities of the dermal components of the hair follicle and two other skin appendages (claw and feather) were compared experimentally. Although a variety of techniques were employed, much of the present study was aimed at clarifying two main points in amniote skin appendage biology. These were: i) the control of the hair follicle cycle, and ii) the interactive abilities of dermal cells in different adult skin appendages. In this chapter the current knowledge in these areas is first discussed, and then with reference to the results presented in the previous chapters I try to make some conclusive comments.

5.1 Hair follicle cycle

The hair follicle cycle is a co-ordinated and complex process and its exact mechanism is so far unknown. However, lengthy investigations into the cycle have led to the identification of a great number of local and systemic factors that appear to be important in controlling its cycle. These factors can be classified into two groups, termed intrinsic and modulatory factors. The intrinsic factors can be defined as the factors and forces which are associated with the cells or cell products in the hair follicle itself. These factors are believed to be common in all follicle types in mammals. The modulatory factors are agents like growth factors and hormones that influence the hair cycle indirectly. In contrast to the intrinsic factors the modulatory factors not only vary in different species, but in different individuals and body sites, and as a result they control the behaviour of many of the hair follicle types in mammals. Unlike the intrinsic factors which are only restricted to the follicular or local cells the modulatory factors are able to act on the follicle cycle from a distance. In this study I concentrated on the intrinsic

influences and provided evidence about the role of cell stemness in the hair follicle cycle. Hence, at the beginning of this chapter I discuss the nature of follicular stem cells and then briefly review the importance of the modulatory factors in the hair follicle cycle,

5.1.1 Stem cells and hair follicles

To unravel the significance of stem cells in the hair follicle cycle an understanding of the general concept and properties of stem cells in biological systems is necessary. Stem cells have long been the subject of intense interest because of their biological properties and importance in many medical fields including cancer. Nevertheless, since different people define stem cells in different ways there is still ambiguity about what exactly constitutes a stem cell. Therefore, it appears to be beneficial to first consider the properties of stem cells and the problems involved in this area before discussing specific questions about follicular stem cells.

Although, many different properties have been ascribed to stem cells, according to most definitions (Lajtha, 1979; Lavker & Sun, 1982; Wright & Alison, 1984; Hall & Watt 1989; Potten & Loeffler, 1990; Morrison *et al.*, 1997) stem cells can be defined as primary or undifferentiated cells capable of proliferation and self-renewal. These cells do not differentiate themselves but produce cells that are able to differentiate into one or several differentiated, functional progenies. They can divide without limit, or at least for the lifetime of individual. When they divide, each daughter cell has a choice; it can either remain a stem cell or can enter on a course, which ends in terminal differentiation (Hall & Watt, 1989; Jones, 1997). The cells that become committed to terminal differentiation are believed to be able to divide rapidly for several rounds before undergoing terminal differentiation. These committed cells have recently been called transient or transit amplifying (TA) cells (Cotsarelis *et al.*, 1990). However, compared to TA cells, stem cells are believed to be the slow-cycling cells (Lavker *et al.*, 1993; Morrison & Weissman, 1994). Nevertheless, there is evidence that suggests that stem cells in some systems (mammalian intestinal crypt and drosophila ovary) divide more rapidly (Potten & Loeffler, 1990; Margolis & Spradling, 1995). Hence, this suggests that slow-cycling may not be a universal property of stem cells (Morrison *et al.*, 1997).

Apart from these compositional and behavioural properties there are some general questions which still remain unresolved: for example, what factor(s) determine

whether a stem cell divides or stays quiescent? What decides whether a given daughter cell remains a stem cell or differentiates? Whether the number of stem cells is constant or varied, and in what ways the differentiation of a daughter cell is regulated after it has become committed to differentiate?

Division or quiescence

Based on the investigation of hematopoietic stem cells Curry and Trentin (1967) proposed a model, which was later extended by others including Schofield (1978). According to this model, also called “the niche” hypothesis, stem cells can only reside in an optimal microenvironment or niche. When a stem cell divides, only one daughter cell can remain in the niche and others will be committed to differentiate unless another niche is available for it. A stem cell within the niche would have a high probability of self-renewal, but a low probability of entry into the cell cycle, and would thus only divide rarely. It is suggested that because the number of niches is limited, the number of stem cells would be limited as well. If the number of stem cells produced is greater than the available spaces the excess cells would differentiate. In fact the behaviour of stem cells in the intestinal crypt (Potten & Loeffler, 1990) and *C. elegans* support this hypothesis, but evidence obtained from other systems does not confirm such a local control mechanism (see Morrison *et al.*, 1997). As a consequence, the concept of the niche hypothesis is not taken as a complete explanation of the determinant of cell fate. Thus, if the niche assumption has some limitation, the fate of a stem cell has to be governed either by intrinsic or other environmental factors. Molecular studies which have been carried out in invertebrates and humans suggest that the Notch gene and its homologs possibly regulate the maintenance or proliferation of stem cells, but it is not clear whether this gene also operates in other systems or not (Morrison *et al.*, 1997). In murine hair follicles it has been suggested that this gene correlates with cell fate determination rather than stem cell maintenance (Kopan & Weintraub, 1993). Likewise, there is evidence from *C. elegans* and mice that indicates that stem cells differentiate as a result of the activation of a small number of master genes that in turn control a regulatory cascade. This cascade finally results in the expression of a number of other genes that determine cell fate (see Hall & Watt, 1989). Given the existence of such master genes which may control the expression of the required genes for differentiation, one important question still remains: what regulates the expression of the master genes?

Inducers of division

A number of growth factors have been shown to stimulate stem cells to undergo proliferation. There are reports that epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) promote proliferation of stem cells in the central nervous system (Reynolds & Weiss 1992, Gritti *et al.*, 1996). The proliferation of stem cells also increases in response to tissue demand (see Forge *et al.*, 1993) which is believed to be due to the release of a mitogen from dying cells, or relief from inhibitors normally produced by healthy cells.

Differentiation

The differentiation of stem cells includes both exit from the uncommitted state and entry into a particular developmental pathway. Evidence from *C. elegans* indicates that these two aspects are independently controlled. In this system exit from the stem cell state requires the loss of PIE-1 protein (Mello *et al.*, 1996a,b). This loss occurs by the asymmetric distribution of PIE-1 to daughter stem cells at each division. However, the absence of PIE-1 in daughter cells is insufficient to initiate a program of differentiation, and extra positive acting regulators are required to promote entry into a particular somatic lineage. It is not clear whether exit from the stemness state and initiation of differentiation are also independently controlled in mammals. One possibility is that differentiation is executed by the stem cell as a default pathway once it is removed from its niche. Another possibility might be the existence of specific signals, which promote differentiation and consequently direct exit from the stem cell state. In the epidermis one possible determining factor might be contact with a basal lamina, with loss of contact triggering the start of terminal differentiation, and maintenance of contact tending to preserve stem cell potential (Watt, 1984; Jones & Watt, 1993; see also Jones, 1997). However, further work is needed to draw a firm conclusion about the determinants of stem cell proliferation.

Cell fate determination

Another significant question is what factor or factors determine the fate of daughter stem cells? In principle, division of a stem cell generates two daughter cells whose fates depend on particular circumstances. One possibility is that the stem cell division could always be asymmetric (see Altman, 1996). This means that one and only one of the daughters would inherit a special character required for the cell to remain as a

stem cell, while the other would be somewhat already altered at the time of its formation in a way that forced it to differentiate and ultimately die. However, if this idea is correct there could never be any increase in the existing number of stem cells, and this is contradicted by the available facts. In skin, if a patch of epidermis is destroyed, the surrounding healthy epidermal cells proliferate and migrate to cover the denuded area and repair the damage. In this process a self-renewing patch of epidermis is re-established, implying that additional stem cells have been generated to make up for the loss. Despite this fact, there are several well-documented examples of asymmetric division in invertebrates, including *Drosophila* and *C. elegans*, where the segregation of daughter cell fates appears to be determined by the proteins Prospero, Numb or PIE-1 which are maternally inherited and asymmetrically distributed to the daughter cells (Altman, 1996; Mello *et al.*, 1996a,b). However, in general there are few examples of asymmetric stem cell division, and the available evidence favours a predominance of symmetric divisions in mammalian stem cell systems (see Morrison *et al.*, 1997).

It should be noted that the difference between stem cells and TA cells may be very small and it is very difficult to draw a rigid border between these two cell populations. There is good evidence in several systems that fully competent tissue regeneration is still possible even after frequent severe damage suggesting, that mature cells can also show stem cell-like properties. Based on this evidence Potten & Loeffler (1990) presented a scheme called "the diminishing stemness spiral" which considers the committed cells as a transition population of proliferating cells situated between the stem cells and the mature functional state, for example dead cells in the surface of the epidermis. Thus the committed cells or TA cells would be expected to share some properties with stem cells and some properties with terminal differentiated cells. The main property that they share with stem cells is the ability to divide. The property they share with mature cells would be the acquisition of one or more differentiation markers. The stem-like properties might be expected to gradually decline as the mature-like properties increase with division. Because TA cells possess stemness properties they are believed to be able to act as stem cells, but whether they do so or not depends on the situation of the cells in terms of the number of divisions that they have undergone and other circumstances. Normally during the lifetime of the animal they do not act as stem cells but over a short period they may satisfy the stemness definition and behave like

stem cells.

Number of cell divisions

Finally, it has been suggested that when a TA cell is committed to differentiate it is able to divide for limited rounds before reaching a final differentiated state. However, the unsolved question is what determines the number of divisions by a committed cell? One idea suggests that there is an internal counting mechanism in which cells are able to count cell divisions, and after the appropriate number, cells undergo terminal differentiation. In the optic nerve of rat, progenitor cells undergo a finite number of divisions in the presence of platelet-derived growth factor (PDGF). In this case it has been proposed that a stable molecule required for proliferation in response to PDGF is diluted with each cell division and when its concentration falls below a certain level the cells stop dividing and differentiate (Temple & Raff, 1986).

5.1.1.1 Hair follicle epidermal stem cells

As mentioned previously according to the bulge hypothesis germinative epidermal (GE) cells are not considered stem cells, however, the evidence provided in this research and others raises the possibility that these cells may represent a stem cell population within the hair follicle. First, it has been shown that these cells display an undifferentiated morphology *in vivo* and *in vitro* (Reynolds *et al.*, 1991) and this, as cited above, is a property of stem cells. The low state of differentiation of GE cells has been also confirmed by immunostaining of follicles against cytokins and integrins. The GE cells demonstrate a remarkable paucity of cytokeratins and are rich in VLA β integrins (see Moll, 1995) which both represent the stemness state. Secondly, it is documented that these cells express telomerase, a ribonucleoprotein enzyme, which is observed in immortal cells like male reproductive cells and most primary tumours (Ramirez *et al.*, 1997). This enzyme plays a role in delaying senescence in these immortalised cells by preventing degradation of DNA (Hastie & Allshire, 1989). Thirdly, my results indicated that these cells have an ability to divide *in vivo* and *in vitro* for longer than would be predicted by the bulge hypothesis. According to this theory, GE cells only can divide for the duration of one anagen phase which takes about 50 days in the rat vibrissa follicle. My plucking experiment found that GE cells continuously divided after plucking and that they were the source of cells that produced a new matrix

and hair. This observation was reinforced in the amputated plucked end bulbs which regenerated new hairs both *in vivo* and *in vitro*. Cell division did not cease in GE cells even after 5 consecutive pluckings which took about 120 days. In chapter 3 the results obtained from epidermal cell culture showed that bulb derived epidermal cells have the capability to continue cell division for more than 5 months, much longer than the duration of one anagen phase. In the latter experiment there is the possibility that the epidermal cells grown in culture were not all derived from GE cells, as a population of ORS cells are also found in this region. However, I consider that at least some of the long dividing cells came from the GE population. Based on these results, a considerable emphasis has been put on the suggestion that the proliferative ability of GE cells extends beyond one hair cycle. Therefore, if the classification of GE cells as TA cells by the bulge theory is based on the proliferative potential of these cells, I believe that my results suggest this is incorrect. In support of this concept recent results from Hebert *et al* (1994) showed that mice homozygous for the *fgf5* allele produce have abnormally long hair, which suggests that GE cells are able to proliferate for longer if they are not inhibited by factors such as FGF5. In fact, further experiments on the proliferative abilities of GE cells are needed to provide deeper insights into the nature of these cells in hair follicles. On the other hand there is documented data that shows that GE cells have abilities which are not observed in other follicular epidermal cells. When rat vibrissa GE or ORS cells were recombined with follicular DS cells, GE + DS recombinants induced hair follicle formation, an event not found in ORS + DS recombinants (Reynolds, 1989; Reynolds & Jahoda, 1996). This indicates that GE cells display a property, which is closely akin to that seen in embryonic stages of appendage development where epithelial cells signal mesenchymal cells to form condensations in the process of hair morphogenesis. In support of this idea it has been demonstrated that bone morphogenetic protein-2A (BMP-2A) which plays a significant role in morphogenesis and pattern formation in vertebrates is only expressed in matrix cells of developing mouse vibrissa, whereas outer root sheath cells do not express this signalling molecule (Lyons *et al.*, 1990).

In conclusion, if we accept the concept that the hair follicle possesses epidermal stem cells, based on the bulk of evidence I believe that GE cells play roles in the development and maintenance of hair follicles and they have characteristics of stem cells,

though to reach a definite conclusion much more investigation is required. I believe that these cells divide during anagen in response to inducing signals from the papilla and they are able to do so as long as these signals continue. Once the papilla stops sending these stimulatory signals, which happens at the end of anagen, the GE cells in turn terminate their division, but this does not mean that they do not retain the ability to divide more. During telogen the GE cells stay quiescent until they are reactivated by the papilla. However, one question that has been put forward is why they are not seen in telogen in the bulb? There is evidence to show that in the rat vibrissa follicle during early catagen, when the hair fibre begins to move up from over the dermal papilla, a small population of GE cells are left behind which may provide a pool from which the next hair fibre derives (Reynolds, 1989). It might be argued that the vibrissa follicle is not a good model for hair follicle stem cell questions but the fact is that much of the evidence which claims that hair follicle stem cells are located in the bulge region of the follicle has come from this system (Kobayachi *et al.*, 1993). To this end, although in this study I did not investigate “the bulge region” epithelium as much as I did GE cells, the importance of the former as stem cells has in no way been undermined. In the context of the follicular epidermal stem cells I believe in the idea of the presence of two populations of stem cells, one GE cells as the source of cells for hair production and another the upper ORS cells as the source of cells in case of follicle or interfollicular epidermal injuries. However, whether this idea is correct or not, it will require future work to clarify the issue. Indeed, finding specific markers for stem cells may bring an end to this controversial subject.

5.1.1.2 Hair follicle dermal stem cells

The follicular dermis is composed of two cell populations; the dermal papilla and the dermal sheath. Although, the role of the dermal papilla as the prime controlling factor in the hair follicle cycle has been shown in many investigations (see Oliver, 1980; Jahoda & Oliver, 1991), there is a good evidence that suggests the dermal sheath cells play the role of dermal stem cells for the hair follicle (see Oliver, 1991) or even dermal skin as a whole. According to this suggestion the dermal sheath cells could act as a cell reservoir to help to maintain and regulate papilla size. It also plays a role in guiding follicle downgrowth and follicle reconstruction during proanagen by interacting with

follicular epidermis to form and maintain the outer root sheath (Oliver, 1991). The evidence for this perspective is derived from Oliver's pioneering work (Oliver, 1966a,b,c) who showed that the dermal sheath are the source of the new dermal papilla in amputated hair follicles. This conception was later reinforced by the same author and others (Oliver, 1967b; Horne *et al.*, 1992), who showed that this dermal tissue is able to induce hair growth in deactivated follicles.

The results presented in this study also provide support for this idea that the dermal sheath cells possibly play the role of stem cells for dermal components in the follicle. As discussed earlier, one of the properties of stem cells is that they are undifferentiated cells. During the process of differentiation they can give rise either to one cell type as in case of the keratinocytes or to several cell types, as in the case of the hematopoietic stem cells. At the present time due to lack of supportive evidence, the growing of different cell types from dermal sheath explants cultured under coverslips, cannot be attributed to the polypotency of dermal sheath cells. However, this observation at least provided evidence that this tissue contains different cell types which possibly play specific roles in follicle. One of the other properties of stem cells is that they possess flexibility and are capable of becoming different cells with different roles. In this study I showed that when implanted into ear wounds dermal sheath cells were capable of incorporating into non-specific follicle types. Thirdly, when they were recombined with ORS cells in chambers they showed a high interactive ability with epidermal cells, a character observed in particular tissues including embryonic mesenchymal cells. In addition to their role in the follicle the results presented here, including the expression of ASMA in the dermal sheath raised the possibility that the dermal sheath cells may also play a role in the maintenance of skin. In other words they could be reservoir of skin dermal cells as well. This concept is supported by the fact that myofibroblasts containing ASMA play an important role in wound healing. It is possible that during the healing of full-thickness wounds, in addition to the contraction of wound area by their cytoskeletal ASMA the dermal sheath cells restore the dermal component of defect skin. Nevertheless, reaching a final answer to these possibilities needs more investigation, for example it would be a good idea to investigate the relationship between wound healing and the dermal component of hair follicles directly.

Before closing the discussion on follicular stem cells there is one point that I

want to put forward. As I discussed in chapter 4, there is documented data (Jahoda, 1992; Reynolds & Jahoda, 1992) that has shown that dermal papillae are capable of stimulating follicle neogenesis in non-follicular epidermis to produce GE cells and other follicle components. If the papilla cells are able of inducing overlying non-follicular epidermis to change then by inference the papilla cells should be able to re-stimulate the ORS or any other adjacent follicular epidermal cells at the beginning of next growth phase. Therefore, the question arising from these data is whether the existence of a stem cell population in the follicle is necessary, when there is the means of re-inducing or replacing the epidermal stem cell properties at the beginning of each anagen cycle? This is true in the same way for dermal cells, as GE cells can signal both DP and DS cells to induce hair follicle neogenesis in non-follicular sites. These are the questions the answers to which beg priority in future work before experiments in the field of follicular stem cells go deeper into molecular complexities.

5.1.2 Hair follicle cycle and modulatory factors

These factors can be divided in two categories: i) factors which are secreted locally and regulate the follicle cycle (extracellular matrix material or growth factors), and ii) systemic factors or hormones which are produced by the endocrine system and reach the follicle via blood vessels acting to stimulate or inhibit the growth of hair.

5.1.2.1 Local factors

Although it has been known for some time that plucking of hairs from resting follicles causes the initiation of new hairs (Collins, 1918, Johnson & Ebling, 1964; Ibrahim & Wright, 1975 & 1978; Hale & Ebling, 1975 & 1979; Wilson *et al.*, 1994a,b), there remains no full explanation of the mechanisms involved. Chase (1955) explained the induction of hair growth after plucking by assuming that an inhibitor accumulated in the hair follicle during the growth period and that this then gradually dissipated during the resting period. According to this idea, plucking the hair will remove the inhibitor, therefore stimulating the follicle to start a new cycle. This concept has much in common with the 'chalone' theory which was used by Bullough (1965) to explain the cyclic growth of hair. Based on this theory, cell division in each tissue is normally inhibited by a combination of a tissue-specific protein or 'chalone'. In the hair follicle this protein is

produced by differentiating parts of the matrix cells, and when its level is low the matrix cells continue to divide, but after reaching a certain concentration it causes their division to cease. Several recent investigations have supported the chalone theory by demonstrating that inhibitory factors are involved in hair follicle cycling. For example, Paus *et al.* (1990) showed that telogen mouse skin contains a factor that inhibits hair growth. This factor, which is not observed in anagen skin, was suggested to be produced by the epidermis. Although in this experiment the nature of the inhibitory factor was not clarified, later experiments did reveal some growth factors capable of inhibiting the hair follicle cycle, one example being epidermal growth factor (EGF). Although this factor is a well-known inducer of epidermal cell proliferation (Pisansarakit *et al.*, 1991) and increases keratinization of epidermal cells in mice, it can also inhibit hair growth and follicle development (see Peus & Pittelkow, 1996). Administration of large doses of EGF to adult sheep causes their follicles to enter to catagen prematurely (see Messenger, 1993), while *in vitro*, the presence of EGF in the medium causes premature cessation of fibre production (Bond *et al.*, 1996). This growth factor also completely abrogates hair growth in cultured human hair follicles (Hoffmann *et al.*, 1996). The inhibitory effect of EGF on hair growth has also been shown in sheep follicles (du Cros *et al.*, 1992). A compelling piece of evidence that strongly supports the inhibitory theory was derived from the expression of fibroblastic growth factor-5 (FGF5) in the hair follicle (Hebert *et al.*, 1994). In mouse hair follicles, it has been shown that FGF-5 is expressed in the ORS of the transient portion (lower one-third) of the follicle. This growth factor has been suggested to control the duration of anagen by inhibition, based on two pieces of evidence; i) FGF-5 knockout mice display delayed onset of catagen, with follicles producing abnormally long hair, and ii) while the steady-state of FGF-5 increases during early anagen VI, it declines around catagen. It has also been demonstrated that the addition of FGF-5 protein to the culture medium changes the behaviour of dermal papilla cells *in vitro*, indicating that they are responsive to FGF5. Therefore, the papilla cells may be the targets for FGF-5 which is produced by the ORS cells. However, there remains some debate as to exactly how FGF-5 may influence the hair cycle. Some believe that FGF-5 is an catagen inducer and therefore it controls duration of anagen (Hebert *et al.*, 1994) while others suggest it may be involved in the process of hair follicle regression (Petho-Schramm, *et al.*, 1996). If we accept that FGF5 is an inducer

of catagen, this could provide an explanation for why plucking initiates hair growth in the follicle. In this study I showed that in addition to removing the fibre, plucking also takes out most of ORS cells particularly in the lower third of the follicle which, as discussed above, is an area that strongly expresses FGF-5. Therefore, continued cell division in the GE cells, and thus hair growth, could be attributed to the removal of the FGF-5 positive cells which would normally inhibit cell division at the end of the anagen phase. In this study it was also demonstrated that the follicles whose hairs had been plucked displayed a similar growth period to unplucked follicles. This might reflect the fact that the follicles need a certain amount of time to build up a critical level of FGF-5 capable of inducing catagen in the follicle. However, since follicles from day 2-3 post plucking showed semi-active states with less GE cell division and condensed papillae, the process of hair cycling is likely to require a more complicated explanation than can be provided by a single growth factor theory.

In contrast to the chalone theory, Swann (1958) proposed that plucking releases an inductive stimulus. This suggestion was reinforced by experiments involving plucking of rat pelage hairs (Johnson & Ebling, 1964). Although in the present study I have not observed any indication that plucking shortens the duration of the growth phase, they reported that plucking of growing hairs shortens the growing period. This is the opposite to what one might expect from the chalone hypothesis. These results led Johnson and Ebling to suggest that plucking induces the release of stimulators which cause initiation of the next follicle cycle. In other words, they proposed that hair growth resulted from the influences of stimulatory rather than inhibitory factors. While these authors were unable to determine the nature of any stimulatory factor, or exactly where it come from, recent investigations have revealed signalling molecules with stimulatory influences on hair growth. Some of the better-characterised factors in this respect are those of the FGF family. In adult life, several members of the FGF family are expressed in the hair follicle at various stages of the hair cycle, which does suggest that they could have functional roles (Rosenquist & Martin, 1996). Two members of the family, acidic and basic FGF (or FGF-1 and FGF-2 respectively) also play important roles in the process of hair follicle morphogenesis and have been localised in skin before and during hair follicle development (Gonzalez *et al.*, 1990; Moore *et al.*, 1991; du Cros *et al.*, 1992). FGF-2 in ovine follicles is associated with cells of the outer root sheath and is localised at the

interface between the bulb matrix and dermal papilla (du Cros *et al.*, 1993). Because this area is adjacent to the proliferative zone of the follicle it has been suggested that it may be involved in regulating mitotic activity in the follicle bulb cells (du Cros, 1993). Keratinocyte growth factor (KGF or FGF-7) is a recently identified member of the FGF family that induces proliferation in a wide variety of epithelial cells, including keratinocytes (see Danilenko *et al.*, 1995). In the skin, KGF stimulates not only epidermal keratinocytes but also keratinocytes within the hair follicle and sebaceous gland. KGFR mRNA has been identified in the developing hair follicles of rats, and when administered subcutaneously or intraperitoneally into mice with alopecia it induces dose-independent hair growth at the site of injection (Danilenko *et al.*, 1995). In adult mouse hair follicles FGF-7 RNA has been localised to the dermal papilla during anagen, but its expression is reduced towards late anagen, and was absent in catagen and telogen (Rosenquist & Martin, 1996). The KGF knockout mouse displayed significant hair coat aberrations, with fur appearing rough and matted (Guo *et al.*, 1996). Based on these findings, it has been suggested that KGF may play a role in the hair cycle by acting on the matrix cells of the follicle (Guo *et al.*, 1996).

In addition to members of the FGF family reports suggest that other factors also stimulate the hair growth. The insulin-like growth factors (IGF-I and IGF-II) are potent promoters of cell growth and survival and are found in variety of tissues including the skin (see Batch *et al.*, 1996). Philpott *et al.* (1994) showed that both IGF-I and IGF-II stimulate human hair follicle growth *in vitro* in a dose dependent manner. However, IGF-I has a stronger influence than IGF-II, which suggests that IGF-I may be an important physiologic regulator of hair follicle growth and the hair cycle (Philpott *et al.*, 1996). This suggestion was supported by the observation that there was a pronounced decline in expression of mRNA for the IGF-I receptor during late anagen and early catagen. In many systems, IGF-I actions are modulated by the IGF binding proteins (IGFBPs) and it has been demonstrated that in human hair follicles IGFBPs mRNA are expressed in the dermal papilla, or at the dermal papilla-epidermal matrix border (Batch *et al.*, 1996). This suggests that IGF-I may play a role in the control of hair follicle growth by acting on the dermal papilla.

In chapter 2, I showed that 2-3 days after plucking follicles entered into a transitional semi-active state during which cell proliferation declined within the GE cells.

If we accept that hair growth is regulated by stimulatory factors, it is easier to explain why this change happened. It could be suggested that plucking could temporarily halt the production of the stimulatory factors in the papilla. According to this idea the GE cells would have continued their proliferation for 2-3 days because during this period of time there was still enough factor present to stimulate cell division. When the level of the stimulator went below a certain point the GE cells would reduce the rate of their division. After about 4-5 days as the effects of plucking were lost the level of the factor would once again become high enough to support rapid proliferation of GE cells as seen in normal follicles.

In addition to inhibitory or stimulatory factors acting to control the hair cycle, there is also evidence to suggest that hair growth could result from the build-up of anti-apoptosis factors, which would prevent the proliferative follicular epidermal cells from undergoing apoptosis. In this respect, Yu *et al.* (1995) using in situ hybridization showed that nexin-1, a serine protease inhibitor, accumulates in rat follicle papillae. This molecule was not observed in the papillae of the catagen follicles. In an earlier study, Stenn *et al.* (1994b) immunolocalised an anti-apoptotic gene (*bcl-2*) product in mouse hair follicles. They showed that the dermal papilla continuously expressed *bcl-2* throughout the cycle, including telogen, but that while the follicular epidermis expressed this protein in anagen, during catagen and telogen it decreased markedly or disappeared (Stenn *et al.*, 1994b). Because it has been shown that overexpression of *bcl-2* prevents lymphoid cells from undergoing experimentally induced apoptosis, the above authors suggested that this molecule might in the same way control the apoptosis occurring normally during the catagen phase of the follicle cycle (see Stenn *et al.*, 1994b). The absence of apoptosis was also confirmed in present study. When plucked follicles were labelled with an antibody, despite the condensation of the papilla no indication of apoptosis was observed in papilla cells.

As well as the molecules and factors mentioned above, over the past two decades an increasing number of locally expressed molecules have been identified that are associated with or are said to play a role in hair follicle activities and to cover all of them would require several books. In this section I have made an attempt to discuss only about the ones that are more relevant to my work and this does not mean that the others are to be ignored. In fact, there are comprehensive reviews which cover the name,

location and possible function of more than one hundred molecules (see Stenn *et al.*, 1994a & 1996; Danilenko *et al.*, 1996). But despite this long list which is the result of many years investigation of hair follicles, ultimately there is still no final answer to the most intriguing question in hair biology, namely, "what does control different stages of the hair follicle cycles?"

5.1.2.2 Systemic factors

Hair follicle activities are not only mediated locally by growth factors but are also influenced by circulating factors. One idea put forward to explain the initiation of hair growth after plucking was that plucking results in the release of growth-promoting hormones in follicles (see Ebling, 1990). When the effects of hormones and plucking on hair growth was studied by Hale & Ebling (1975 & 1979) it was shown that estradiol and propylthiouracil prolong the time between the plucking and eruption of a new hair from the skin surface, whereas this period is shortened by thyroxine (Hale & Ebling, 1979). Similarly, in a recent work Oh and Smart (1996) showed that topical treatment of plucked follicles with 17- β estradiol stops hair growth by arresting the follicles in telogen. Topical treatment of these inhibited follicles with an estrogen receptor antagonist, caused the hair follicles to exit telogen and enter anagen. This receptor has been expressed in the follicle dermal papilla in a stage-dependent manner with the highest level at telogen. By inference, it has been suggested that an estrogen receptor pathway within the papilla regulates the telogen-anagen transition (Oh & Smart, 1996).

Among the systemic hormones, androgens have been considered to play a significant role in the regulation of the hair follicle cycle. The importance of androgens in human hair follicles was first recognised by Hamilton (1942) who observed that men castrated before puberty neither grew beards nor became bald unless they were treated with testosterone (see Messenger, 1993). The androgens circulating in blood arrive at the follicle via the follicular vascular system but the mechanism and site action of androgens in the hair follicle is complicated. The metabolism of testosterone to the more active androgen 5 α -dihydrotestosterone (DHT) by the enzyme 5 α -reductase appears to be a main step in many androgen-responsive tissues like prostate. Men deficient in this enzyme have testes and normal level of testosterone, but as well as having abnormal external genitalia they do not grow a normal beard or male pattern body hair (Randall,

1994b). These men have a female distribution of pubic and axillary hair growth indicating these two regions can respond to testosterone but the full expression of male-pattern hair elsewhere on the body requires only DHT (see Randall, 1996). It has been hypothesised that androgens exert their action on the hair follicles via the their dermal papilla (Jahoda, 1982; Randall *et al.*, 1991; Randall, 1994a & 1996). This idea is based on different supportive results: for example, androgen receptors have been localised in the nuclei of human dermal papilla cells (see Randall, 1996). Moreover, beard human dermal papilla cells have been shown to metabolise testosterone to DHT (Itami *et al.*, 1990; Randall *et al.*, 1992; Hamada *et al.*, 1996).

If the androgens exert their actions via the papilla how does the papilla respond to them? It has been proposed that androgen in the papilla react with androgen receptors altering the expression of specific genes for regulatory molecules. Any alteration in growth factors or extracellular matrix in the papilla would effect the activity of other follicular components to initiate the follicle response to androgen (Randall, 1996). However, the question as to whether this theory is correct or not requires further investigation.

In addition to the above mentioned systemic and local factors there are others factors which have been reported to have a role in hair growth. For example, due to the fact that the hair follicle is a densely innervated organ (Jahoda & Oliver, 1984a; Hashimoto *et al.*, 1990; Halata, 1993), neural factors have been put forward as playing role in the hair growth control (Paus *et al.*, 1997b). They possibly exert trophic roles on the hair follicle by regulating the supply of nutrients and oxygen to the hair follicle. Neural signals have also been suggested as influencing the proliferation, differentiation and apoptosis of hair follicle epidermal cells (Paus *et al.*, 1997b). Diet has been demonstrated that have effects in hair growth, and there are reports that nutritional changes change influences the rate of fibre production in sheeps (Hynd *et al.*, 1986). Likewise, Bradfield (1971) showed that in men the scalp hair root diameter was decreased after only a short period (11 days) of protein deprivation. Moreover, there is evidence that malnutrition causes abnormal hair growth in humans (see Randall, 1994a). In summary, there has been a great deal of investigation aimed at unravelling the mechanisms that control the follicle cycle. These investigations have resulted in some considerable successes, and in this respect a vast number of factors have been nominated

as influences in hair growth. However, despite all these efforts, to date we are not able to tell exactly what factor(s) control the hair cycle. Nevertheless, they have provided key indicators, which will be vital for future investigations, aimed at elucidating these regulatory mechanisms.

5.2 Properties and inductive ability of dermal papillae in adult skin appendages

In chapter 4, I attempted to clarify the properties of the dermal components of three cutaneous appendages in mammals and birds using different methods. Although there was a difference in the *in vitro* aggregative behaviour of dermal papilla cells derived from different origins (hair follicle, claw and feather follicle) more or less all the appendages displayed this trait. Cell aggregation and clumping in these cells is possibly an intrinsic behaviour which they retain from embryonic time but the size and intensity of the aggregations could be related to appendage function or as Reynolds (1989) suggested, to appendage size. However, more investigations are required to clarify this issue. All three appendages displayed a common cellular property *in vitro* with respect to ASMA expression, but since it has been previously shown that the papilla cells of the hair follicle do not express this cytoskeletal element *in situ* (Jahoda *et al.*, 1991), the presence of ASMA in the papilla of feather follicles *in situ* was interesting. The inductive ability of the hair follicular papilla cells in heterotopic sites has been well established (Reynolds & Jahoda, 1991a), and this trait also has been demonstrated in the case of tooth papilla cells (Reynolds, 1989). The recombinations performed in this study have confirmed this capability for the hair follicle dermal papilla. However, in recombinations of claw papilla cells with heterotypic epidermal cells, the claw papilla cells failed to induce any changes. This could be because i) unlike the papilla cells of the hair follicle, the claw cells may do not retain their embryonic potential, and indeed claws do not display the same degree of regeneration as hair follicles in maturity, ii) they may have lost their inductive properties *in vitro* prior to the recombination, iii) *in situ* they are in close proximity to digit bone, hence, they may need specific signals from other tissues to show their inductive ability and iv) perhaps they never had such ability. As there is little known about the claw, future work may reveal some interesting insights into the properties of cells from this appendage. The recombinations of feather papilla with epidermal cells with rat follicle epidermis again did not induce any appendage

morphogenesis. This type of recombination has been shown to be successful with embryonic tissues, which initiate hair or feather formation (see Sengel, 1976). Therefore, this suggests that these cells may have lost part of their potential in the adult life. The recombination of adult hair follicle papilla cells with feather epidermal cells will clarify whether the reciprocal potential persists?

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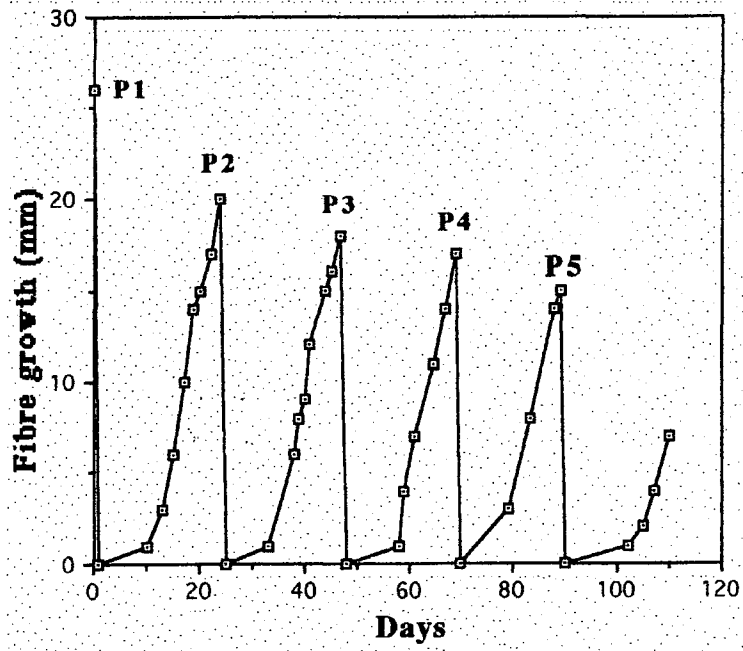
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Appendix

From chapter 2)

Fig. 1) Hair growth following 5 consecutive pluckings. As seen by the slope of the curves the fibre grew consistently at an average rate of 1.2-1.4 mm/day.



From chapter 2)

Table 1) Detailed data from repeat plucking experiment. The total length of fibre that was produced by a follicle (Tot. pro. Len.) was calculated as the sum of the length of original growing fibre, and the successive regenerated fibres. (p) plucking.

Fol. iden	Club len. at 1 st p mm	Time before emerg day	Grow rate after 1 st p mm/d	Fib. len. at 2 nd p mm	Time before emerg day	Grow rate after 2 nd p mm/d	Fib. ren. at 3 rd p mm	Time before emerg day	Grow rate after 3 rd p mm/d	Fibre len. at 4 th p mm	Time before emerg day	Grow rate after 4 th p mm/d	Fibre len. at 5 th p mm	Time before emerg day	Grow rate after 5 th p mm/d	Fibre len. at 6 th p day	Tot. pro. len. mm	Incr. over club %	Mean grow rate mm/d
1a/dr	41	10	1.3	18	9	1.4	19	11	1.2	13	11	1.2	11	12	1.2	12	107	160	1.3
2a-dr	43	13	0.9	10	9	0.92	13	11	1	11	11	1.3	12	12	1.3	13	84	95	1.3
3a/dr	46	10	1.2	17	10	1.4	18	10	1.4	17	10	1.5	14	13	1.2	9	107	132	1.4
4a/dr	51	8	1.3	20	9	1.3	18	9	1.2	17	9	1.3	15	13	0.8	12	107	109	1.2
1a/dl	38	10	1.3	18	9	1.35	19	11	1.1	12	11	1.2	14	13	1.2	11	108	184	1.2
2a/dl	36	9	1.1	17	10	1.15	15	11	1	11	10	1.3	15	12	1.2	12	93	158	1.2
3a/dl	40	9	1	15	10	1.3	17	11	0.9	10	11	1.3	16	13	1.15	10	99	147	1.1
4a/dl	45	10	1.35	19	9	1.4	19	10	1.4	19	11	1.4	18	12	1.2	12	112	148	1.35
3a/dr	41	8	1.4	21	10	1.3	23	10	1.4	19	11	1.1	14	-	-	-	95	131	1.3
1a/bl	31	11	1.25	10	11	1.4	19	12	0.9	15	12	1.3	18	-	-	-	89	187	1.3
1b/bl	29	9	1.3	13	12	1.2	14	12	1.15	13	11	1.2	14	-	-	-	75	158	1.2
1c/bl	20	10	1.1	11	11	1.5	16	12	1.1	13	9	1.3	17	-	-	-	73	265	1.2
4a/gr	50	9	1.3	14	10	1.3	18	10	1.5	17	11	1.4	15	-	-	-	90	80	1.4
1c/gr	21	10	1	12	10	0.9	15	11	1.1	11	12	1.25	10	-	-	-	64	204	1.1
4a/fr	52	9	1.4	14	9	1.3	10	9	1.35	19	-	-	-	-	-	-	74	42	1.35
3a/fr	52	8	1.5	18	9	1.3	10	9	1.3	18	-	-	-	-	-	-	78	43	1.4
3a/fr	48	10	1.3	15	11	1.2	9	9	1.3	18	-	-	-	-	-	-	71	47	1.3
3a/dr	35	9	1.3	20	11	1	15	12	0.9	35	-	-	-	-	-	-	87	148	1.1
1a/al	32	9	1.15	23	11	1.1	12	-	-	-	-	-	-	-	-	-	63	96	1.1
1b/al	30	9	0.95	19	11	0.8	9	-	-	-	-	-	-	-	-	-	48	60	0.9
1c/al	20	10	0.9	19	11	1.1	12	-	-	-	-	-	-	-	-	-	44	120	1
1a/hr	34	9	1.2	19	9	1.1	17	-	-	-	-	-	-	-	-	-	64	88	1.15
2a/hr	35	9	1.25	20	10	1.3	20	-	-	-	-	-	-	-	-	-	69	97	1.3
3a/hr	38	8	1.3	22	10	1.35	21	-	-	-	-	-	-	-	-	-	74	94	1.35
2a/el	42	9	1.2	22	-	-	-	-	-	-	-	-	-	-	-	-	58	38	1.2
3a/el	49	9	1.2	20	-	-	-	-	-	-	-	-	-	-	-	-	64	30	1.2
4a/el	50	9	1.3	21	-	-	-	-	-	-	-	-	-	-	-	-	65	30	1.3
1b/fr	30	9	1.3	26	-	-	-	-	-	-	-	-	-	-	-	-	50	66	1.3
2a/fr	34	10	1.25	25	-	-	-	-	-	-	-	-	-	-	-	-	53	55	1.25

From chapter 3)

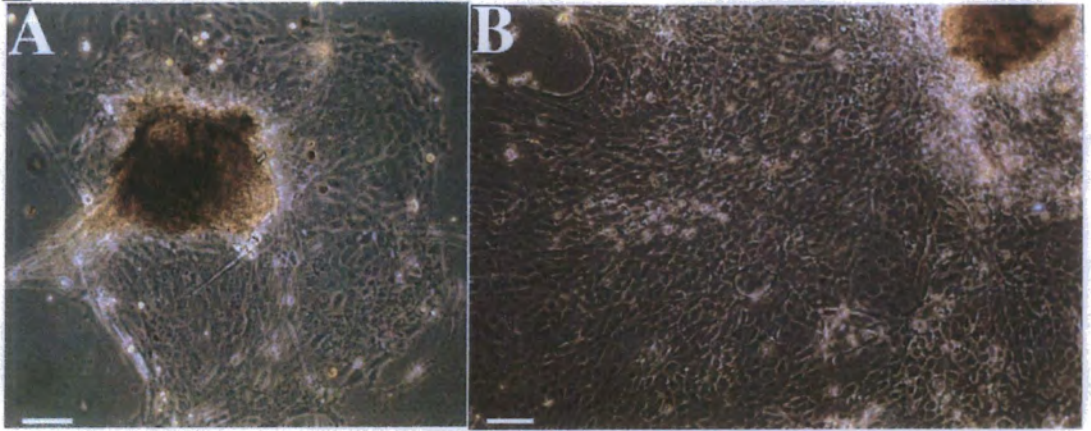
Fig. 2) Phase contrast micrographs showing outgrowth of epidermal matrix cells around a tissue explant. The tissue was a combination of dermal papilla attached to lower epidermal matrix dissected from a follicle vibrissa. After dissection the tissue was directly (without trypsinisation) cultured on a plastic dish without any supporting layer. (A) 10 and (B) 17 days after cultivation. Scale bars 100 μ m

Fig. 3) Micrograph shows a pair of culture dishes containing epidermal colonies 30 days after seeding. Epidermal cells from two regions of bulb (left) and upper follicle (right) were dissected and cultivated on the dishes following trypsinisation. Compared to the bulb, epidermal cells from the upper follicle produced more colonies at a higher growth rate. Scale bar 9mm.

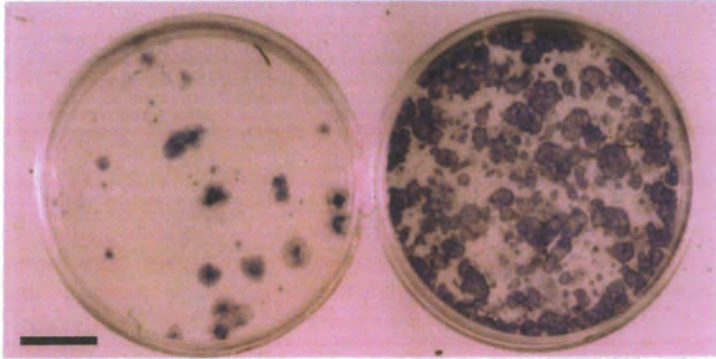
Fig. 4) Another pair of culture dishes from the bulb (left) and the upper follicle (right) 34 days after cells were seeded. In this case nearly all of the bulb epidermal cells terminated their growth after a short time and differentiated. Scale bar 9mm

Fig. 5) Micrograph showing a culture dish containing epidermal colonies established from bulb cells 36 days post-cultivation. Two large colonies are visible in the culture. Scale bar 6.7mm

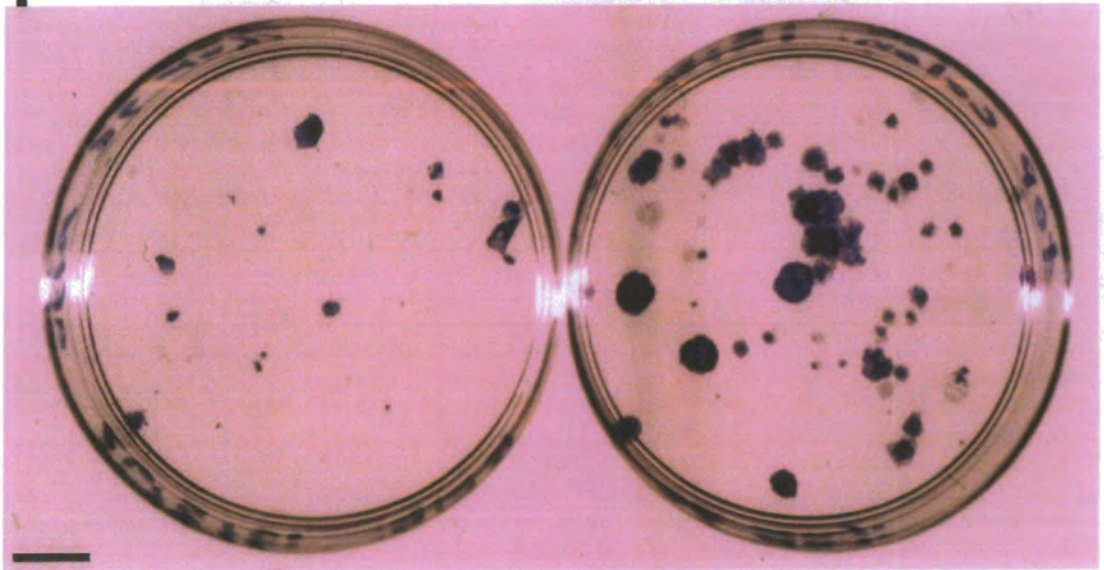
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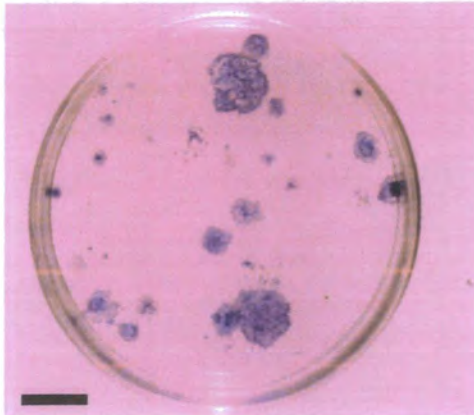
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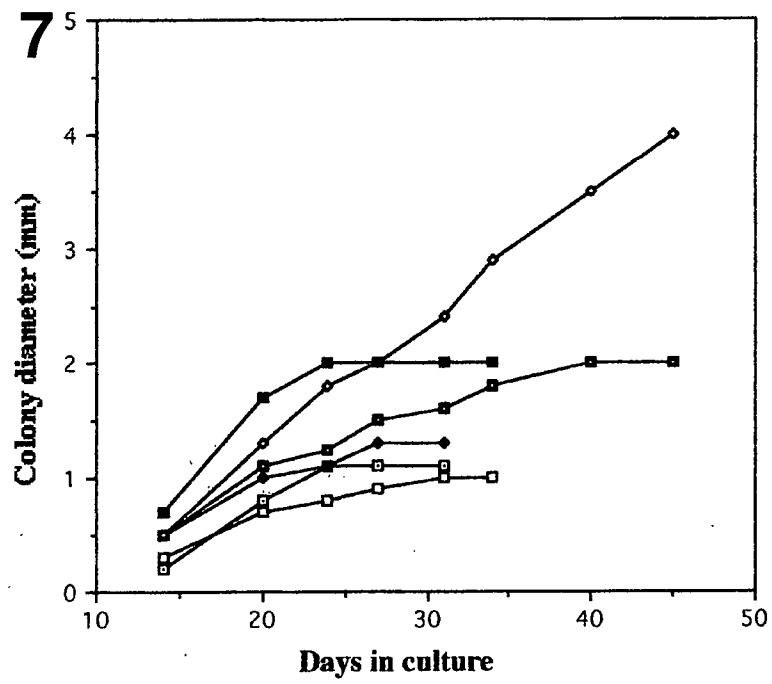
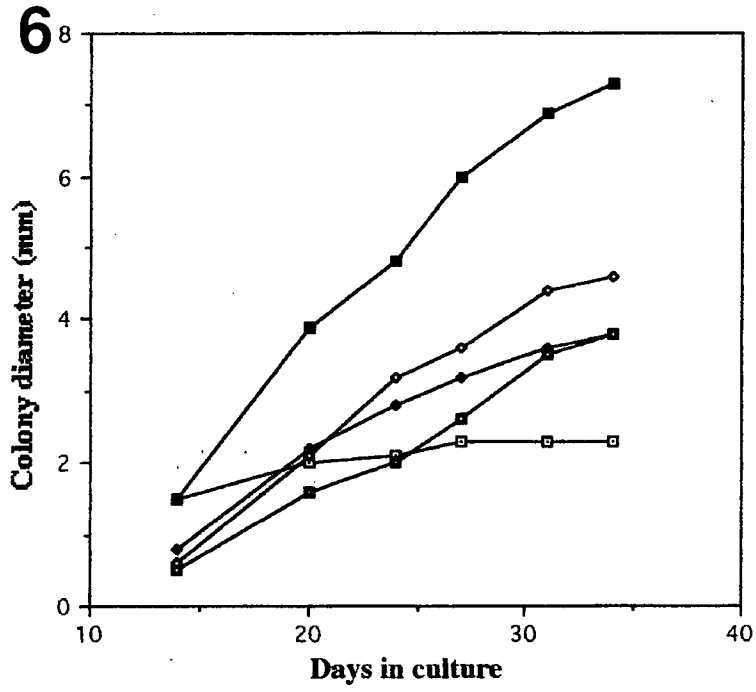
5



From chapter 3

Fig. 6) Growth curves of different upper follicle (UF) epidermal colonies derived from single founding cells. Each curve was derived from the same colonies measured consecutively.

Fig. 7) Growth curves of different bulb epidermal colonies derived from single founding cells. As mentioned in chapter 3, compared to the upper follicle, most of the bulb colonies ceased their growth and differentiated at early stage therefore, it was possible to measure their growth for longer periods of time. In comparison to the UF long-lived colonies (curve UF5 in Fig. 6) colonies in the bulb region (curve bulb 4) grew slower.



From chapter 3

Table 2) Numbers of epidermal colonies from bulb and UF regions counted at different times post-seeding. On average the number of the UF colonies was three times higher than that of the bulb region.

Date	No of colony: UF	No of colony: Bulb
1/10/96	87	22
14/1/97	82	23
16/2/97	40	32
23/2/ 97	52	7
15/5/97	69	18
Average	66	20

From chapter 4)

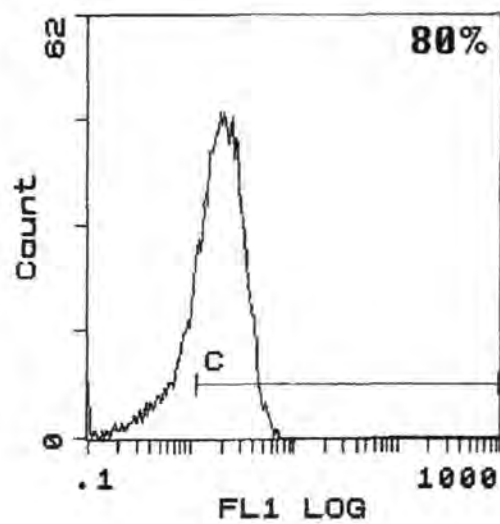
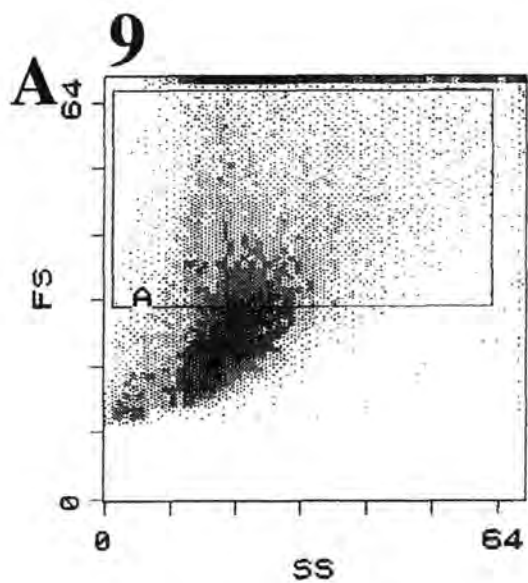
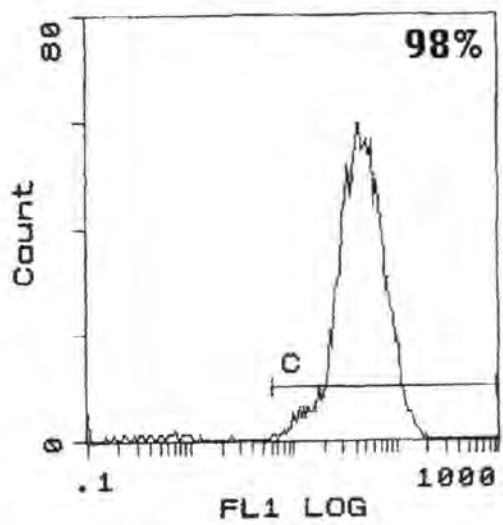
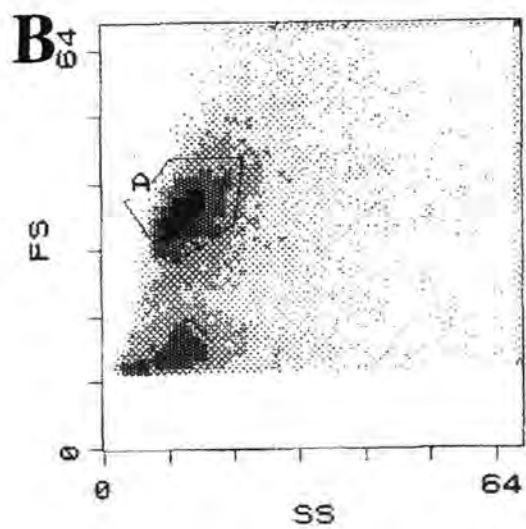
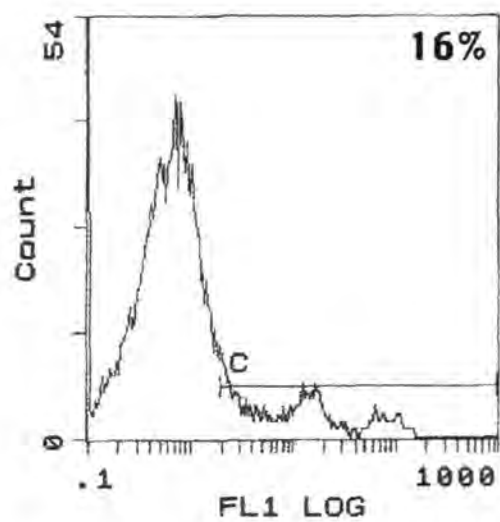
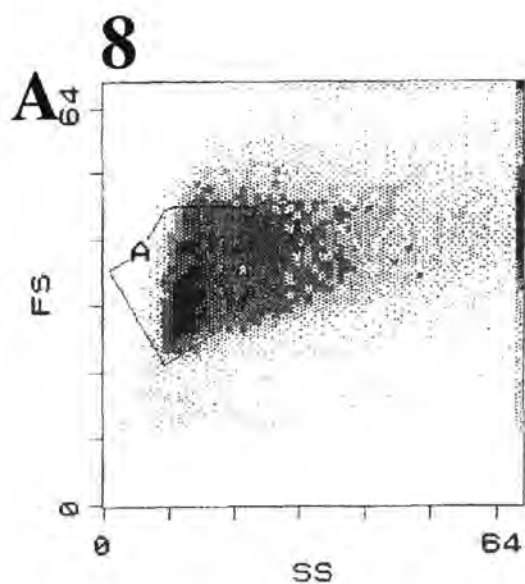
Table 3) Comparison of cell growth between dermal sheath cells derived from the upper and lower regions of rat vibrissa follicles. Originally equal number of cells (1.92×10^5) were seeded in culture dishes. Two days later the number of cells in dishes was counted.

Cell type	Replicate	Original cell number	Cell number after 48-hrs	Cell number mean	Proportion
Lower DS	1	1.92×10^5	3.41×10^5	3.41×10^5	1.11
	2		3.36×10^5		
	3		3.46×10^5		
Upper DS	1		3.07×10^5	3.08×10^5	1
	2		3.04×10^5		
	3		3.13×10^5		

From chapter 4)

Fig. 8) Flow cytometric histograms showing the pattern of distribution (left) and expression of MHC-I (right) in rat splenocytes. Only a small population of red blood cells (16%) was labelled with the antibody (A) but the MHC-I was expressed in nearly all (98%) lymphocytes (B). FS= cell size, SS= cell granularity.

Fig. 9) Flow cytometric histograms showing expression of MHC-I among different phenotypes of follicle dermal sheath cells. The cells were derived from the upper region of rat vibrissa follicles. A) 80% of the cells with large size and higher granularity labelled with anti-MHC-I.



From chapter 4)

Fig. 9B) Flow cytometric histograms showing expression of MHC-I among different phenotypes of the follicle dermal sheath cells. Compared to the large and highly granular cells (see A) a fewer number of small and non-granular cells (42%) expressed the MHC-I. FS= cell size, SS= cell granularity.

