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*Hair follicle dermal cells: a morphological,  
behavioural and molecular study*

Matthew Alexander Sleeman

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**"Hair Follicle Dermal Cells:  
A Morphological, Behavioural and Molecular Study"**

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by

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**thesis submitted for the degree of Doctor of Philosophy  
in the University of Durham**

**Department of Biological Sciences**

**February 1995**



**08 DEC 1995**

This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

M.A. Sleeman

February, 1995

## ABSTRACT

Anatomy and smooth muscle  $\alpha$ -actin expression in hair follicles from a variety of animal species (Mink, Polecat, Meerkat, Grey squirrel and stoat) was investigated. Smooth muscle  $\alpha$ -actin expression was related to follicle activity. Expression was greatest during anagen, with a marked reduction in expression during telogen. Follicular dermal cells were cultured from the above animal species. *In vitro* grey squirrel dermal papilla (DP) and dermal sheath (DS) cells both expressed smooth muscle  $\alpha$ -actin.

Rat dermal papilla cell *in vitro* aggregative behaviour was characterized, by proliferation, chemotactic and molecular studies. Aggregation behaviour in these cells was not attributed to focal proliferation. However, fluctuations in cell motility correlated with the aggregation process, with the greatest motility between subconfluent and clumped cells. Motility was terminated within the clumped cells. Furthermore, DP cells *in vitro* secreted molecules that enhanced motility in subconfluent DP cells and a variety of other cell types. As yet the type and specificity of this medium borne component is unknown. TGF $\beta$ , bFGF and aFGF were all used as a comparison to the unknown molecule, however migration was rarely similar in magnitude to the response of the DP cell medium.

Mouse cDNA probes of BMP 2 and BMP 4 were used to isolate rat homologues from a cDNA library. Wholemount *in situ* hybridization of BMP 4 expression was consistent with the data in the mouse, however BMP 4 was also expressed in adult rat telogen vibrissae. Molecular expression within *in vitro* DP cells was studied using a differential screen of a cDNA library. A number of clumped DP specific clones were differentially expressed, one of which having high homology with migration inhibitory factor. These results are discussed, and a hypothetical model is proposed to describe how DP aggregation occurs with reference to dermal condensation *in vivo*.

## ABBREVIATIONS

|               |  |
|---------------|--|
| aFGF          | acidic fibroblast growth factor                  |
| AG            | aggregating                                      |
| AG DP CM      | aggregating dermal papilla conditioned medium    |
| bFGF          | basic fibroblast growth factor                   |
| BMP 2         | bone morphogenetic protein 2                     |
| BMP 4         | bone morphogenetic protein 4                     |
| bp            | base pair  |
| Confl         | confluent  |
| CL            | clumped  |
| CL DP CM      | clumped dermal papilla conditioned medium        |
| DEPC          | diethyl pyrocarbonate                            |
| DNA           | deoxyribonucleic acid                            |
| DP            | dermal papilla                                   |
| DS            | dermal sheath                                    |
| DS CM         | dermal sheath conditioned medium                 |
| EDTA          | ethylenediaminetetra-acetic acid (disodium salt) |
| Fi            | dermal skin fibroblast                           |
| FCS           | foetal calf serum                                |
| kb            | kilobase   |
| MEM           | minimal essential medium                         |
| $\mu$ M       | microlitre                                       |
| mM            | millimolar                                       |
| M             | molar  |
| nm            | nanometre  |
| OD            | optical density                                  |
| PBS           | phosphate buffered saline                        |
| PDGF          | platelet derived growth factor                   |
| RNA           | ribonucleic acid                                 |
| rpm           | revolutions per minute                           |
| SC            | subconfluent                                     |
| SC DP CM      | subconfluent dermal papilla conditioned medium   |
| SDS           | sodium dodecyl sulphate                          |
| smaa          | smooth muscle $\alpha$ -actin                    |
| TGF- $\beta$  | transforming growth factor- $\beta$              |
| TNF- $\alpha$ | tumour necrosis factor- $\alpha$                 |
| Tris          | tris(hydroxymethyl)methylamine                   |
| UV            | ultra violet                                     |

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# Chapter 1

## Introduction

### 1.1 Hair

After the crucial events of early embryogenesis; mesoderm induction, gastrulation and neurulation, some of the most intriguing and complex interactions in later embryogenesis occur during the development of integumental appendages. Formation of skin appendages, examples of which include feathers, scales, hair, nails and claws, involves two distinct tissues, the epidermis and dermis, which in turn are derived from the ectoderm and mesoderm of the developing triploblast. Skin appendages have widely differing functions and consequently are morphologically very diverse.

The development of these appendages occurs soon after differentiation of the epidermis and dermis (Sengel, 1976). During morphogenesis of these structures, their initial characteristics are similar, with a thickened epidermal placode and subjacent dermal condensation. However, divergent activities soon after these events produces developmental structures representative of the final phenotype. The mechanisms used to create these appendages are complex, and involve multifarious aspects of cellular activity, from cell proliferation and migration to biosynthesis of extracellular matrix and production of cell adhesion molecules. The final result is a complex array of differentiated epithelial and mesenchymal tissues within the respective appendage.

The hair follicle has been investigated for many years, being one of the most easily accessible and numerous of the skin appendages. Hair is a primary feature of all mammalia, although this may not be immediately apparent, as in the case of the Pinnepedia (Seals, Sea-Lions and Walruses) and Cetacea (Whales, Dolphins and Porpoises). Furthermore, the hair follicle has a varied phenotype and function, depending on the animal type and position on the body. An obvious example of this is the contrast between the small and more numerous solid medullate pelage follicles over

the body of the animal, and the fewer much larger hollow medullate sinus follicles generally confined to the mystacial pad (Dry, 1926, Davidson and Hardy, 1952)

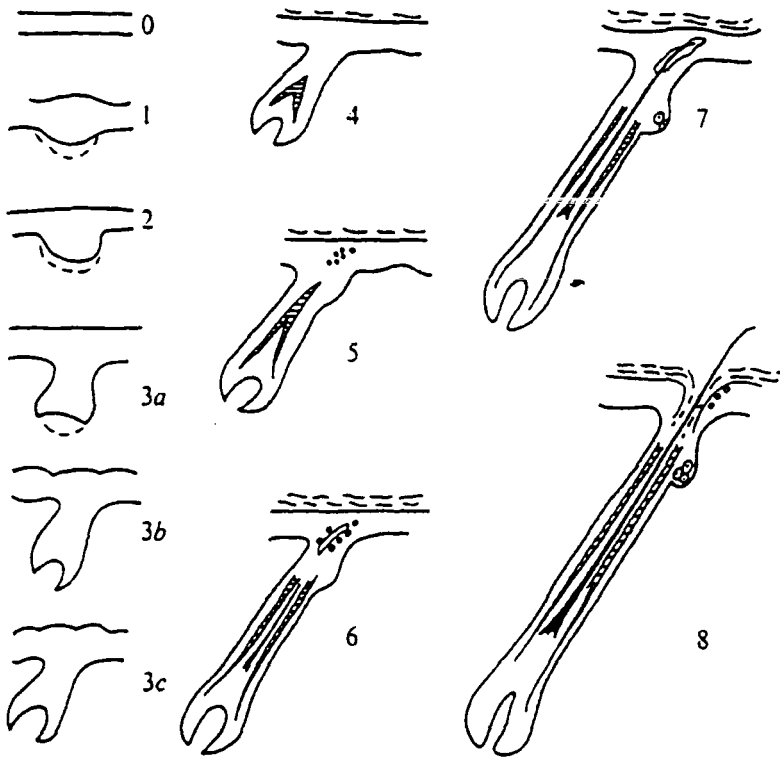
Follicles are not only present as a means of controlling temperature, but can have a sensory tactile role; act as a means to aid hearing; play a defensive role; and in some animals be a means of communication. Furthermore, pigmentation, or lack of it within the hair fibre, can be used to great effect for camouflage. Within human development the hair follicle appears to have a very minimal role, and yet in children and adults, hair plays a major role in human psychology and culture. Hair follicles have also been shown to be involved in healing the glabrous skin between follicles after wounding events (Eisen et al., 1955, Krawczyk, 1971, Pang et al., 1978). Therefore, the hair follicle plays a very important and crucial role, not only in skin physiology but also in human and animal behaviour.

## **1.2 Hair development**

Initial histological signs of hair production can be seen by a thickening of cells in the epidermis, called a placode, and a parallel condensation of dermal cells subjacent to the placode. This whole structure is termed the hair germ (Pinkus, 1958). As time progresses, the epidermal constituent divides and begins to penetrate the dermis. This downgrowth of epidermal tissue continues through the hair bud (1), hair bulb (3) and bulbous peg (5), stages of development (Sengel, 1976; Fig 1.). As the epidermal tissues move down they partially enclose the apparently undifferentiated mesenchyme in a ball like structure termed the dermal papilla. Once this occurs, epidermal cell division and differentiation produces the upward growing fibre which erupts at stage 8. Basally and laterally around the hair follicle epidermis, the mesenchyme is distinguishable as a specific layer termed the dermal sheath. A thick basal lamina, called the glassy membrane separates the outer root sheath cells of the epidermis from the dermal sheath cells of the follicular connective tissue. A schematic diagram of hair development, cited from Sengel (1976), can be seen in figure 1.

**FIG 1**

Summary of the development of the pelage hair follicle in the mouse, Engel (1976).



- Stage 0 - Prior to appearance of hair follicle rudiments
- Stage 1 - Initial hair bud
- Stage 2 - Hair nodule
- Stage 3 - Hair bulb and formation of dermal papilla
- Stage 4 - Hair cone
- Stage 5 - Formation of the sebaceous gland rudiment and insertion knob of the erector muscle
- Stage 6 - Formation of the inner root sheath and hair shaft
- Stage 7 - Tip of the hair reaches the pilary canal
- Stage 8 - Hair eruption

One of the interesting features of hair follicles is that they cycle throughout the lifespan of the adult organism, fluctuating between the growing anagen phase and the resting telogen phase, with the transitory stage from growing to resting being termed catagen (Kligman, 1959, Straile et al., 1961, Montagna, 1962). A great deal of research has been done on the precise movements and functions of the cells involved during the various stages in the hair cycle. In particular, expression studies have yielded an interesting array of data on cell and tissue specific distribution of molecules within the follicle (Stenn et al., 1994a). In part of this thesis the changing anatomy of hair follicles during the various stages of the cycle from different animal species is investigated, to try and gain further insights into the morphogenetic changes during the different stages of the hair cycle.

### **1.3 Dermal papilla cells *in vitro* and *in vivo***

The dermal papilla has long been thought to be one of the major group of cells controlling hair growth and cycling. (Geary, 1952; Chase, 1955). As the follicle changes in the cycle (anagen to telogen), so does the morphology of the dermal papilla. The anagen papilla is large, with cells producing copious extracellular matrix (ECM) including proteoglycans and glycoproteins, such as fibronectin, laminin and type IV collagen (Couchman et al., 1991, Messenger et al., 1991, Jahoda et al., 1992). In complete contrast to this the telogen papilla is greatly reduced in size, and ECM production is at a low level. This reduction in papilla size and activity coincides with a cessation in keratin and fibre production. Early experiments by Cohen (1961) and Oliver (1966a,b), using microdissection and rat vibrissa papilla re-implantation, were the first to ascertain the role of the dermal papilla. It was discovered that removal of the papilla terminated hair growth but that the follicle was able to regenerate a new dermal papilla, which in turn re-organised the epidermis to form a fibre producing germinative epidermal matrix. This regeneration was still possible if up to the lower third of the follicle was removed, but the regenerated papilla was smaller and the

resultant fibre was shorter in this case. Regeneration ceased if more than one third of the follicle was removed. In subsequent experiments Oliver (1967a), implanted other regions of the hair follicle, comprising combined tissues of the epidermal outer root sheath and dermal sheath in the form of tubes into ectopic sites. Follicle production only occurred if these tissues were derived from within the lower third of the follicle. Moreover, active follicles could only be re-established after a new papilla had been generated. Histology revealed that the dermal sheath provided the main source for the new papilla. In another study, isolated dermal papillae were able to induce a new active epidermal matrix and subsequent hair growth, when re-implanted into follicles that had been cut in half (Oliver, 1967b). For this to happen the papilla had to be in contact with epidermis, which was otherwise incapable of making hair. Oliver (1970, 1973) recombined dermal papilla with sheets of epidermis from ear, hairless scrotal sac and oral mucosa, and inserted them into ear pinna. As long as the dermal papilla was in contact with the epidermis, follicles could be induced. Work done later by Jahoda (1992) produced follicles by inserting whole dermal papilla into ear wounds, and again follicles were stimulated if they were in contact with overlying wound epidermis. These follicles also produced fibres of the size and character of a vibrissa or sinus follicle. Therefore, adult rat vibrissa dermal papillae retain the ability to induce vibrissa specific follicles.

Jahoda and Oliver (1981) were the first to grow dermal papilla cells in culture and were able to show that the cultured cells retained the inductive properties of the intact DP (Jahoda et al., 1984, Horne et al., 1986). However, in some of these experiments it was not possible to rule out the action of additional influences from established hair follicles in the vicinity. In a complex recombination, Reynolds and Jahoda (1992) were able to tackle this problem by implanting rat footpad skin, a naturally glabrous area, with cultured rat pelage dermal papilla cells, inserted between the footpad dermis and the epidermis. These footpad dermal-epidermal recombinations were then put into a granulation pocket on the back of rats, and further isolated from the surrounding follicular skin by silicone chambers. Pelage type follicles were

induced. This protocol removed the possibility that follicles were being formed with the help or cellular contribution of surrounding follicles, and demonstrated that dermal papilla cells have the ability to reprogramme overlying epithelium, even if that epithelium does not naturally produce hair.

Another interesting property of adult hair follicle dermal papilla cells is that they are able to form three dimensional structures *in vitro* which are morphologically similar to the early dermal condensations in the embryo (Jahoda and Oliver, 1984). Thus, it appears that dermal papilla cells are able to maintain some of their embryological properties whether *in vivo* or *in vitro*, and that their specific inductive abilities can be switched on when in conjunction with an overlying epidermis. When intact dermal papillae, or cultured dermal papilla cells are implanted into the dermis without being in contact with the epidermis, the cells are unable to produce follicles (Jahoda, 1992, Jahoda et al., 1993). However, rather than disappear into obscurity within the dermis, the cells still maintain close links and form distinct aggregates and condensations.

It therefore appears that not all the interesting phenomenon are due entirely to epidermal-dermal interactions, but that certain functions of the dermal papilla cells are possibly controlled within, or intrinsic to, the dermis. As one of the first signs of hair follicle production is a condensation of the dermis to form the papilla anlage (Pinkus, 1958), this phenomenon of aggregating *in vitro* and *in vivo* (intradermally) has the potential to be a useful model for isolating molecules specific to the very early events in hair follicle differentiation and morphogenesis, with possibly broader significance. Part of this thesis investigates the aggregative properties of these dermal papilla cells, how aggregations are formed and maintained *in vitro*, and if these aggregates can be linked in any way to their *in vivo* counterparts?

## 1.4 Models and theories of mesenchymal condensation

Condensation events in the developing embryo are of crucial importance to the correct morphogenesis of the animal. Condensation, an increasing of cell density in a particular area, precedes more complex organogenesis. Feather, hair, bone, kidney, somites and teeth are all whole or partial derivatives of a condensation event (Hamburger & Hamilton, 1951, Grobstein, 1955, Pinkus, 1958, Serri & Cerimele, 1990, Thorogood & Hinchcliffe, 1975, Saxen, 1987, Jacobson, 1988, Thesleff et al., 1989, ). Even though these organs are functionally different, the early condensation events are morphologically, and as recently shown, often molecularly similar. In the following section, mesenchymal condensation in bone, feather, teeth and hair are discussed in detail.

### 1.4.1 Chondrogenesis

Chondrogenesis begins with the appearance of a mesenchymal condensation, the inner part becoming cartilage and the outer region developing into the perichondrium (Archer et al., 1983). The cell density within these condensations increases by about 60%, with cells becoming rounded and depositing cartilage specific extracellular matrix (Zanetti & Solursh, 1984).

Chondrogenesis has been one of the most thoroughly studied of condensation events. The main reason for this stems from the finding that the initial events of chondrogenesis *in vivo* (Fell, 1925) could faithfully be represented *in vitro* (Ahrens et al, 1977). In addition, Urist et al., (1965) pioneered bone induction in non-bone forming regions, using demineralized bone. Therefore a set of systems exist with which to tackle the earliest events in chondrogenesis.

The technique of micromass culture of prechondrocytes produced changes that morphologically resembled early bone development (Ahrens et al., 1977) and cells that were capable of cartilage production, depending on the time at which the pre-

chondrogenic cells were removed from the embryo. However, it was also discovered that cartilage could be induced in early, stage 19 derived cells, if cAMP was added, and it was later shown that cAMP levels naturally increased during the formation of avian limb chondrogenesis (Solursh et al., 1979). Although cAMP has a role in cartilage formation, it is unclear if it has a role in condensation formation. Cartilage production did not occur unless an aggregate of pre-chondrogenic cells had formed (Solursh & Reiter, 1980). Solursh et al., (1978) postulated three possible mechanisms for formation of aggregates *in vitro*, and by inference *in vivo*. 1) as a clone derived from precursor cells, 2) as a local condensation of cells in the mass culture and 3) from a specific group of cells that had sorted out from a mixture of cell types already present in the early limb. The concept of precursor or stem cell populations is one of the most heavily investigated areas in developmental biology; however, in many systems it is very difficult to isolate cells with stem cell-like properties. Nevertheless a number of markers for a variety of cell lineages are currently under investigation (Heath et al., 1990, Graham and Pragnell, 1992, Stemple and Anderson, 1992, Morrison-Graham and Takahashi, 1993). For the precursor cell theory to apply to chondrogenic condensation, differential proliferation would have to occur and yet the mitotic index was not found to be different between unaggregated and aggregated regions *in vitro* (Solursh et al, 1978).

The second hypothesis proposes a condensation event within the mass of the culture. Archer et al., (1982) noted that the rounded structure of cells within cultured chondrogenic aggregates was analogous to the structure of precartilaginous condensations *in vivo* (Thorogood and Hinchliffe, 1975). Therefore, it appears likely that these micromass cultures form via a similar condensation event to that which takes place *in vivo*.

The final hypothesis is supported by Steinberg (1970) whereby cells of different types in culture can sort out into homospecific groups depending on the relative cell adhesiveness between heterospecific cell populations. In early limb development, cells are from two different lineages, the myogenic lineage or muscle cell precursors

migrating from the somites, and pre-chondrocytes originating from the somatopleure (Chevallier et al., 1977, George-Weinstein et al., 1988). It would seem unlikely therefore that these two cell lineages would mix together and then sort out again to form distinct structures. It would seem more logical that the muscle cell precursors remain in their distinct groups throughout their migration, and then differentiate separately. The weight of evidence therefore supports the idea that chondrogenesis is mediated through a condensation event. However, what are the mechanisms involved in forming this condensation event. In an early study, Ede and Agerbak (1968) used the *talpid<sup>3</sup>* chick mutant, an embryonic lethal mutant whose defects are the result of incorrect mesenchymal condensation, to investigate the properties of mesenchymal condensation in the limb bud. They argued that condensation *in vivo* was comparable to cell reaggregation of dissociated cells *in vitro*. Furthermore, they went on to show that *in vitro* aggregates of limb bud mesenchymal cells were smaller than their normal chick counterparts, and that this was attributable to greater adhesion between the *talpid<sup>3</sup>* mutant cells. Earlier research (cited from Hampe, 1960) had suggested that individual cell movement was the mechanism involved in condensation formation, taking this into consideration, Ede and Agerbak (1968) postulated that the defective condensation in the *talpid<sup>3</sup>* mutant was a result of an increased adhesion between the cells, which would then reduce cellular motility. Furthermore, they went on to speculate that as condensations occur at definite locations within a limb bud, these foci would produce a morphogenetic substance, similar to that described for slime mould amoebae (Bonner, 1963), that would either have a chemotactic effect on other cells or that it would trap cells by immobilisation. Ede and Flint (1975) went on to study motility between the *talpid<sup>3</sup>* mutant and normal cells. What they discovered was that normal cells travelled faster than *talpid<sup>3</sup>* mutant cells, and that the *talpid<sup>3</sup>* mutant cells spent more time at rest. Moreover, this difference in motility was also reflected in cell morphology. Since other groups had previously indicated a role for cell adhesiveness in condensation events (Abbott and Holtzer, 1966, Holtfreter, 1968, Toole, 1972), and the fact that no investigators had indicated a local increase in cell

proliferation, Ede and Flint (1975) felt that this and their research supported the case for an antagonistic effect between the strength of cell adhesion and reduced motility in the incorrect morphogenesis of the chondrogenic condensation in *talpid*<sup>3</sup> chick mutants. Therefore, there seems to be a strong evidence for cell motility as a means of forming a condensation event. Subsequently, recent research has isolated many molecules within the chondrogenic condensation, that could only be speculated upon at the time Ede was conducting these early works. In the following paragraphs, these recent molecular discoveries will be brought into context

As mentioned previously cAMP was found to have a significant effect on enhancing the expression of the cartilage phenotype in these early condensation models. However, since the discovery of a variety of cell adhesion molecules, growth factors and extracellular matrix molecules associated with this phenomenon, the complexity of the system is only now being unravelled.

As the differentiated phenotype of the *in vitro* model is identified by the presence of cartilage specific collagen type II, and cartilage specific proteoglycan (Dessau et al 1980, Kimata et al. 1986), the variety of extracellular matrix molecules that are involved will be considered first. Work by Kosher et al., (1975) provided early evidence for the role of extracellular matrix in condensation and chondrogenesis by enhancing *in vitro* somitic vertebral chondrogenesis using procollagen and collagen. Glycosaminoglycans such as chondroitin sulphate and hyaluronic acid (Kosher et al, 1973, Kujawa & Caplan, 1986) have also been implicated in stimulation of chondrogenesis. More recently tenascin, a commonly expressed ECM molecule at the sites of epidermal / mesenchymal interactions *in vivo*, has also been shown to enhance the number of cartilaginous nodules, and therefore by inference, the number of condensation events, when prechondrocyte cells were grown on a substrate coated with this molecule (Mackie et al., 1987). Tenascin is of further interest because it has been shown to bind to proteoglycans (Chiquet & Famborough 1984). However, when Mackie et al. (1987) treated their tenascin substrate cultures with hyaluronidase, they still had a chondrogenic effect, if somewhat reduced, which implies that the

chondrogenic effect is not the result of bound hyaluronate. Not all extracellular matrix (ECM) molecules enhance chondrogenesis in culture. Fibronectin, a widely expressed ECM molecule in the developing embryo, has an inhibitory effect on chondrogenesis (Pennypacker et al., 1979, West et al., 1979, Mackie et al., 1987). Mackie et al., (1987) also noted that prechondrocyte cell attachment to fibronectin was greater than attachment to the tenascin substrate, and that tenascin treated cells were rounder and less fibroblast-like. This relationship between cell shape and differentiation had previously been postulated by Solursh et al., (1978). From this data it appears that this type of condensation event could be simply controlled by a variety of ECM molecules. However, further investigations have yielded yet another level of molecular complexity involving growth factors.

Growth factors are a wide variety of peptide signalling molecules that have been shown to have a great significance in cell and developmental biology. They are capable of stimulating and inhibiting cell growth, altering cell behaviour *in vivo* and *in vitro*, and inducing tissue differentiation in embryogenesis (Sporn & Roberts, 1990 a,b). The range of growth factors expressed *in vivo* during chondrogenesis is also wide, from members of the transforming growth factor- $\beta$  super family (Heine et al 1987, Pelton et al., 1989, Pelton et al., 1990, Millan et al., 1991) to those of fibroblast growth factors (Gonzalez et al., 1990). A number of these molecules have demonstrated significant effects, when used in conjunction with the *in vitro* model of chondrogenesis

Transforming growth factor- $\beta$ 's (TGF $\beta$ ) are distributed *in vivo* throughout chondrogenesis (Heine et al 1987, Pelton et al., 1989, Pelton et al., 1990, Millan et al., 1991), however their exact function *in vivo* could only be speculated on. TGF  $\beta_2$ ,  $\beta_3$  and  $\beta_4$  are expressed by differentiated chick chondrocytes *in vitro* (Jakowlew et al., 1991, Thorp et al., 1992, Roark & Greer, 1994), and it has been shown in many studies that TGF  $\beta$ s can promote chondrogenic differentiation. Seyedin et al., (1986, 1987) were able to stimulate cultured fibroblastic cells to express the cartilage phenotype using TGF  $\beta_1$  and TGF  $\beta$ , both of which were later shown to promote differentiation of chondrocytes within embryonic limb mesenchymal cells *in vitro*

(Carrington and Reddi, 1990, Schofield and Wolpert, 1990, Leonard et al 1991). In the most recent, study Roark and Greer (1994) monitored the steady state levels of expression of TGF  $\beta$ 2,  $\beta$ 3 and  $\beta$ 4, and discovered that all three genes were expressed before or during the cellular condensation process, and that changes in intensity of expression occurred sequentially during the condensation events. (TGF  $\beta$ 2 and  $\beta$ 4 from 3 to 15hrs and TGF  $\beta$ 3 from 15-24 hrs). It therefore appears that the TGF $\beta$ s may in some way be involved in early chondrogenic condensation activities. However, if chondrogenic condensation only requires TGF $\beta$ s what then are the functions of the other peptide growth factors that are expressed at this critical time *in vivo* ? A number of groups have tackled this problem by investigating these different peptides individually and synergistically. In a series of experiments Gospodarowicz et al., (1987) and Globus et al. (1988) found that basic fibroblast growth factor (bFGF) had a positive mitogenic effect for chondrocytes *in vitro*. Frenz et al., (1994) carried this work further and investigated the synergistic interaction of bFGF with the well documented effects of TGF  $\beta$ , when alone, TGF  $\beta$ 1 was incapable of initiating otic chondrogenesis (Frenz et al., 1992). Basic FGF could elicit a limited chondrogenic response, however addition of TGF  $\beta$ 1 and bFGF to the periotic mesenchyme was found to stimulate full chondrogenesis. Furthermore, antibodies to either of these molecules could also block epithelial induced chondrogenesis. It therefore appears that at least in the case of periotic mesenchyme, a synergistic action between these molecules is required to ensure correct chondrogenesis.

One of the most interesting groups of peptide growth factors involved in chondrogenesis are the bone morphogenetic proteins (BMP). Bone morphogenetic proteins are members of the TGF $\beta$  super family and one of their most interesting properties is their ability to induce cartilage formation when injected into ectopic sites (Sampath et al., 1987, Wozney et al., 1988, Wang et al., 1990). The micromass model has recently been used to investigate the role of these growth factors. BMP-4 was found to stimulate the expression of chondrogenic phenotype in both micromass and explant cultures of articular chondrocytes (Luyten et al., 1994) by increasing the expression of

type II collagen and the proteoglycan aggrecan. Nevertheless, this level of expression was rarely greater than that found in the parallel experiments for TGF  $\beta$ 1. Bone morphogenetic protein 2, a peptide growth factor very closely related to BMP 4, has also been investigated with respect to its role in chondrogenesis. Roark and Greer (1994) showed that BMP 2 could increase the accumulation of ECM molecules in micromass cultures, however BMP 2 had the greatest effect on already condensed cultures. It was therefore postulated that the function of BMP 2 might be to accumulate ECM molecules after the initial condensation events.

Activin A, a known inducer of mesoderm (Smith et al., 1990, Thomsen et al., 1990, Smith, 1993) and member of the TGF $\beta$  super family, is another growth factor distributed throughout the developing cartilage *in vivo* (Roberts et al., 1991). It is expressed temporarily during chondrogenesis of the snout, mandible and limb. Again using the micromass culture system, activin was found to increase the size of the precartilaginous condensations and enhance chondrogenesis of the limb bud cells and articular chondrocytes (Jiang et al., 1993; Luyten et al., 1994). Moreover, this molecule increased the expression of neural cell adhesion molecule (NCAM) within the condensation (Jiang et al., 1993). The fact that activin upregulates a cell adhesion molecule in this manner makes this type of interaction a prime candidate for controlling this condensation event, particularly as both molecules have similar expression regions within the *in vivo* precartilaginous condensation (Edelman, 1988, Chong, 1990). However, this is not the only cell adhesion molecule expressed with *in vivo* chondrogenesis. Oberlander & Tuan (1994) have revealed that N-cadherin, a member of the Ca<sup>2+</sup> dependant cell adhesion super family (Takeichi, 1990), is also expressed during chondrogenesis of the limb *in vivo* and *in vitro*. Moreover, the greatest level of expression of N-cadherin corresponds with active cell condensation. Furthermore, these authors were able to perturb condensation *in vivo* and *in vitro* by using a monoclonal antibody against the binding region of N-cadherin. Therefore these results provide evidence that N-cadherin is a component critical for condensation and chondrogenesis.

From the above it is clearly apparent that there are a range of different categories of molecules, whether extracellular, growth factor or cell adhesion, that appear at the time of chondrogenesis and that a number of these molecules have the ability to stimulate or, if blocked by antibodies, inhibit chondrogenesis. The above examples also imply that these molecules have a role in all chondrogenic events even though the majority of research using micromass cultures involves the limb bud system.

However, this is not always the case. Retinoic acid, a well known teratogen, when used in physiological excess, is also an essential component in regulating skeletal development and pattern under normal concentrations (Thaller & Eichele, 1987; Dencker et al., 1990). However, during craniofacial development, Sakai and Langille (1992) discovered a differential and stage dependent effect of retinoic acid on two facial chondrogenic regions in the chick, the mandibular and frontonasal mesenchyme. Whilst retinoic acid stimulated the chondrogenic process on micromass cultures of mandibular mesenchyme, the same result was not true of the frontonasal mesenchyme. Retinoic acid receptor distribution has been well documented (Brand et al., 1988, Giguere et al., 1987, Petkovich et al., 1987, Zelent et al., 1989, Tabin, 1991) and the discovery of differential expression of these receptors within craniofacial development (Dolle et al., 1989, Maden et al., 1991, Osumi-Yamashita et al., 1990, Ruberte et al., 1990, Ruberte et al., 1991) is probably the main reason for differences between the action of retinoic acid on events in mandibular and frontonasal chondrogenesis. It is therefore clear that not all the events in chondrogenesis are uniform for different parts of the body.

It is apparent from the above that the volume of research in this field has given many insights into the molecules involved in the condensation process. In the past these studies were restricted to single molecules but it is obvious that to answer the question of condensation formation, research may have to look at the coordinate action of the extracellular matrix, growth factors and cell adhesion molecules.

## 1.4.2 Feather dermal condensation

Dermal condensation with respect to histogenesis of avian skin and feather is one of the most structured and ordered events in development. On approximately the sixth day of chick embryo formation small regularly spaced spots become apparent on the spinal lumber region and thereafter the thoracic regions of skin (Wessells, 1965, Wessells and Evans, 1968). This dorso ventral progression down the embryonic flank is under strict temporal and spatial control (Davidson, 1983 a, b). This dense spotting consists of an epidermal placode and condensed dermis. Although feather development is the result of instructive and permissive inductions between the epidermis and the dermis, this section will be restricted to the events within the dermis.

Cell density within the dermal condensation increases from 2.6 nuclei/1000  $\mu\text{m}^3$  to 5.52 nuclei/1000  $\mu\text{m}^3$  (Wessells, 1965). How this increase in density occurs is still a matter of some controversy. Early research by Wessells (1965) discovered that tritium labelled DNA was diluted in the centre of the condensation in comparison with the periphery of the condensed tissue, thus indicating that focal proliferation could be a possible candidate. However histological evidence illustrates that cell orientation within the dermal condensation is strictly aligned (Wessells and Evans, 1968). Between the neighbouring condensations in the process of formation, the dermal cells have a bipolar fusiform shape, with their axis aligned according to lines of force between two attracting poles, possibly representing sites of future condensation. These lines of force may be set up by a pre-established lattice of collagen fibres (Stuart and Moscona, 1967), therefore cells may migrate to the centre of a condensation via contact guidance. However, Ede et al., (1971) found that this collagen lattice occurred during cell orientation rather than than before the emergence of this cellular pattern. Furthermore, they proposed that the cells determined this collagen lattice rather than vice versa. Although, Wessells and Evans (1968) indicated a slight increase in mitotic activity, Ede et al., (1971) believed that cell movement had the predominant role in producing these dermal condensations. It was speculated that cells within the condensation were

relatively immobile, and chemotactic to cells not already within the dermal thickening. Furthermore, the orientation of remaining cells between the condensations could be set up if all the cells were intrinsically motile, and tended to move between and around the condensations. Therefore, the collagen lattice described by Stuart and Moscona (1967) could be formed.

As with chondrogenesis, there is evidence that the ECM has a distinct function within events in feather dermal condensation. Kitamura (1981) discovered that there were increases in concentrations of lectin and fibronectin within early condensations. In conjunction with this Kitamura (1987) later showed that the expression of the proteoglycan chondroitin sulfate was linked with these condensation events. As chick skin could be cultured *in vitro* it meant that the role of these molecules could be tested. Using para-nitrophenyl- $\beta$ -D-xyloside, a molecule that disrupts glycosylation of the core protein of the proteoglycan, Goetinck and Carlone (1988) disrupted the feather pattern. Feather rudiments often become fused, however disruption due to xyloside occurred only during establishment of the rudiments and not afterwards, therefore suggesting that proteoglycans have a prominent role in differentiation of feather dermal condensations.

If increased adhesivity among cells is involved in the mechanism of condensation, then cell-cell adhesion molecules would be expected to play a role in condensation formation. Choung and Edelman (1985a) discovered that expression of N-CAM is spatially and temporarily distinct within the condensed mesenchyme of the feather rudiment. When antibodies to N-CAM were used to block development *in vitro*, the dermal condensations became unevenly sized, forming distorted hexagonal patterns (Jiang and Choung, 1992). Therefore N-CAM also has a conspicuous role in controlling dermal condensation. Although this introduction has concentrated on the dermis, the action of cell adhesion molecules in the epidermis cannot be ignored in this context. Gallin et al. (1986) found that blocking of L-CAM, a cell adhesion molecule expressed within the epidermal placode, by its antibody, altered feather pattern in the skin. Furthermore, these changes involved the dermis as well as the epidermis, with the

dermal condensations fusing and becoming horizontal stripes. This type of interaction supports the concept that the early stages (day7-8) in chick feather condensation are epidermally induced (Sengel, 1976). An excellent review of the events in feather development and in particular the role of the extracellular matrix and cell adhesion molecules can be found in Choung (1993).

### **1.4.3 Tooth dermal condensation**

As with the feather, the tooth forms as the consequence of interactions between the two components of the integument, the epithelium and oral mesenchyme. However, in contrast to other skin appendages, both the epithelium and oral mesenchyme contribute to the hard structure. The tooth initially occurs as a local proliferation of oral epithelia and a condensing of the underlying mesenchyme (Thesleff and Hurmerinta (1981), Ruch (1987), Thesleff, 1991).

As in previously described systems, ECM expression is again crucial at times during odontogenic condensation. In particular, two molecules are of specific interest, syndecan, a cell surface proteoglycan ( Bernfield & Sanderson , 1990) and tenascin. Both of these molecules have been well documented within tooth development (Chiquet-Ehrismann et al., 1986, Thesleff et al., 1987, Thesleff et al., 1988, Thesleff et al. 1990). However, in an interesting expression study involving both of these molecules, Vainio and Thesleff (1992) were able to show that a sequential induction of syndecan occurred slightly prior to that of tenascin in the dental mesenchyme, with both molecules becoming colocalized in the mesenchymal condensate at the bud and cap stage. They also noted that in the bud stages there was an increase in proliferation within this mesenchymal condensate. While investigating syndecan some interesting properties have come to light. Firstly, syndecan can bind to ECM molecules including tenascin (Bernfield & Sanderson, 1990, Salmivirta et al., 1991) Moreover the same molecule has also been shown to bind growth factors such as bFGF and TGF  $\beta$  (Kiefer et al., 1990, Rapraeger , 1989). Growth factors are known to enhance extracellular

matrix production and cell proliferation (Sporn and Roberts, 1990 a, b), therefore syndecan could be a prime candidate in controlling and coordinating dental mesenchyme condensation (Thesleff & Vaahtokari, 1992).

Growth factor expression within the developing tooth has also been well characterised for a number of different peptides, a large number of which occur prior to or within the condensed dental mesenchyme (Heine et al., 1987, Lyons et al., 1990, Gonzalez et al., 1990, Vaahtokari et al., 1991). However, their coordinate action and function is only now being determined. Harada et al. (1990) developed a similar micromass culture method to Ahrens et al., (1977) to monitor pulp cell differentiation and condensation. In preliminary experiments, Nakashima et al. (1994) used this method in an attempt to determine the function of many of the previously described growth factors, in particular TGF  $\beta$ , BMP 2 and BMP 4. On day 14 of these tooth micromass cultures, cell proliferation was reduced in control cultures and extracellular matrix formation increased. By day 21 cell proliferation had ceased when condensation events *in vitro* first became visible. ECM in the form of type III, type I collagen and fibronectin increased two to three fold on day 14, however by day 21 ECM production had declined. During this time scale TGF  $\beta$  was maximally expressed during active proliferation, being down regulated on day 14, whilst BMP 4 was only detectable on day 14. BMP 2 expression occurred at day 28, the time of nodule formation. Roark and Greer (1994) showed that BMP 2 accumulated ECM molecules after chondrogenic condensation in the chick limb. Therefore, it is possible that the later expression of BMP 2 in this tooth model equivalent may have a similar function to that described by Roark and Greer (1994).

The transient expression of the BMP 4 molecule at the time of condensation formation may have a significant role, as it reflects the timing of BMP 4 expression *in vivo* (Vainio et al., 1993). Here, this molecule is present during thickening of the epithelial arch (day 11), but there is a subsequent shift of expression to the condensed mesenchyme from day 13, during bud formation. As with the above *in vitro* model, BMP 2 expression occurs later, at day 14-15 in the dental papilla. Furthermore TGF  $\beta$

expression also shows unanimity between the *in vitro* and *in vivo* system (Vaahtokari et al., 1991). It therefore appears that the sequential expression of TGF $\beta$ , BMP4 and BMP2 has a distinct spatial and temporal function in the differentiation of tooth cells *in vitro* and *in vivo*

During *in vitro* chondrogenic condensation, the presence of type II cartilage specific collagen has been used as a marker of differentiation. However, as no analogous differentiation product has been isolated in the dental micromass cultures, it is difficult to characterise the exact roles of the growth factors, TGF- $\beta$ , BMP2 and BMP-4, with respect to *in vitro* differentiation of mesenchymal cells. Nevertheless, it is encouraging that the molecules expressed have close similarities to events *in vivo*, and the use of this type of system should lead to greater insights into tooth condensation events.

#### **1.4.4 Hair dermal condensation**

Hair formation, like that of tooth and feather, occurs as an interaction of the epidermis and dermis. Likewise the dermis condenses subjacent to the thickening of the epidermal placode. The majority of investigations to date have involved expression studies within the developing human, rat or mouse embryo, with only a few studies being performed on organ or tissue culture of developing hair follicles. As a result of this there is a considerable wealth of data on molecular expression in hair follicle development, and in particular for the sinus follicles.

Syndecan, the cell surface proteoglycan, is expressed within the developing epithelium and mesenchyme of the follicle, and becomes reduced as development proceeds (Trautman et al. 1991). It is first present within the overlying epidermis at day 10 (murine development). As the dermis condenses (day 13.5 in mouse) syndecan expression shifts to the mesenchyme with labelling absent from the epidermis.

Concomitant with this, tenascin expression is apparent within the later stages of sinus follicle dermal condensation, at the early hair peg stages (Chiquet-Ehrismann et al.,

1986, Choung et al., 1991). This parallels the sequential expression discovered in odontogenesis. Tenascin expression in human follicle development is similar to that exhibited by the rodent, however prior to the hair bulb stage ( the hair bud) tenascin is present within the dermal epidermal junction between the placode and condensing mesenchyme (Kaplan & Holbrook, 1994). Chondroitin sulphate proteoglycans are expressed throughout the dermal-epidermal junction (Couchman et al., 1990, Fine and Couchman, 1988) and the condensed dermis (Kaplan & Holbrook, 1994). This is unsurprising as chondroitin sulphate proteoglycan binds and co-distributes with tenascin *in vivo* (Chiquet & Famborough, 1984, Hoffmann et al., 1988). N-CAM, previously shown to be distributed in the condensing mesenchyme of the feather papilla, was recently found to be expressed in similar regions within the developing hair follicle. Labelling is particularly dense within the condensed dermis. (Choung et al., 1991, Kaplan & Holbrook, 1994).

Growth factor expression is equally complex. Examining the data from the transforming growth factor  $\beta$  super family alone, TGF  $\beta$ 1, TGF  $\beta$ 2 and BMP4 are all localised in the condensing mesenchyme (Heine et al. 1987, Pelton et al., 1989, Lyons et al., 1989, Lyons et al., 1990, Jones et al., 1991). Both isoforms of fibroblast growth factors, acidic and basic, are colocalized within events during the hair germ formation, however bFGF distribution is restricted to the dermal-epidermal junction, whereas aFGF is present within the hair germ (Gonzalez et al., 1990, Du Cros et al., 1993). Moreover, what is more interesting is the distribution of the receptors to these growth factors. FGF receptor 1 has a mesenchymal distribution whereas FGF receptor 2 an epidermal distribution (Peters et al., 1992), furthermore FGF receptor 1 can bind both aFGF and bFGF (Lee et al., 1989) and with equal affinity (Dionne et al., 1990). Recently, however research has shown that different isoforms of FGF receptors occur as a result of alternative splicing and that these display alternative binding affinities (Johnson et al., 1991, Eisemann et al., 1991). For example, an isoform of FGF receptor 2 binds aFGF with fifteen times greater affinity than bFGF ( Miki et al., 1991).

Therefore, a system exists for sorting out the action of growth factors where similar molecules have overlapping domains. With respect to the TGF  $\beta$  super family, receptors to the above molecules are only just being characterised. However, to add to the complexity of trying to interpret this data, recent research has shown that receptors to the TGF  $\beta$  super family can functionally replace each other during early embryonic inductive events, operating similar signalling pathways (Bhushan et al., 1994).

### **1.5 Mesenchymal condensation**

How all these growth factors operate within the confines of the extracellular matrix is unclear, since growth factors are capable of so many different functions (Sporn and Roberts, 1990a, b). However, from the previous few pages it becomes apparent that there are striking molecular similarities between the examples given. Cell adhesion molecules alter during the time of the condensation event, appearing focused at the time of condensation. Cell surface proteoglycans often appear prior to aggregate formation, with evidence that they may control the expression of ECM molecules and growth factors, both of which are expressed previous to, and during condensation formation. Moreover, the same molecules tend to be expressed in different systems, for example TGF $\beta$ s. Is it possible then that condensation events in a variety of organs all undergo the same molecular steps required for formation and that the organ type specific molecules still remain to be discovered?

Although much is known about molecular expression within condensations, the problem still arises as to what exact cellular mechanism creates these important morphogenetic building blocks. Focal proliferation is a prime candidate for aggregation and yet, hair and bone do not show signs of increased BrdU labelling and DNA labelling within the condensing mesenchyme. Tractional forces between cells and cell populations are also possible mediators of condensation, with what appears to be a strict sequence of expression between syndecan and tenascin. However, do they promote physical forces between cells or are they acting as better substrates for cell

motility, providing a means for focal migration? Finally, what is the role of the cell adhesion molecules, are they present to stimulate and create the condensation events or are they just a means of maintaining the stability within the already condensed tissue?

Current lines of approach have been to look at the expression of previously discovered molecules within the cellular condensation event. These molecules are isolated, often from totally unrelated systems, and then at a later date are looked for within embryonic development. Therefore, it is often by chance that these molecules become associated with an embryonic condensation event, and the resulting hypothesis is moulded to fit the expression pattern discovered.

Models have been developed with which to study condensation *in vitro*, by using cell culture in the case of chondrogenesis or organ culture with respect to feather and tooth development. However, the research adopted tends to look at a) the changing molecular expression, again with molecules that have been isolated from different systems or b) how these molecules can alter the condensation event, whether by blocking by using antibodies, or by adding the molecule in physiological or non-physiological concentrations prior to the production of the condensation.

In this thesis a model of hair follicle dermal condensation has been developed using the cells isolated from adult sinus follicle dermal papillae. Using this model, the question of condensation formation is addressed directly by examining how the cells interact during the formation of the condensation. However, molecular expression has not been ignored with respect to this model, but rather than looking for or using known molecules to alter the condensation formation, differential screening of a cDNA library has been used in an attempt to isolate the genes specifically involved in its formation, in an unbiased fashion.

## **Chapter 2**

### **Follicle Anatomy**

#### **Introduction**

##### **2.1.1 Pelage follicle anatomy**

Mammalian hair anatomy has been characterised in laboratory (Dry, 1926; Hardy, 1949; Chase, 1954; Mann, 1962; Butcher, 1951); agricultural (Wildman, 1932; Hardy & Lyne, 1956, Lyne & Heideman 1959, 1960); and to a lesser degree wild animals (Scheffer, 1962; Ling & Thomas, 1967; Dolnick 1959; Gibbs 1938; Lyne, 1957; Lyne 1970). A basic description of pelage development in the mouse was first defined by Hardy (1949) and later expanded by Hardy & Lyne (1956). The first appendages to develop in the pelage are known as the primary follicles (Hardy & Lyne, 1956), their exact morphogenesis following the same stages described for other follicles (Davidson & Hardy, 1952). Adjacent to the primary follicles, secondary follicles develop to create a triplet arrangement. This basic trio pattern has been discovered in over 200 species (cited by De Meijere, 1894 and more recently reviewed by Noback, 1951). More follicles bud off from the upper outer root sheath, forming bundles of secondary follicles. The result of this neogenesis is that multiple fibres protrude from a single opening. This phenomenon is seen in a variety of different species (Hardy & Lyne, 1956; Dolnick, 1959; Lyne, 1957) with probably the most complex occurring in the Chinchilla, where 75 hairs can emerge from a single orifice (Wilcox, 1950). The number of secondary follicles produced is quite varied even within closely related species (Korhonen, 1988).

Hairs from primary and secondary follicles have different functions within the integument. Secondary follicles, also known as underfur, provide the thermoregulatory function, whereas primary follicle fibres provide protection, being larger, thicker and protruding above the secondary follicles fibres. Hedgehogs and porcupines spines represent the most extreme examples of primary follicles (Ling, 1970). Pelage follicles

can also have limited sensory function. Sensory follicles, known as tylotrichs (Straile, 1960), have a thickened epidermal pad adjacent to the follicle that is innervated in a similar manner to that of the ringwulst region in the sinus follicles (Vincent, 1913). In studies of moult in Black tailed deer, Cowan and Raddi (1972) showed that primary and secondary follicles could also cycle at different times.

### **2.1.2 Sinus follicle anatomy**

Sinus follicles are highly specialized hairs, generally located on the face, however they have also been described on the ventral side of red squirrels (Hyvarinen et al., 1977). They are large in size and enclosed in a dense collagen capsule. Their primary function is a sensory one, with each vibrissa being highly innervated (Vincent, 1913, Patrizi and Munger, 1966, Stephens et al. 1973, Dykes, 1975).

This sensory function is tactile, however recent hypotheses indicate a possible audio function in conjunction with echolocation in the Ringed seal (Hyvarinen, 1989). In the majority of species the vibrissae are the first follicles to develop, however in some marsupial species this is reversed and the pelage develop before the vibrissae (Lyne, 1959). It is in the Pinnepedia that the greatest hair specialization is seen, with extremely large vibrissae and an absence of pelage follicles over much or all of the body (Ling, 1970).

The pattern and function of these follicles are therefore studied by neurophysiologists and developmental biologists alike. A schematic representation of the basic anatomy of a rat sinus follicle, taken from Jahoda (1982), can be found in diagram (1).

### **2.1.3 Smooth muscle alpha-actin labelling in follicles**

The use of cytoskeletal markers has been widely used in the identification of a broad range of cell types. For example, the intermediate filament desmin is generally

expressed within muscle cells ( Debus et al., 1983) whereas neurofilaments are used as markers for nerve cells (Laseke et al., 1979). In contrast to this, vimentin, another intermediate filament, has a broad expression in mesenchymal cells (Franke et al., 1979). However, one of the earliest identified cytoskeletal markers is actin.

Actin was originally isolated in muscle cells by Straub (1942). It was thought to be a component specific to muscle until it was isolated from non-muscle cells by Hatana et al. (1966). The appearance of this molecule in a variety of different vertebrate cells showed its widespread occurrence (Ishikawa et al., 1969), and it is now known to be present in every plant and animal cell, representing 5-10% of total cell protein. Actin is highly conserved globular protein of 375 amino acids and a molecular weight of 42KD (Vandekerckhove and Weber, 1978). In the higher eukaryotes, several isoforms have been isolated and these fall into three classes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), as detected by isoelectric focusing (Garrels and Gibson, 1976, Whalen et al., 1976). Furthermore, these actin isoforms appear to be specific to closely related cell groups such as myocardial, striated or smooth muscle cells (Vandekerchove & Weber, 1979, Skalli et al. 1987).

Skalli et al. (1986) noted that some hair follicle dermis stained positive for the monoclonal antibody smooth muscle alpha-actin. In later work Jahoda et al (1991) isolated this labelling to the dermal sheath *in vivo* , and dermal papilla and dermal sheath cells *in vitro*. Jahoda et al (1991) noted that the labelling of smooth muscle alpha-actin altered with different stages in the hair cycle. In this paper it was proposed that smooth muscle actin may be used as a marker for these cell populations. However, as yet the investigation only extended to human and rat follicles, and the relationship between this marking and the hair cycle mechanism is still to be established.

#### **2.1.4 Aims of follicle anatomy research**

Although hair function is varied, the basic morphology between follicle types is similar. In this chapter hair follicle morphology is considered and compared in several animal species in an attempt to understand further the cellular changes of the hair cycle. Current investigations involve the screening of follicles from a variety of species with smooth muscle alpha-actin to test the hypothesis that this could be used as a marker for dermal sheath cells *in vivo* . In particular, smooth muscle alpha-actin labelling was monitored throughout the hair cycle, to determine whether there was a relationship between its expression and follicular behaviour that would imply a functional role.

## **Materials and methods**

### **2.2.1 Follicle samples**

Tissue was obtained from recently deceased animals from Camperdown Wildlife Park, Dundee. Tissue was generally isolated from the mystacial pad and skin, however the exact age, sex and anatomical position of skin samples was unknown. Samples were from the mink (*Mustela vison*), polecat (*Mustela putorius*), stoat (*Mustela erminea*), meerkat (*Suricata suricatta*) and the grey squirrel (*Sciurus carolinensis*). Isolated sinus follicles were dissected from the mystacial pad region as described by Oliver (1966a). Sample preservation and embedding was carried out as described in section (2.2.2).

### **2.2.2 Histology of isolated skin and follicles**

Three to five sinus follicles were prepared for histology from each species depending on the availability of the specimens. Samples were fixed in formol saline and dehydrated through a series of alcohols, 50%, 70%, 90%, 95% and 100%, after which the tissue was cleared with three overnight washes in HistoClear (National diagnostics). To remove HistoClear and permeate with wax the tissue had several changes in liquid paraplast at 60°C prior to embedding. Tissues were embedded and orientated before hardening of paraplast. 10 µm sections were cut using a microtome (Leitz 1512). Sections were then stained using a combination of alcian blue, Wiegert's haematoxylin and Curtis Ponceaus.

### **2.2.3 Polylysine coating slides**

Slides were incubated in 0.1% poly-L-lysine solution (Sigma) for 30 minutes, then removed and allowed to air dry overnight.

#### **2.2.4 Immunofluorescent Histochemistry of samples**

Follicles and skin samples were snap frozen over liquid nitrogen after embedding in tissue tek (Miles). Five micron sections were cut using a cryostat (Bright) and lifted up on poly-l-lysine coated slides. Sections were washed in PBS before application of the first antibody. Follicle and skin sections were incubated with a monoclonal antibody to smooth muscle  $\alpha$ -actin (Skalli et al. 1986) in a 1:10 dilution of phosphate buffered saline (PBS) , pH 7.4 for 30 minutes at room temperature. After several washes in PBS, mouse IgG rhodamine, (Boehringer Mannheim) diluted 1:9 with PBS was applied to sections and incubated for 30 minutes in the dark. Sections were then washed in PBS and mounted in Citifluor (Agar aids). Sections were then viewed using an Axiovert microscope (Zeiss) with epillumination. Controls were carried out by removing one of the antibodies and replacing with PBS. For comparison some sections were double labelled with a polyclonal antibody that recognizes all actin isoforms (Skalli et al ., 1986). The secondary antibody used in conjunction with the total actin antibody was rabbit IgG FITC conjugate. (Wellcome).

## **Results**

### **2.3.1.1 Mink Hair follicle anatomy**

When isolating the sinus follicles for sectioning and cell culture, it was noted that the sinus follicles contained a single fibre, unlike the sinus follicles of the rat mystacial pad, where at any given time, many follicles contain two fibres, a growing hair, and a fully grown or club hair. A single externally visible fibre indicates that a follicle is either late in the growing phase; a transition from growing to resting (catagen) or not growing at all. Histology revealed that the majority of sinus follicles were in the non-growing telogen stage of the cycle.

### **2.3.1.2 Mink Anagen Sinus Follicle**

Consistent with previous anatomical reports (Butcher, 1951, Dolnick, 1959, Ling and Thomas, 1967, Ling, 1970) the overall structure of the mink anagen sinus follicle was found to be similar to other mammalian follicles. Follicles were enclosed in a dense collagen capsule, with the nerve penetrating this structure half way up the anagen end bulb region, opposite the dermal papilla (Fig. 2b).

In the anagen sinus follicle the dermal papilla was large and onion shaped, producing glycosaminoglycan (GAG) rich extracellular matrix, as shown by alcian blue staining . Within the papilla were an abundance of capillaries. In one interesting follicle the proximal edge of the dermal papilla tissue was highly convoluted, indicating that the follicle was in late anagen/early catagen. Continuous with the stalk was a thick layer of dermal sheath cells. The glassy membrane separating the epidermis from the dermis was barely visible. The dermal papilla was surrounded by the epidermal cells of the end bulb (Fig 2c,d). In the germinative region of epidermis encircling the dermal stalk two distinct cell layers were visible at this stage. Germinative cells were present in the upper portion of the bulb epidermis, extending around the edge of the epidermis

opposing the dermal stalk. This is in contrast to the morphology of the rat vibrissa end bulb (Fig 2a), which illustrates only a single cell layer in the region of the germinative matrix. Below this were the cells of the inner root sheath. The inner root sheath cells nearer the germinative population appeared less differentiated than those further up the end bulb (Fig 2d), the former cells having smaller nuclei and less cytoplasm.

Further up the end bulb, the epidermal cells were more differentiated with the distinct layers of inner root sheath, cuticle and cortex more apparent (Fig. 2d). The glassy membrane was thin around the end bulb of the follicle but increased in thickness proximally (Fig 2e), becoming broadest opposite the ring sinus and ringwulst, after which it decreased in thickness in the upper isthmus (Fig 2f).

#### **2.3.1.3 Mink Catagen Sinus Follicle**

In the mink catagen sinus follicle gross morphological changes had occurred from the anagen state. The dermal papilla had flattened into an anvil shape and shrunk due to a reduction in volume of extracellular matrix. The biggest visible change had occurred in the epidermis, which was detached from the lower dermis (Fig 3a). However, this movement was not uniform since, part of the matrix was still in contact with the dermal papilla, whereas the other side of the matrix had detached and begun proximal migration (Fig 3b). What was interesting was that the matrix had buckled and become convoluted (Fig 3a). Consistent with observations by Jahoda (1982) on the rat sinus follicle, an histological asymmetry was apparent within the epidermal cells.

#### **2.3.1.4 Mink Telogen Sinus follicle**

Continuation of the changes initially observed during catagen, meant that the morphology of the telogen sinus follicle had altered further. The papilla was compacted, with loss of alcian blue staining indicating an apparent termination in GAG production (Fig 4 a,b). The ringwulst was tapered through its circumference (Fig 4a).

From the apparent size of the dermal stalk and relative position of nerve entry, it appeared that the main elements of the follicle had been drawn up inside the capsule (Fig 4b). Within the dermal stalk, large capillaries that supplied the anagen papilla were still visible (Fig 4b). Above the dermal papilla was a plug of ORS like cells. Abutting the basement and glassy membrane was a single cell thick palisade layer of ORS cells. Between the DP and ORS was a thin basement membrane, however between the DS and the ORS the glassy membrane had enlarged and thickened over much of its length. At the base of the epidermis was a distinct bottleneck that maintained a link between the ORS and the papilla (Fig 4b). In the most proximal region of the follicle was the sebaceous duct and gland, in which were large bundles of vacuolar cells. The sebaceous gland was maintained throughout the different stages of the hair cycle (Fig 4c).

#### **2.3.1.5 Immunohistochemistry of Mink Sinus follicles**

Smooth muscle alpha actin labelling of catagen follicles was tissue specific. Dermal sheath stained positively, with label detected from the mid end bulb dermal sheath region to  $1/4 - 1/3$  the length of the follicle (Fig 5a). Staining was not present around the base of the end bulb. Along the dermal sheath smooth muscle alpha actin labelling was punctuated, rather than a single continuous line of expression (Fig 5b). Capillaries within the dermal papilla and external to the dermal sheath also stained positive (Fig 5a). There was also faint labelling on the inside edge of the collagen capsule adjacent to the dermal sheath in the end bulb region (Fig 5c).

Smooth muscle alpha actin labelling of telogen sinus follicles also displayed distinct tissue specificity. However, overall labelling was reduced compared to the anagen/catagen follicle. Dermal sheath cells were positively labelled in the cell layers immediately adjacent to the epidermal outer root sheath plug above the dermal papilla (Fig 5d,e). Labelling of the dermal sheath extended from halfway up the end bulb to just above a visible constriction in the epidermis. Staining was again not present around

the base of the follicle. Capillaries in the DP and adjacent to the DS also stained positive (Fig 5d,e). Total actin labelling was widespread with particularly intense marking in the epidermal outer root sheath (Fig 5f,g). Controls of the sections illustrated no auto fluorescence.

#### **2.3.1.6 Mink Pelage follicles**

Mink pelage consists of two follicle types, larger guard follicles and many more smaller underfur follicles. Both follicle types penetrate deep into the dermis of the skin, with guard follicles passing into the fat region (Fig 6a). Large lipid packed cells could be seen surrounding the end bulb and lower follicle regions of the guard hairs (Fig 6b). Endbulbs from the secondary follicles merely abutted the fat layer.

Both follicle types were distinctly linked and arranged in tight bundles. Secondary follicles were approximately at the same depth within each rosette (Fig 6b), and each bundle of secondary fibres emerged from a single orifice in the skin. Near the surface, each bundle of follicles was supported by a large sebaceous gland. In some bundles sweat glands were also present (Fig 6a). These bundles were separated from each other by a dense layer of collagen (Fig 6d). Sweat glands were present at all levels peripheral to the follicular bundles. All follicles were in the anagen phase of the cycle, no telogen follicles were observed (Fig 6c).

A primary follicle could be associated with three or four bundles of secondary follicles arranged in the trio pattern (Noback, 1951; Dolnick, 1959). Each bundle of secondary follicles was separated by a thin collagen membrane. Counting revealed that bundles each contained between 15 and 24 secondary follicles (Fig 6d).

#### **2.3.1.7 Immunohistochemistry of mink pelage follicles**

Labelling of pelage follicles revealed that smooth muscle  $\alpha$ -actin was restricted to the dermal sheath regions of the follicle (Fig 7a). However, unlike the sinus follicles,

marking of the dermal sheath was continuous around the base of the end bulb in the pelage follicles. In one section a single large guard hair was found to have labelling more akin to that of the vibrissa (Fig 7a). Consistent with Jahoda et al., (1991), certain follicles revealed a banding of the smooth muscle alpha actin around the follicle (fig 7b). Total actin labelling was spread broadly throughout the follicles (Fig 7c).

### **2.3.2.1 Polecat Hair Follicle Anatomy**

Sinus fibres in the polecat were similar to that described for the mink (sections 2.3.1.2, 2.3.1.4), however converse to the mink data there was a preponderance of anagen follicles among the specimens examined.

### **2.3.2.2 Polecat Anagen Sinus follicle**

Polecat anagen follicle anatomy was similar to that described previously for the mink (section 2.3.1.2.) with the dermal papilla conspicuous as a large 'onion' shaped bundle of GAG rich DP cells (Fig 8a,b). However, at the opening of the follicle were a pair of single lobed sebaceous glands, with ducts emptying into the follicle just below the surface of the skin (Fig 8c). In addition to these glands a series of multi-lobed sebaceous glands were positioned deeper in the dermis (Fig 8d). Ringwulst was prominent on both sides of the sinus, each section of which was enclosed in an equal sized ring sinus (Fig 8g). The glassy membrane was thin, but thickened slightly in the region of the ringwulst (Fig 8 a,b,g). Epidermal layers of ORS, IRS, cuticle and cortex were all conspicuous (Fig 8b). Within the inner root sheath trichohyalin granules were discernible (Fig 8e). The cuticle of the epidermis consisted of a single layer of palisade cells (Fig 8e,f). Melanin production begun in the epidermal matrix at the level of the widest circumference of the papilla (Fig 8f).

### **2.3.2.3 Polecat Telogen Sinus follicle**

The anatomy of the polecat telogen sinus follicle was similar to that of the mink telogen follicle. The glassy membrane was enlarged and enclosed the ORS cells (Fig 9). The epidermis tapered to constriction above a relatively indeterminable group of papilla and dermal sheath like cells. As is typical of sinus follicles, the whole structure was embedded in a thick collagen capsule (Fig 9). It was unclear whether these follicles had undergone the shortening described for the mink sinus follicle, because nerve entry points into the collagen capsule could not be compared from both stages in the cycle.

### **2.3.2.4 Immunohistochemistry of Polecat Sinus follicles**

Sinus follicles of the polecat revealed smooth muscle alpha actin labelling in a tissue specific manner. In the mid anagen follicle, smooth muscle  $\alpha$ -actin staining was again restricted to the dermal sheath cells surrounding the epidermal constituent of the hair follicle (Fig 10a). Labelling began halfway up the end bulb and terminated within the lower third of the follicle (Fig 10a). Rather than being a continuous line of labelling, staining was punctuated (Fig 10b). Labelling was also present in capillaries within and beneath the dermal papilla (Fig 10a,c). A few dermal papilla cells were also positive for smooth muscle alpha actin (Fig 10 a,c). Antibody to total actin revealed a widespread distribution, with intense staining occurring within the outer root sheath of the epidermis (Fig 10d,e). Telogen vibrissa follicles were unmarked with only the capillaries staining positive for smooth muscle alpha actin (Data not shown). The dermal sheath and the dermal papilla had no obvious expression.

### **2.3.2.5 Polecat Pelage Anatomy**

Polecat pelage was essentially similar to that of mink (section 2.3.1.7), with primary follicles associated with bundles of secondary follicles (Fig 11). One difference was

that sebaceous glands were more abundant in polecat, and the dermis was also thinner than that of the mink (Fig 6a). Moreover, both primary and secondary follicles penetrated into the fat layer subjacent to the dermis (Fig 11). It is also apparent that the overall follicular density is less in the polecat than that of the mink, as can be seen by comparing the micrographs Fig. 6 and Fig. 11.

### **2.3.3.1 Meerkat Anagen Sinus follicle**

Tissue preservation in these samples was particularly poor, however, as with other anagen follicles, the epidermis was differentiated into the layers of the matrix and outer root sheath. The dermal papilla was large, and GAG rich. The follicle was surrounded by a large sinus, and the whole structure was enclosed in a dense collagen capsule (Fig. 12).

### **2.3.3.2 Meerkat Telogen Sinus follicle**

Morphologically the meerkat telogen sinus follicle resembled the mink equivalent. In the end bulb region the papilla was reduced and spherical (Fig 13a), and embedded in a large area of connective tissue, which extended around the follicle (Fig 13b). The papilla was fixed to the collagen capsule via a short dermal stalk (Fig 13b). The dermal sheath tissue was extremely thick for the distal third of the follicle, but thinned out proximally (Fig 13a,b). The glassy membrane, separating epidermis from dermis was broad, but was reduced in thickness in the region of the ringwulst (Fig 13a). The ringwulst was differential in size through out its circumference with one side distinctly larger than the other. Nerve entry occurred at the base of the collagen capsule adjacent to the endbulb (Fig 13a), but nerves entered the dermal sheath above the level of the dermal papilla. The epidermis consisted of ORS and non growing hair fibre (Fig 13a).

This fibre had asymmetry in the club region, with the 'brush like fibre' tailing off diagonally on the nerve side of the follicle, and the other side of the fibre remaining

flush (Fig 13a). Adjacent to the glassy membrane was a palisade layer of ORS, however this was not as regimented as that of the mink telogen sinus (Fig 4b), and the palisade arrangement degenerated nearer the endbulb (Fig 13b). Where the ORS opposed the dermal papilla, projections of epidermis occurred around the papilla (Fig 13c). Proximal to the ringwulst, the ORS thickened to form a distinct bulge region of cells. The sebaceous gland in the upper isthmus was small and single lobed (Fig 13d). The whole of the follicle was contained within a large cavernous sinus (Fig 13a).

#### **2.3.4.1 Stoat Pelage Anatomy**

Stoat pelage consists of primary and secondary follicles. Unlike the mink, secondary hairs were arranged in bundles of only a few follicles, with some secondary follicles arranged singly. Interspersed amongst the secondary follicles were large primary follicles. Large sebaceous glands were present in the upper dermis and associated with the bundles of secondary follicles were sweat glands. Distinct bulge regions were present in the outer root sheath at the level of the upper isthmus of primary follicles. Follicles did not penetrate into the fat layer distal to the dermis (Fig 14).

#### **2.3.4.2 Immunohistochemistry of Stoat pelage follicles**

Smooth muscle alpha actin label was restricted to the dermal sheath cells of the pelage (Fig 15). Consistent with the mink data the stain was continuous around the base of all the follicles. Dermal sheath label occurred in the lower 2/3 of the follicles. Autofluorescence in these sections was at a relatively high level.

### **2.3.5.1 Grey Squirrel anagen sinus follicle**

The anagen sinus follicle of the grey squirrel had very similar morphology to that of the rat sinus follicle. The follicle was embedded in a dense collagen capsule. At the base, the end bulb consisted of a large GAG rich DP surrounded by all the typical layers of the hair follicle epidermal matrix (Fig 16a). The DP was connected to a thin DS layer via a short and thin stalk. In pigmented follicles a dense layer of melanin was visible adjacent to the papilla in the epidermal matrix (Fig 16a).

One follicle displayed an unusual feature in the mid-proximal region, where the epidermis was invaginated with dermal sheath cells (DS). This invaginated DS was surrounded by its own basal lamina, which was considerably thinner than the glassy membrane (Fig 16b,c). Further sectioning revealed that this invagination was related to the presence of another anagen follicle. This smaller follicle had been induced in the mid proximal-distal region adjacent to the original fibre. The new follicle was embedded in the epidermal outer root sheath cells of the upper isthmus (Fig 16d).

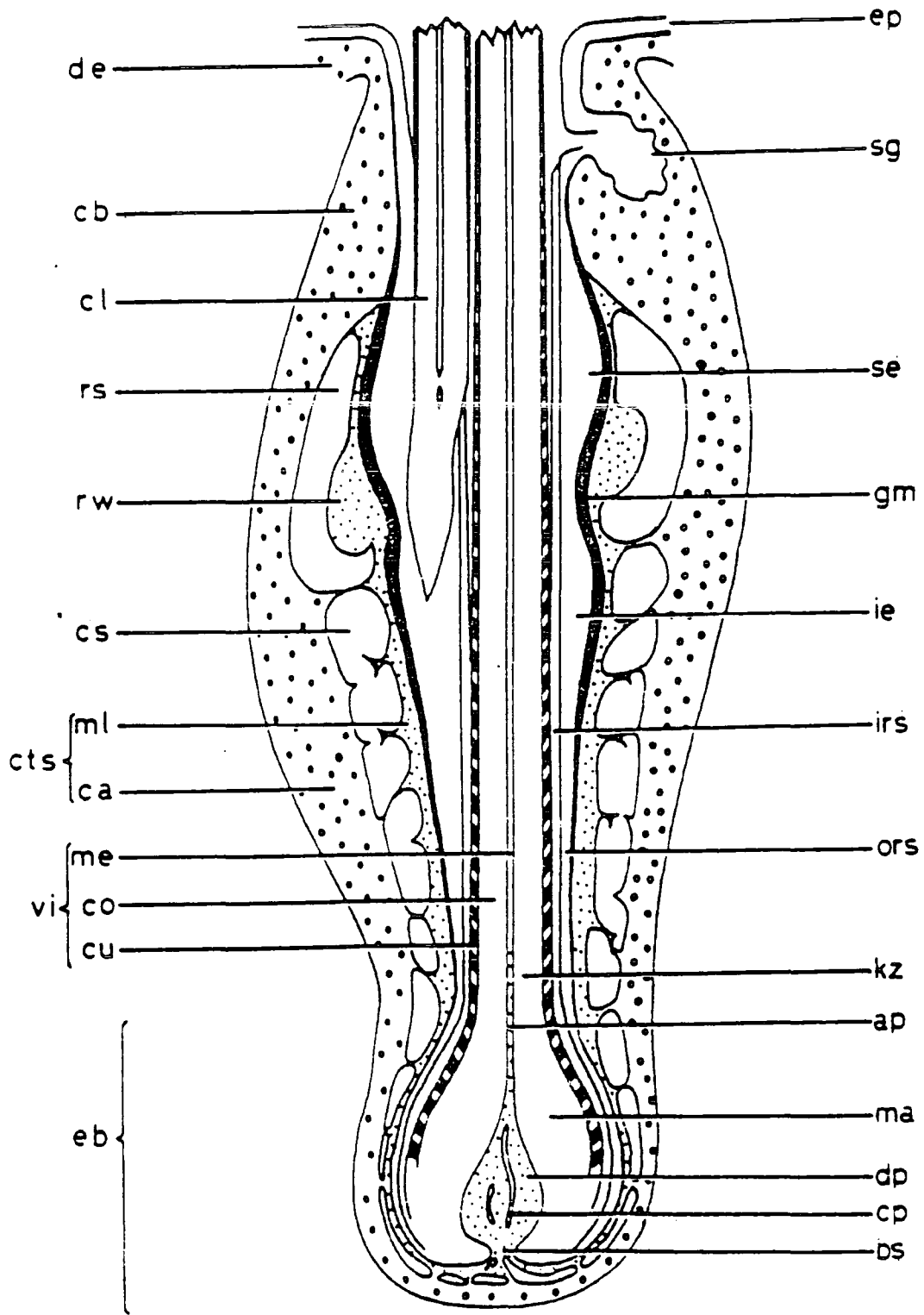
### **2.3.5.2 Grey Squirrel telogen sinus follicle**

As in previous cases, the telogen follicle morphology had greatly changed. The dermal papilla was reduced with low GAG production, and the epidermal tissue consisted of outer root sheath-like cells only (Fig 17a,b). In contrast to the anagen stage the dermal papilla was connected to the collagen capsule via a long broad dermal stalk. It appeared that the lower follicle components had lifted slightly during the telogen phase (Fig 16a). Moreover, the DP was embedded in a thick dermal sheath (Fig 17a). A small sebaceous gland was visible in the upper isthmus of the follicle (Fig 17c).

**Table 1** Tabulation of the anatomical data described in chapter 2. (**X**) tissue not available, ( $\checkmark$ ) tissue processed. u DS upper dermal sheath, m DS mid dermal sheath; l DS lower dermal sheath; (+++), a high intensity of positive marking of smooth muscle  $\alpha$ -actin labelling; (++) , an intermediate intensity positive marking of smooth muscle  $\alpha$ -actin; (+), a low intensity of positive marking of smooth muscle  $\alpha$ -actin; (-), no positive marking of smooth muscle  $\alpha$ -actin.

| Animal species | Follicle types                                      |                     |  |   |
|----------------|---|---------------------|--|---|
|                | Histology of vibrissae, and stage in the hair cycle | Histology of pelage | Smooth muscle $\alpha$ -actin labelling on vibrissae | Smooth muscle $\alpha$ -actin labelling on pelage |
| Mink           | Anagen, Catagen and telogen                         | $\checkmark$        | m,l DS (++)<br>l DS (+)                              | u,m,l DS (+++)                                    |
| Polecat        | Anagen and telogen                                  | $\checkmark$        | m,l DS (++)<br>DS (-)                                | <b>X</b>  |
| Stoat          | <b>X</b>  | $\checkmark$        | <b>X</b>   | u,m,l DS (++)                                     |
| Meerkat        | Anagen and telogen                                  | <b>X</b>            | <b>X</b>   | <b>X</b>  |
| Grey squirrel  | Anagen and telogen                                  | <b>X</b>            | <b>X</b>   | <b>X</b>  |

Diag.1



#### **Figure 5d**

Low power micrograph of anti-smooth muscle  $\alpha$ -actin labelling of a mink telogen sinus follicle. Dermal sheath cells (**DS**) and capillaries (**Ca**) are all positively labelled for the antibody. Labelling of the dermal sheath (**DS**) is restricted to the level at which the epidermis becomes constricted. (**DP**) dermal papilla. (Magn. x74).

#### **Figure 5e**

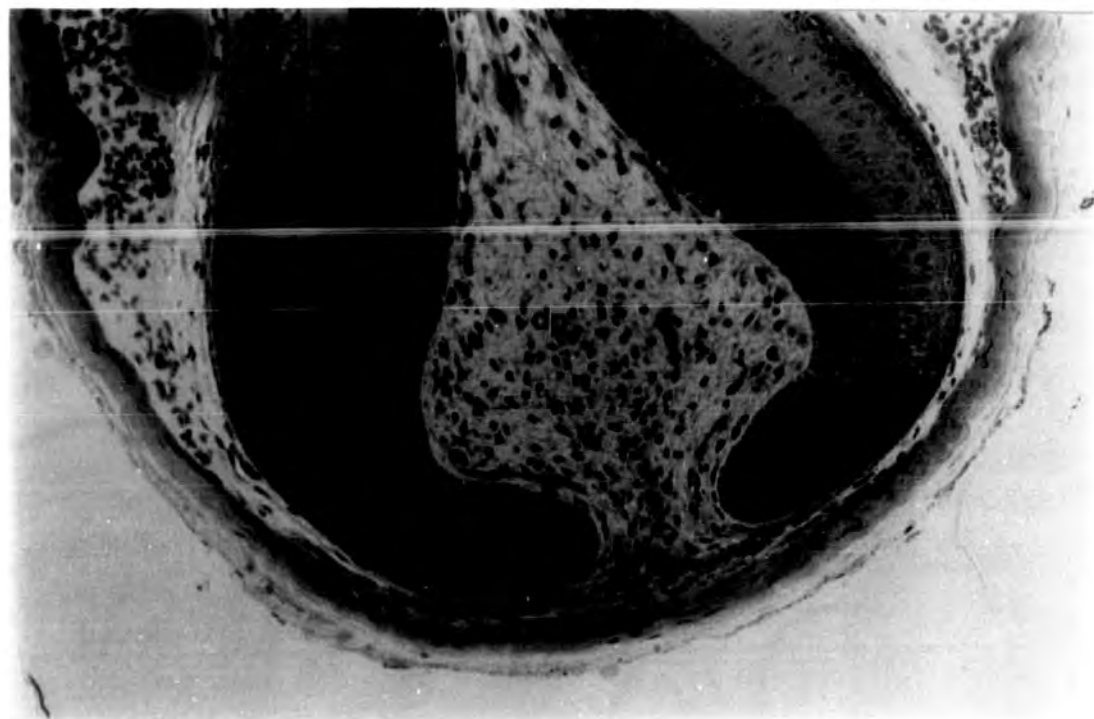
High power micrograph of anti-smooth muscle  $\alpha$ -actin labelling of a mink telogen sinus follicle. Dermal sheath (**DS**) labelling was outside the end bulb, and constricted region of the outer root sheath. Staining was not present around the base of the sinus follicle. (**DP**) dermal papilla. (Magn. x103).

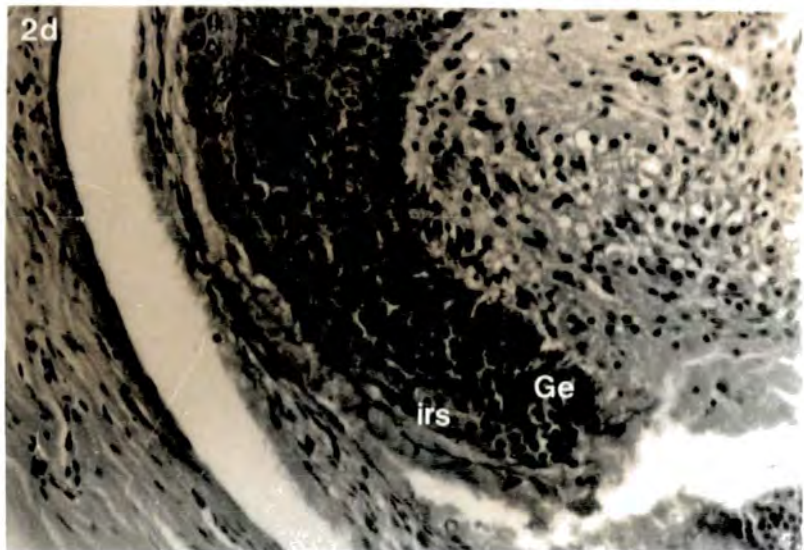
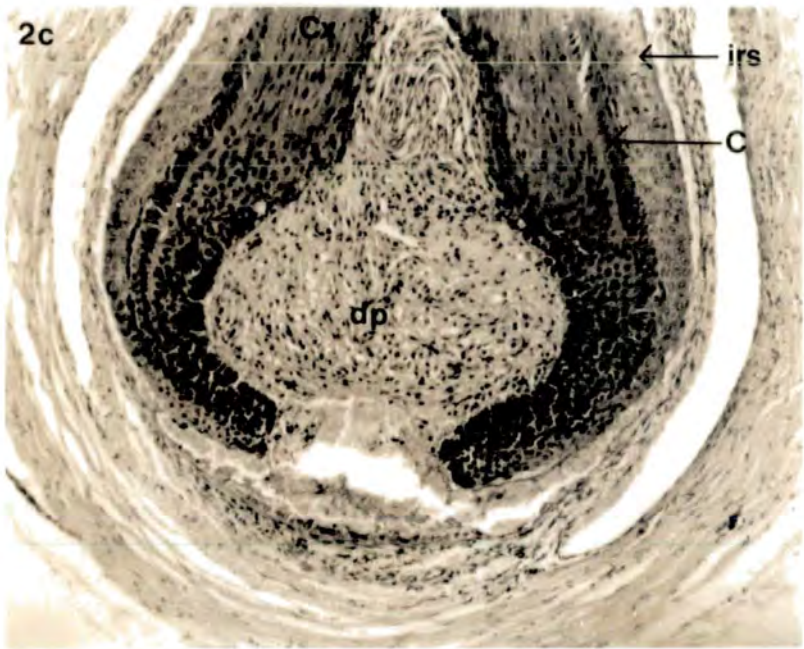
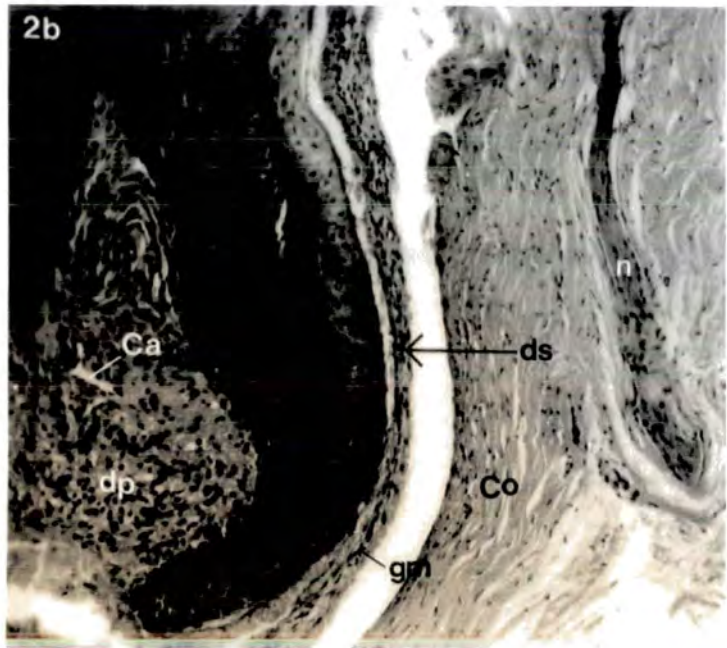
#### **Figure 5f**

Low power micrograph of labelling of a mink telogen sinus follicle with the antibody to all actin isoforms. Follicle morphology is clearly apparent with particularly intense stain restricted to the outer root sheath (**ORS**) cells of the epidermis. (Magn. x74).

#### **Figure 5g**

High power micrograph of labelling of a mink telogen sinus follicle with the antibody to all actin isoforms. Follicle morphology is clearly apparent with particularly intense stain restricted to the outer root sheath (**ORS**) cells of the epidermis. (**DP**) dermal papilla. (Magn. x103).



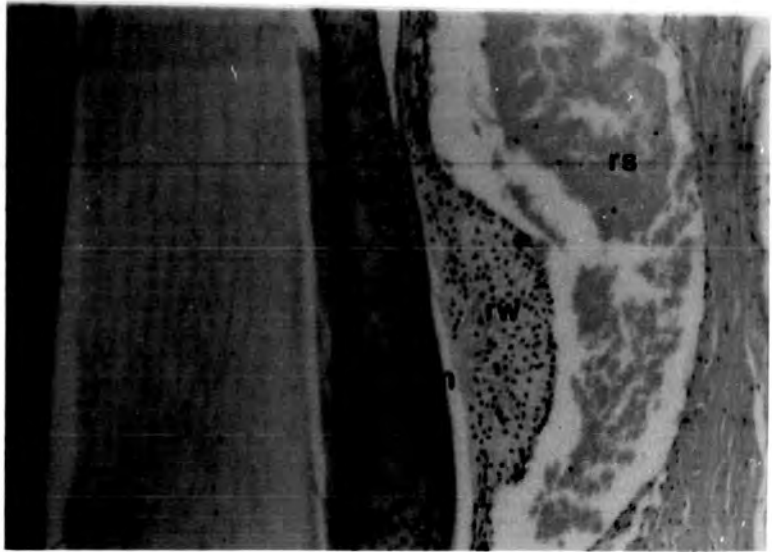
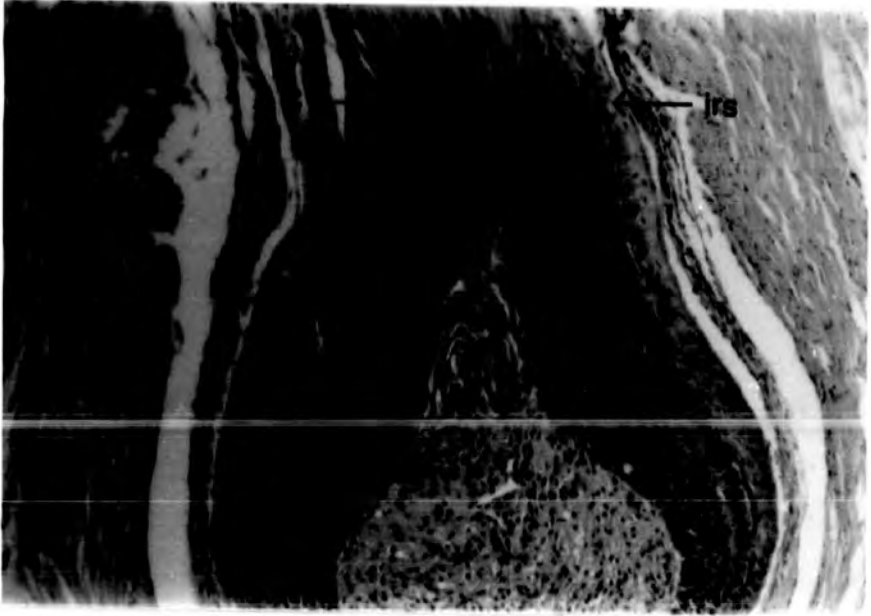


### **Figure 2e**

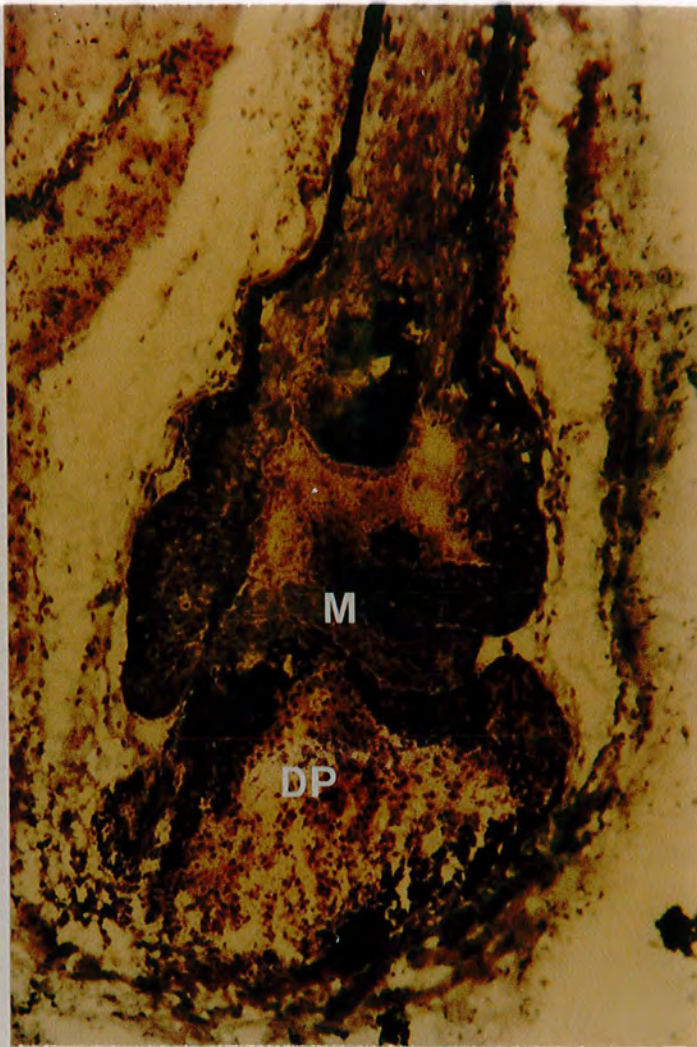
Low power micrograph of the upper end bulb region of a mink anagen sinus follicle. Epidermal differentiation has progressed, with inner root sheath (**irs**), cuticle (**C**) and cortical (**Cx**) layers all clearly defined. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn x98).

### **Figure 2f**

Light micrograph of wax embedded section of the mink anagen sinus follicle. The micrograph is in the mid proximal-distal region of the follicle. The ringwulst (**rw**) is large and embedded within the ring sinus (**rs**). Separating the outer root sheath and the dermal tissue is a thick glassy membrane (**gm**). Thickness is greatest in the region of the ringwulst, decreasing in width in the proximal region of the follicle. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn x100)



3a



3b

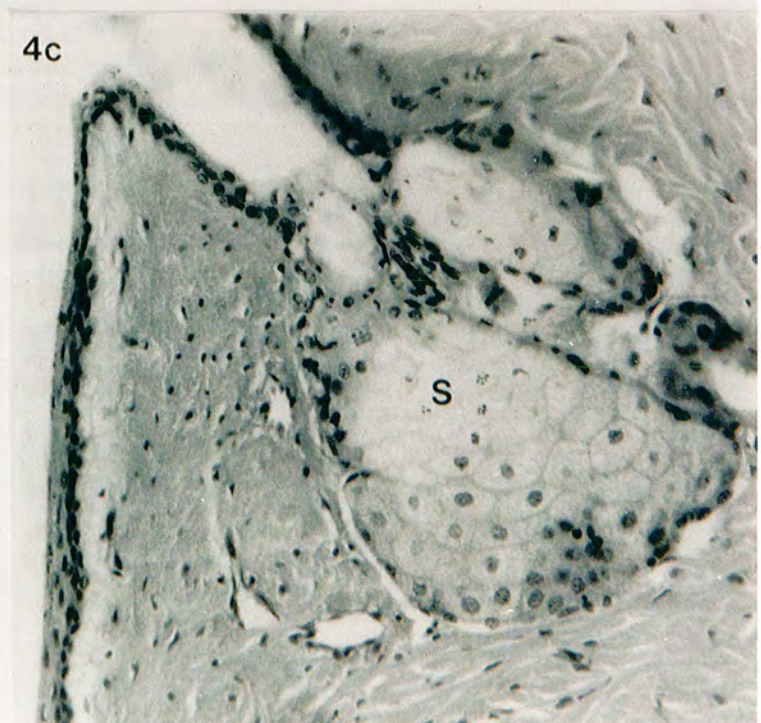
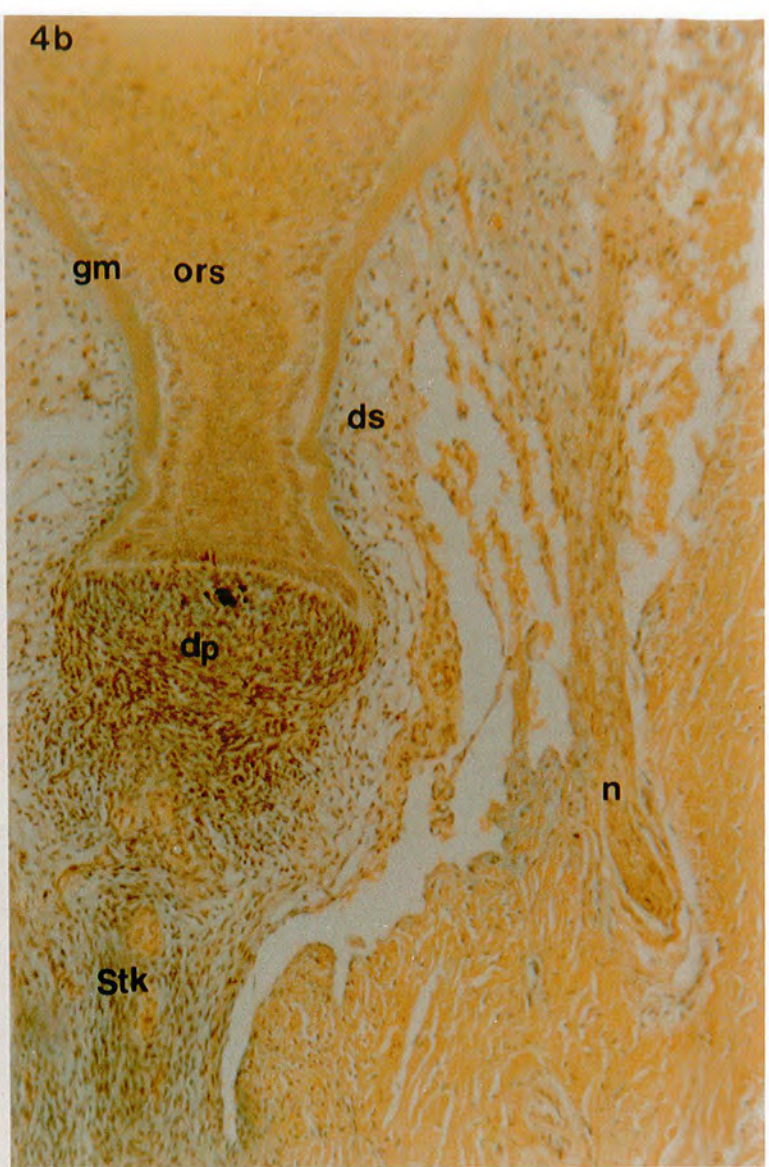


### **Figure 3a**

Light micrograph of a cryostat section of the mink catagen sinus follicle. The dermal papilla (**DP**) is reduced and become anvil-shaped. The matrix (**M**) has become detached and begun proximal migration. The matrix has become buckled and convoluted. The section is stained with toluidine blue. (Magn x 115).

### **Figure 3b**

Light micrograph of a cryostat section of the mink catagen sinus follicle. The matrix is convoluted (**M**) and has differential proximal migration. The section is stained with toluidine blue. (**DP**) dermal papilla (Magn x 94).



#### **Figure 4a**

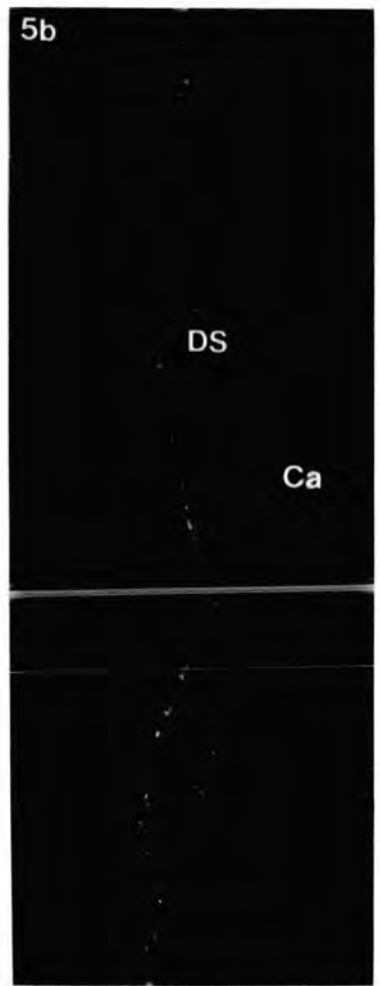
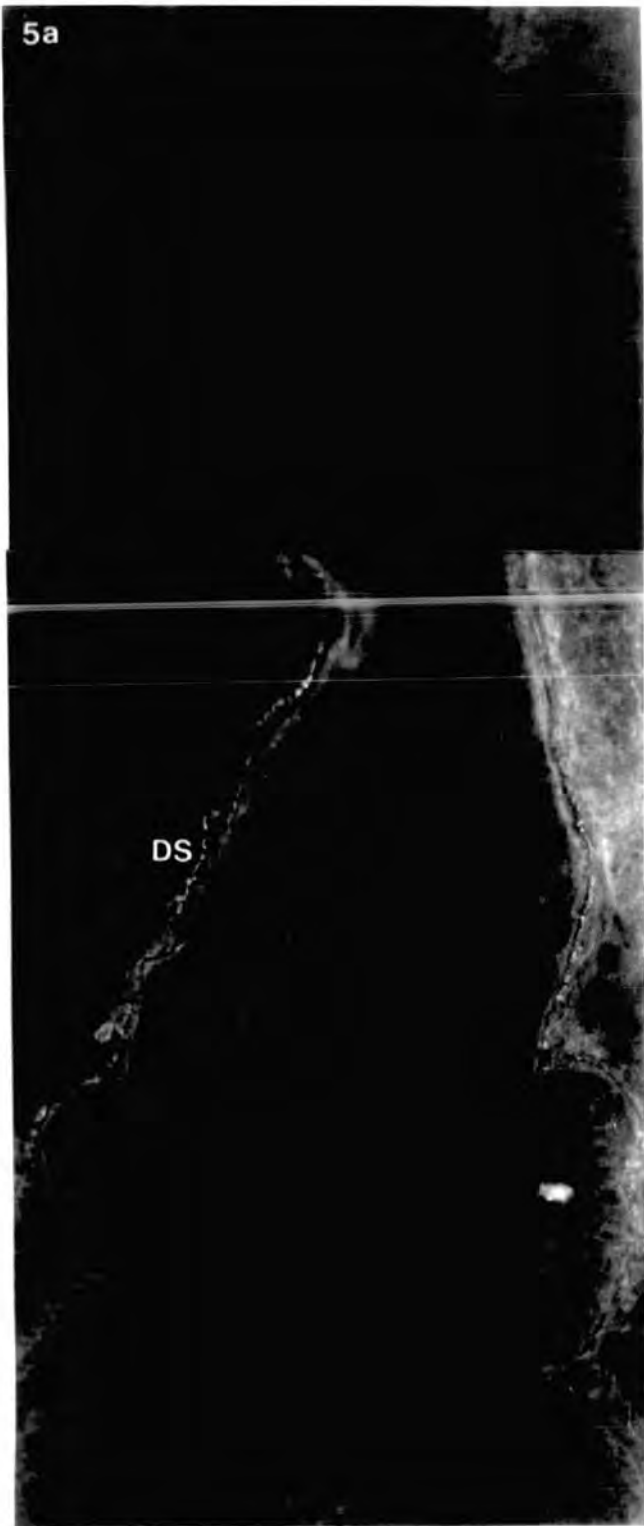
Light micrograph of wax embedded section of the mink telogen sinus follicle. The dermal papilla (**dp**) has compacted and can be distinguished from the mesenchyme but has no clear boundary. The dermal stalk (**Stk**) is elongated, indicating that the whole follicle has moved upwards. (**n**) nerve, (**Co**) collagen capsule, (**cf**) club fibre, (**rs**) ring sinus, (**rw**) ringwulst and (**S**) sebaceous gland. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn x51)

#### **Figure 4b**

Light micrograph of wax embedded section of the mink telogen sinus follicle in the end bulb region. The outer root sheath cells (**ors**) about the dermal papilla (**dp**), being separated by a thin glassy membrane. Outer root sheath cells adjacent to the glassy membrane have a palisade arrangement. (**n**) nerve, (**ds**) dermal sheath, (**Stk**) dermal stalk and (**gm**) glassy membrane. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn x115).

#### **Figure 4c**

Light micrograph of wax embedded section of the mink telogen sinus follicle. Micrograph illustrates the morphology of the sebaceous gland (**S**). Cells within gland are large and vacuolar. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn x85).



### **Figure 5a**

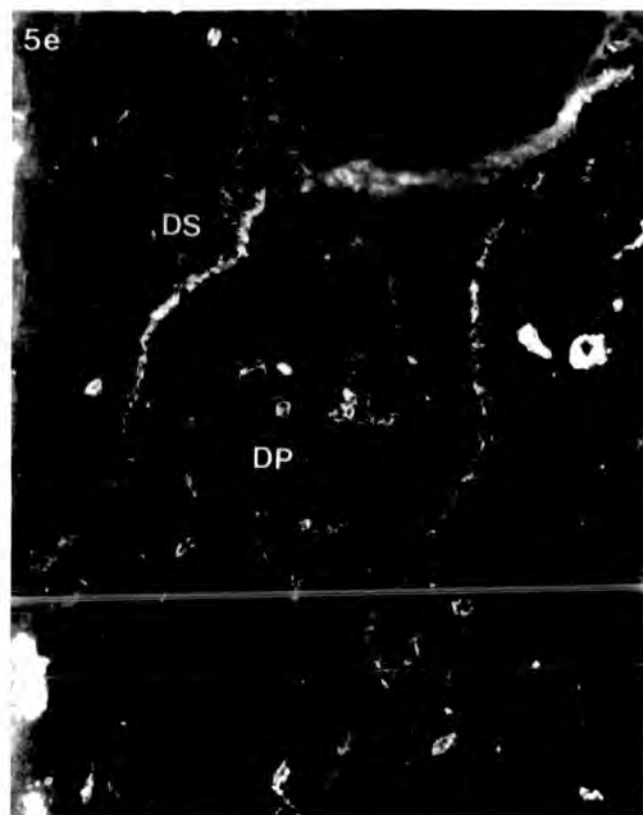
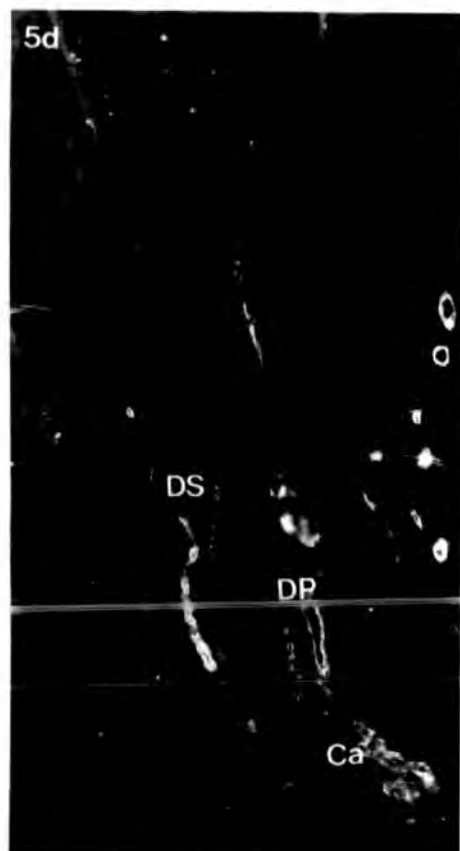
Immunofluorescent micrograph of a cryostat section of a mink catagen sinus follicle. Tissue was labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. Label was restricted to the cells of the dermal sheath (DS) and capillaries around the follicle. (Magn. x98)

### **Figure 5b**

High power micrograph of the labelling of the dermal sheath (DS) in the mink catagen sinus follicle with anti-smooth muscle  $\alpha$ -actin. Rather than being continuous, expression is punctuated along the length of dermal sheath distribution. Capillaries (Ca) adjacent to the DS were also positive. (Magn. x170).

### **Figure 5c**

High power micrograph of anti-smooth muscle  $\alpha$ -actin labelling of the dermal sheath in the end bulb region of the mink catagen sinus follicle. Expression was restricted to the dermal sheath, with the inner facing edge of the collagen capsule (Co) also labelling slightly positive. (Magn. x170)



### Figure 13a

Micrograph of a meerkat telogen sinus follicle. The dermal papilla (**dp**) is reduced and embedded in a dense layer of dermal sheath. The glassy membrane is thick in the lower third of the follicle. The ringwulst (**rw**) is differential in size throughout the circumference of the follicle. The epidermis consists of outer root sheath and the resting club fibre (**cf**). Nerve (**n**) entry occurs at the base of the collagen capsule, at a level opposing the end bulb. (**cs**) cavernous sinus, (**b**) bulge cells. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x90).

### Figure 13b

High power micrograph of the end bulb of a meerkat telogen sinus follicle. Nerve (**n**) entry occurs at the base of the collagen capsule, at a level opposing the end bulb. (**dp**) dermal papilla, (**ds**) dermal sheath, (**Stk**) dermal stalk. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x136).

### Figure 13c

Micrograph of the meerkat telogen sinus follicle. Outer root sheath (**ors**) cells occurred in finger like projections on either side of the dermal papilla (**dp**). Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x136).

### Figure 13d

Sebaceous gland (**S**) in the upper isthmus of the meerkat sinus follicle was small and singled lobed. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x136).

### **Figure 6d**

Low power light micrograph of a transverse section through the mink pelage. Large bundles of secondary follicles ( $2^{\circ}$ ) are associated with a single primary follicle ( $1^{\circ}$ ). Groups of secondary and primary follicles are separated from adjacent groupings by barriers of collagen (Co). Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x25).

### **Figure 7a**

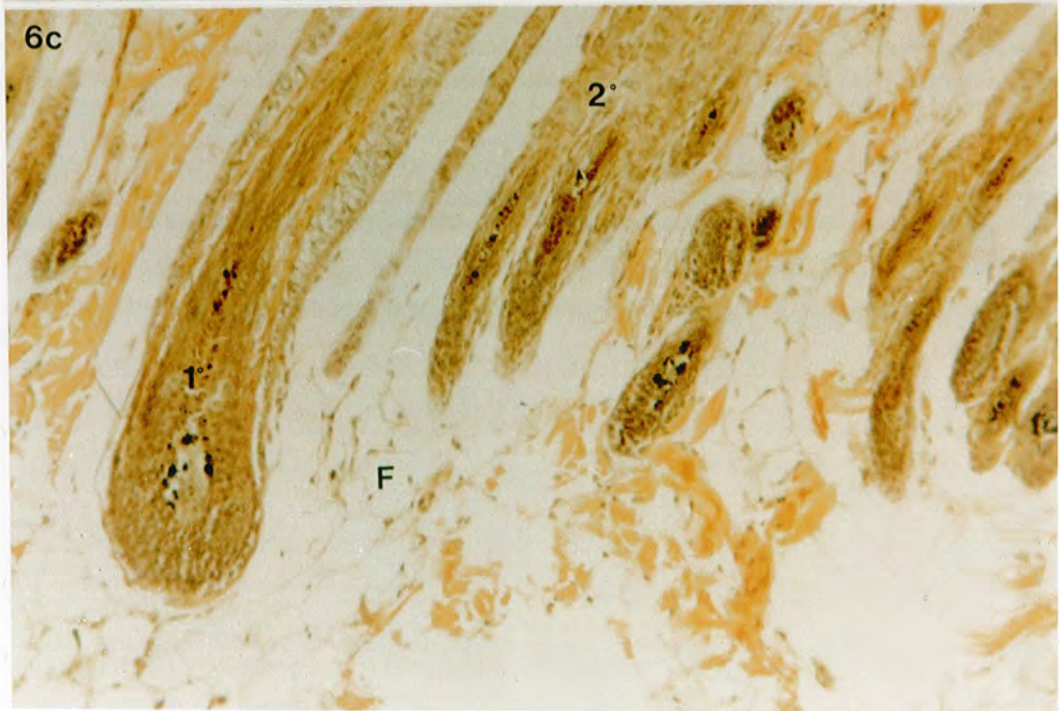
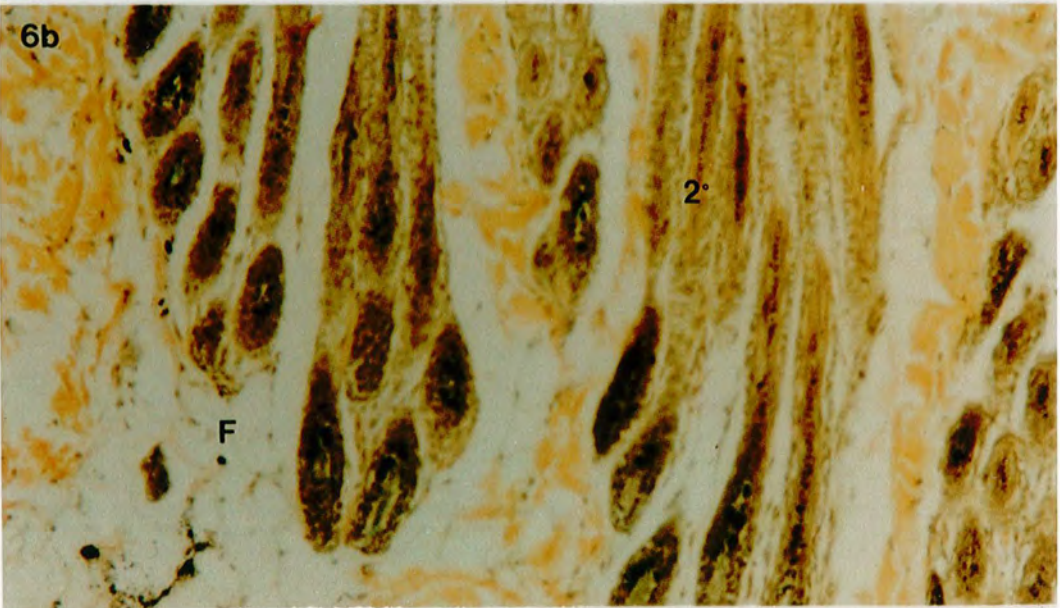
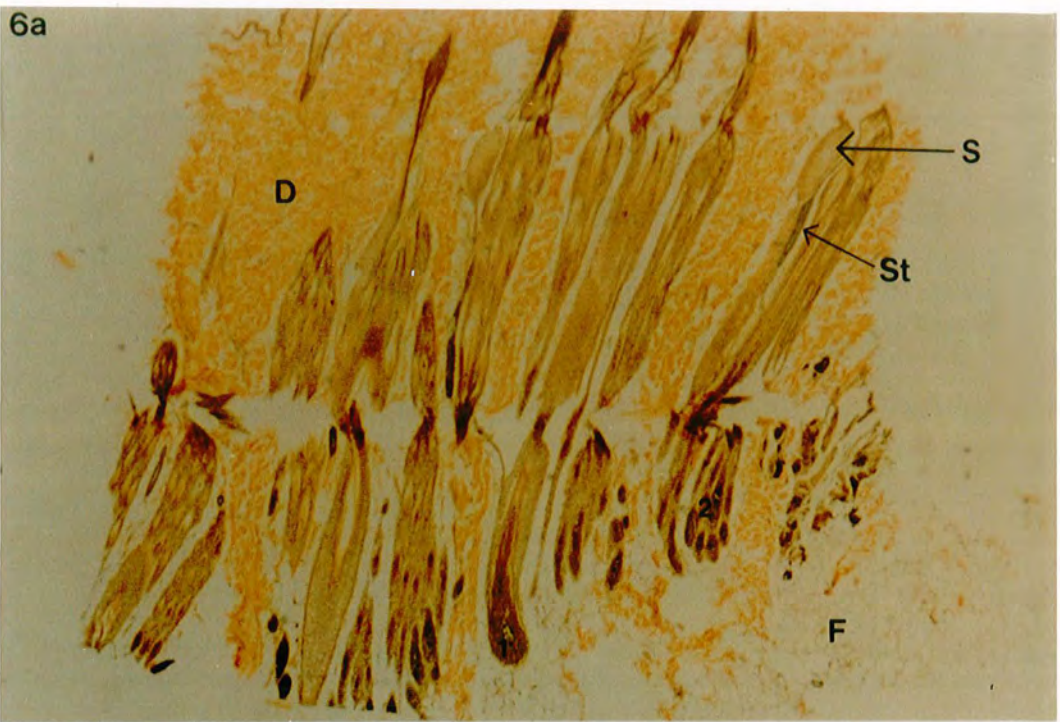
Immunofluorescent labelling of mink skin pelage with the antibody for smooth muscle  $\alpha$ -actin. Labelling is restricted to the dermal sheath of both primary ( $1^{\circ}$ ) and secondary follicles ( $2^{\circ}$ ), with expression extending around the base of the follicle. Magn x(112).

### **Figure 7b**

Immunofluorescent labelling of mink skin pelage with the anti-smooth muscle  $\alpha$ -actin. In some sections the labelling pattern is present in bands throughout the dermal sheath. (Magn. x112).

### **Figure 7c**

Immunofluorescent labelling of mink skin pelage with an antibody that recognises all isoforms of actin. Labelling was non specific, with all tissue layers staining positive for the antibody. (Magn. x112).

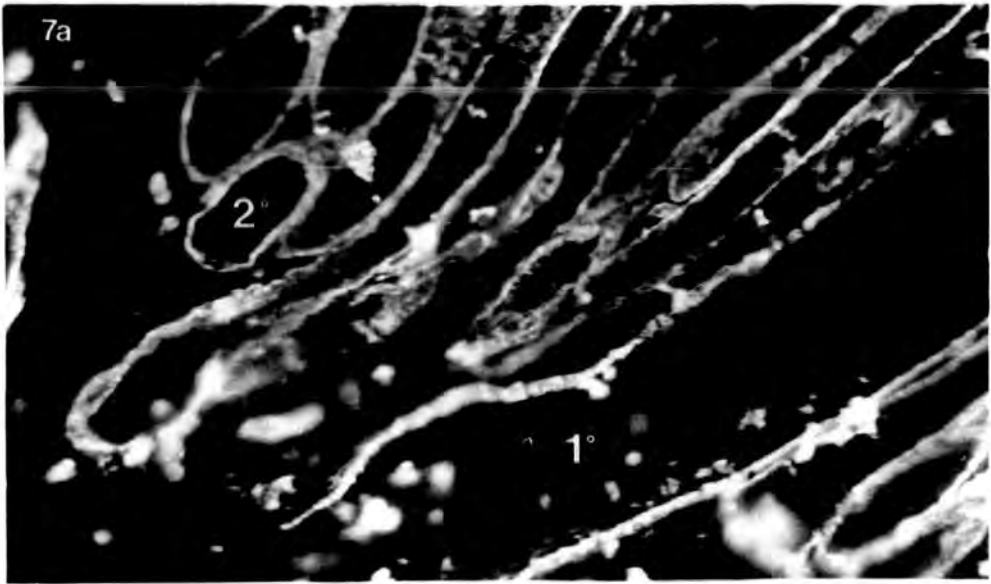
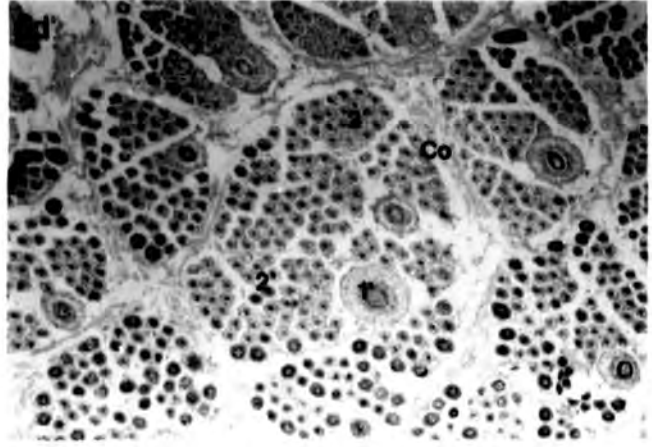


### **Figure 6a**

Low power light micrograph of a wax section through the pelage of the mink. Two follicle types are apparent, large primary or guard follicles (**1<sup>o</sup>**), adjacent to which are bundles of secondary follicles (**2<sup>o</sup>**). The dermis (**D**) is thick with an abundance of sebaceous glands (**S**) and sweat glands (**St**). Below the dermis is the fat layer (**F**) with large lipid packed cells. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x30).

### **Figure 6b,c**

High power light micrograph of a wax section through the pelage of the mink. Secondary follicles (**2<sup>o</sup>**) are flush with the edge of the fat layer. Most follicles are in the anagen stage of the hair cycle. (**F**) fat layer. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x 85).



### **Figure 10d**

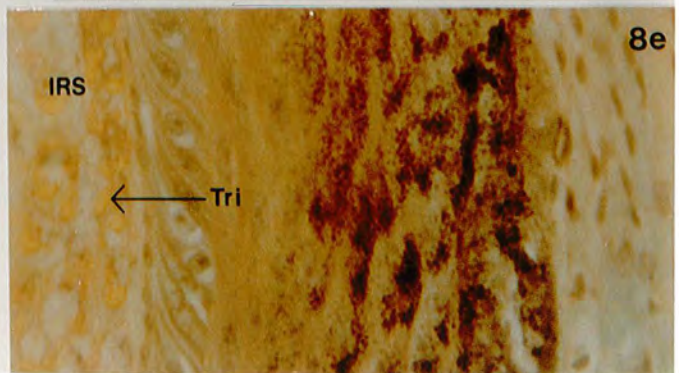
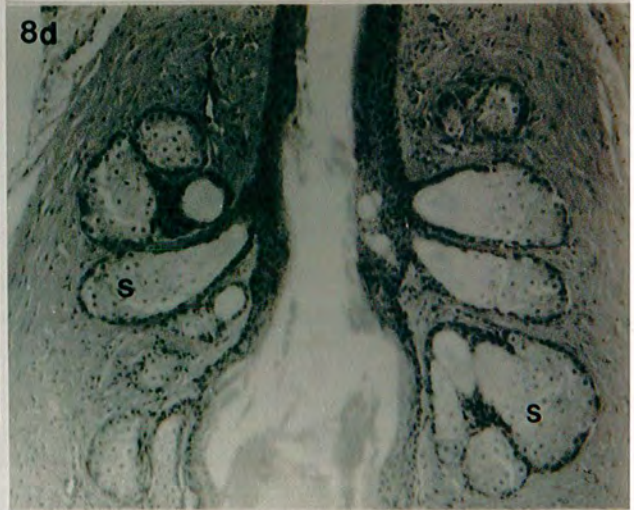
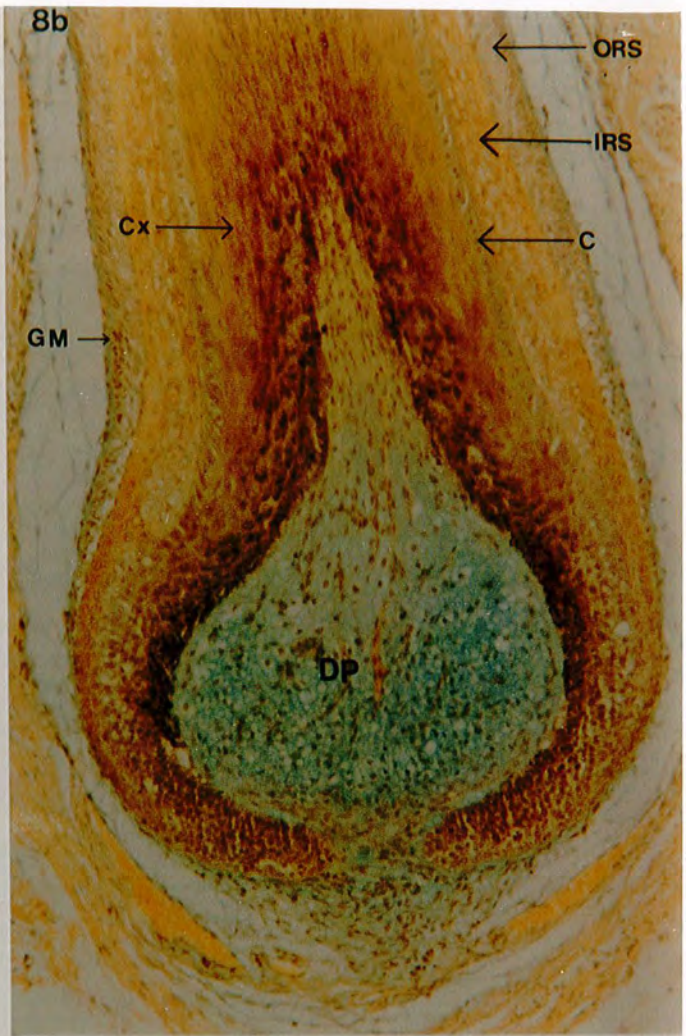
Low power micrograph of a polecat anagen sinus follicle labelled with a polyclonal antibody that recognises all actin isoforms. Follicular morphology is apparent, with stronger labelling in the outer root sheath. (Magn. x96).

### **Figure 10e**

High power micrograph of a polecat anagen sinus follicle labelled with anti-total actin. Micrograph is of the upper epidermal region of the follicle. Outer root sheath (ORS) labelling stands out. (Magn. x153).

### **Figure 11**

Low power light micrograph of a wax section through the pelage of the polecat. Two follicle type are apparent, large primary or guard follicles (1°) adjacent to which are bundles of secondary follicles (2°). The dermis is thick with an abundance of sebaceous glands (S). Below the dermis is the fat layer (F) with large lipid packed cells. (D) dermis. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x44).



### **Figure 8a**

Low power light micrograph of the polecat anagen sinus follicle. The dermal papilla is large, being enclosed in the differentiated layers of the epidermis. The whole follicle is confined in a dense collagen capsule. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x35).

### **Figure 8b**

High power light micrograph of the polecat anagen sinus follicle. The layering of the epidermis is apparent. The dermal papilla is greatly enlarged, with alcian blue staining indicating a high production of glycosaminoglycans. (**DP**) dermal papilla, (**irs**) inner root sheath, (**C**) cuticle, (**ors**) outer root sheath, (**GM**) glassy membrane and (**Cx**) cortex. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x115).

### **Figure 8c**

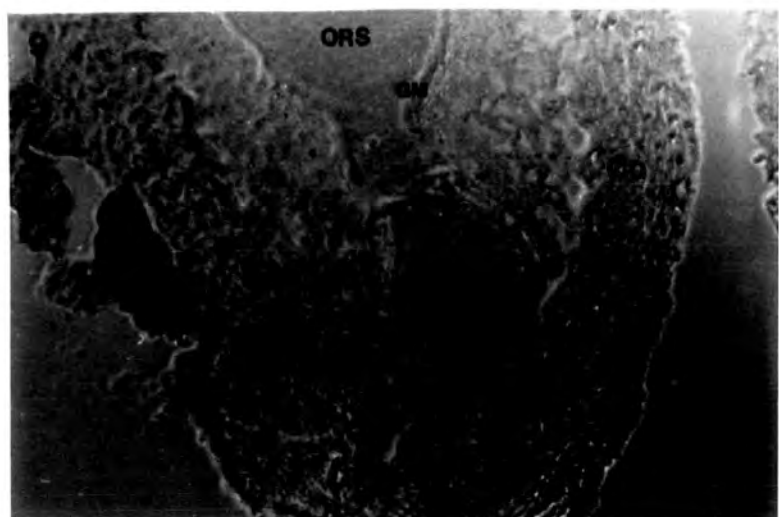
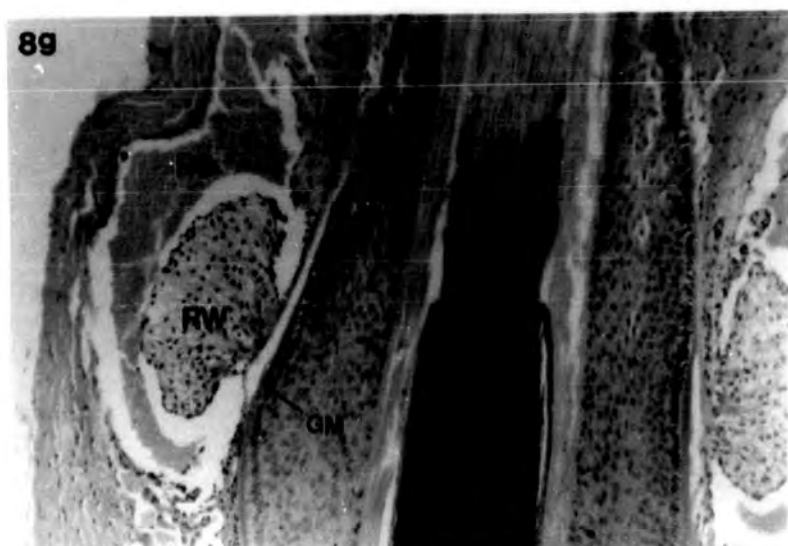
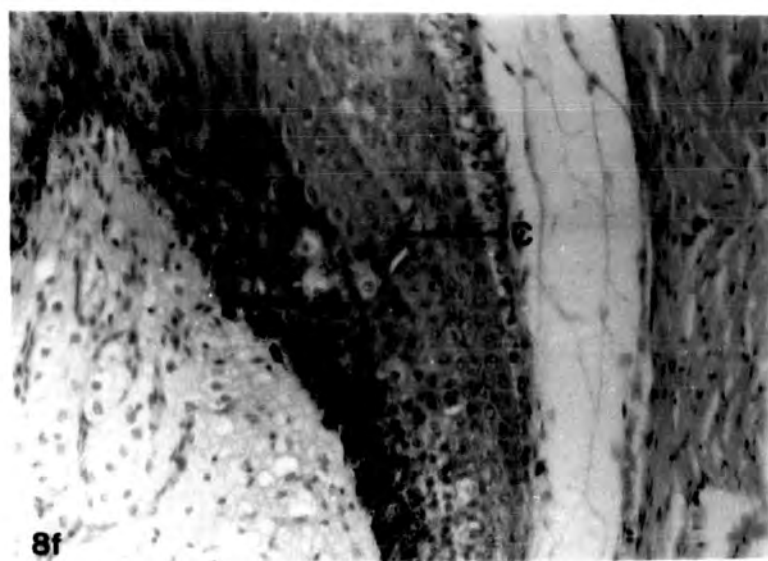
High power light micrograph of sebaceous glands of the polecat sinus follicle. Two pairs of sebaceous glands (**S**) were present, ducting into the follicle. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x87).

### **Figure 8d**

High power light micrograph of sebaceous glands of the polecat sinus follicle. Two pairs of multi-lobed sebaceous glands (**S**) are present in the upper isthmus of the follicle. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x87).

### **Figure 8e**

High power micrograph of the layering of the polecat anagen sinus follicle epidermis. Trichohyalin granules (**Tri**) are present within the inner root sheath (**IRS**). Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x400).



### **Figure 8f**

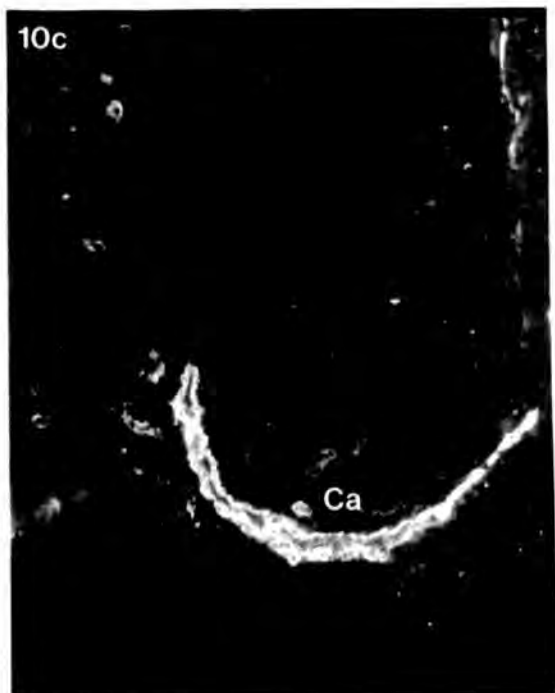
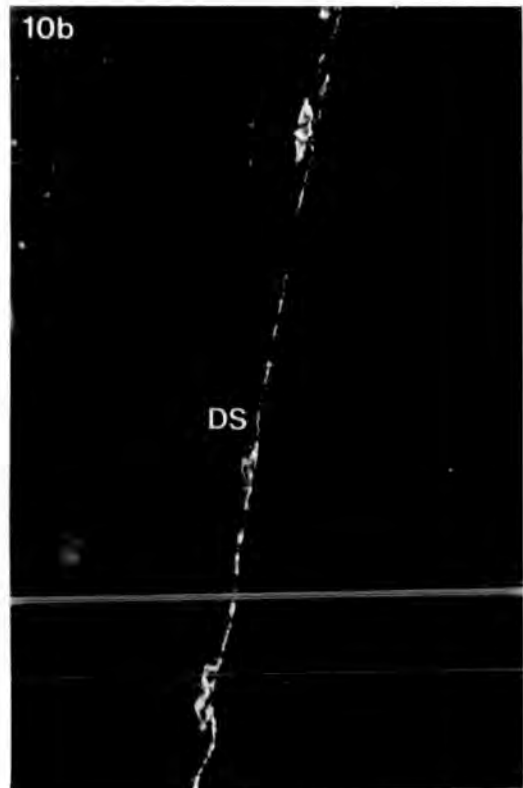
High power micrograph of the edge of the polecat sinus follicle end bulb. Melanin production is apparent by a dark band within the epidermis, adjacent to the dermal papilla. Melanin production is occurring within the top half of the end bulb. (C) cuticle. Section was stained with Alcian blue, Weigert's Haematoxylin and Curtis Ponceau. (Magn. x162).

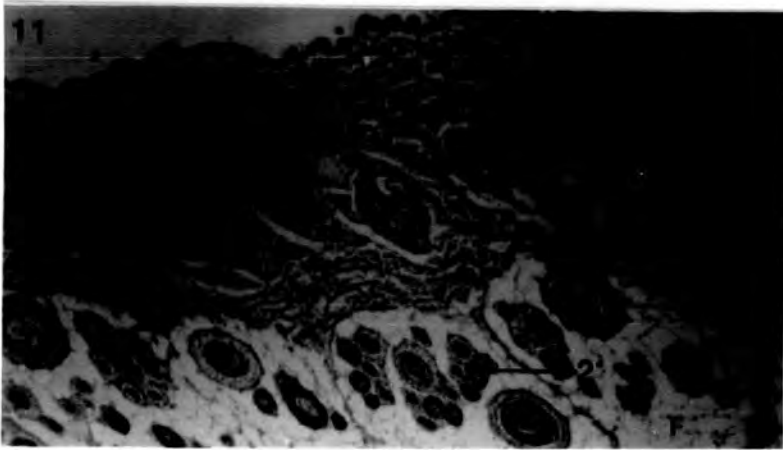
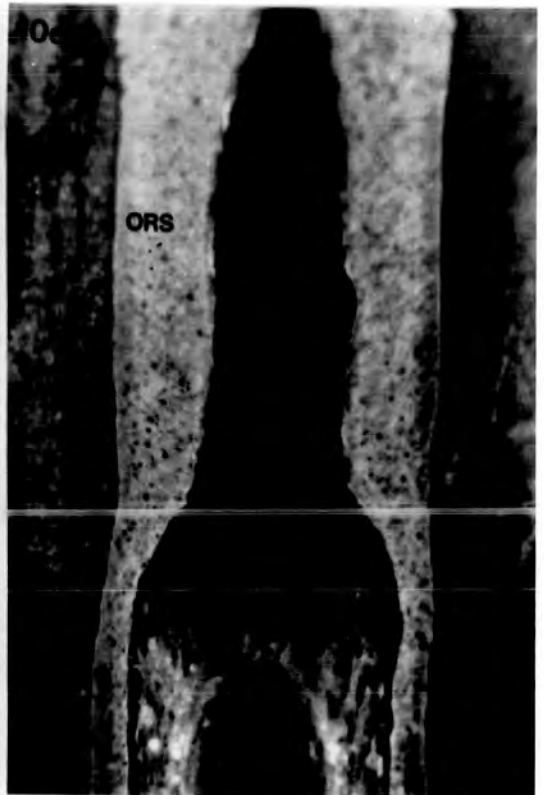
### **Figure 8g**

Micrograph of the mid region of the polecat sinus follicle. Ringwulst (rw) are apparent on both sides of the fibre. Ringwulst are enclosed in the blood filled ring sinus (rs). (GM) glassy membrane. Section was stained with Alcian blue, Weigert's Haematoxylin and Curtis Ponceau. (Magn. x93).

### **Figure 9**

Low power micrograph of a polecat telogen sinus follicle. Follicle embedded in a dense collagen capsule (Co). Glassy membrane (GM) is enlarged over much of its length. Dermal papilla (dp) is small and reduced. (Co) collagen capsule. Tissue was sectioned on a cryostat and viewed under phase contrast. (Magn. x85)





### **Figure 10a**

Immunofluorescent micrograph of a cryostat section of a polecat anagen sinus follicle. Tissue was labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. Label was restricted to the cells of the dermal sheath (**DS**) and capillaries (**Ca**) around the follicle. (Magn. x96)

### **Figure 10b**

High power micrograph of labelled dermal sheath with anti-smooth muscle  $\alpha$ -actin. Labelling is punctuated rather than continuous along the dermal sheath (**DS**). Capillaries adjacent to the dermal sheath are also positive. (Magn. x153)

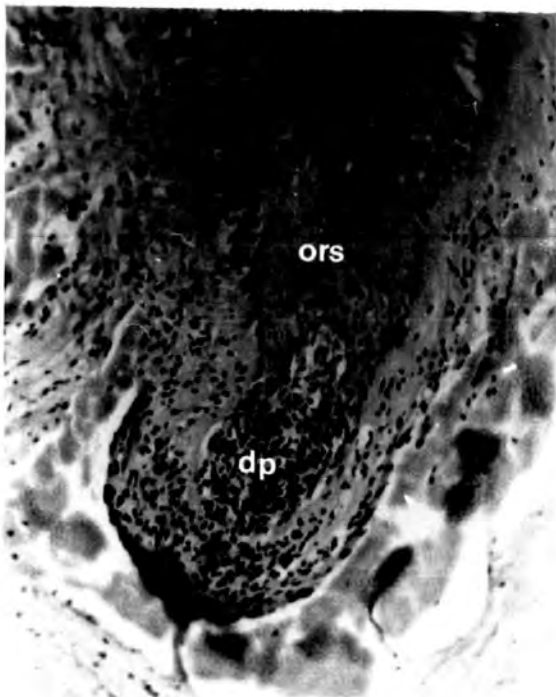
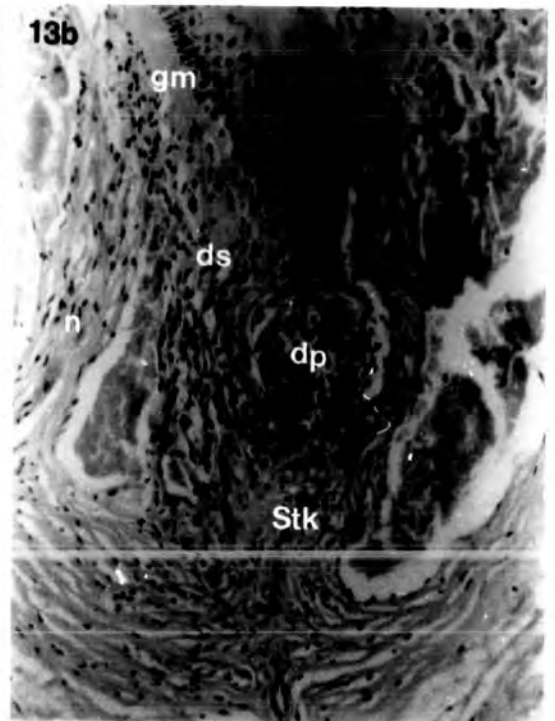
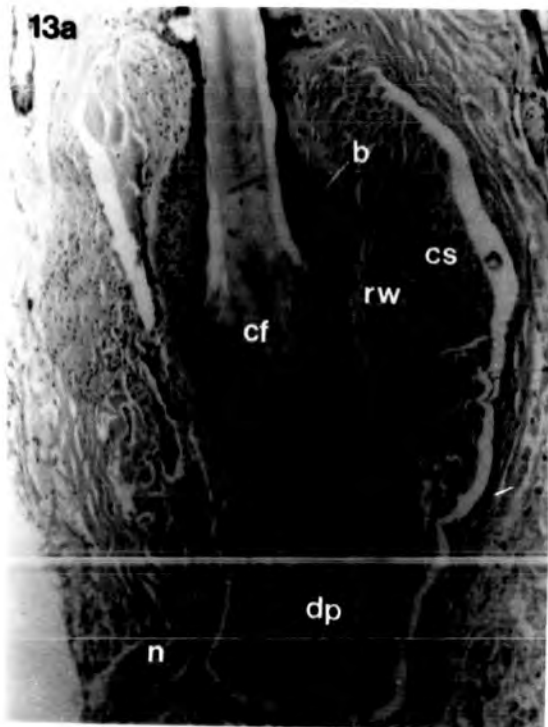
### **Figure 10c**

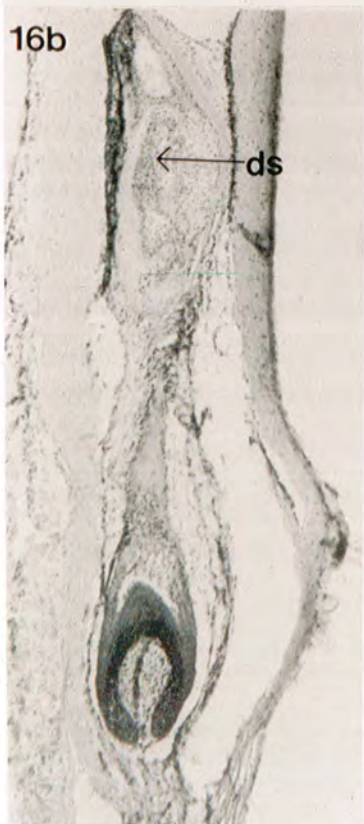
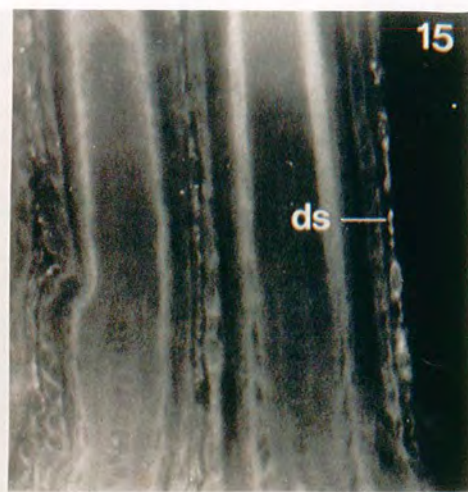
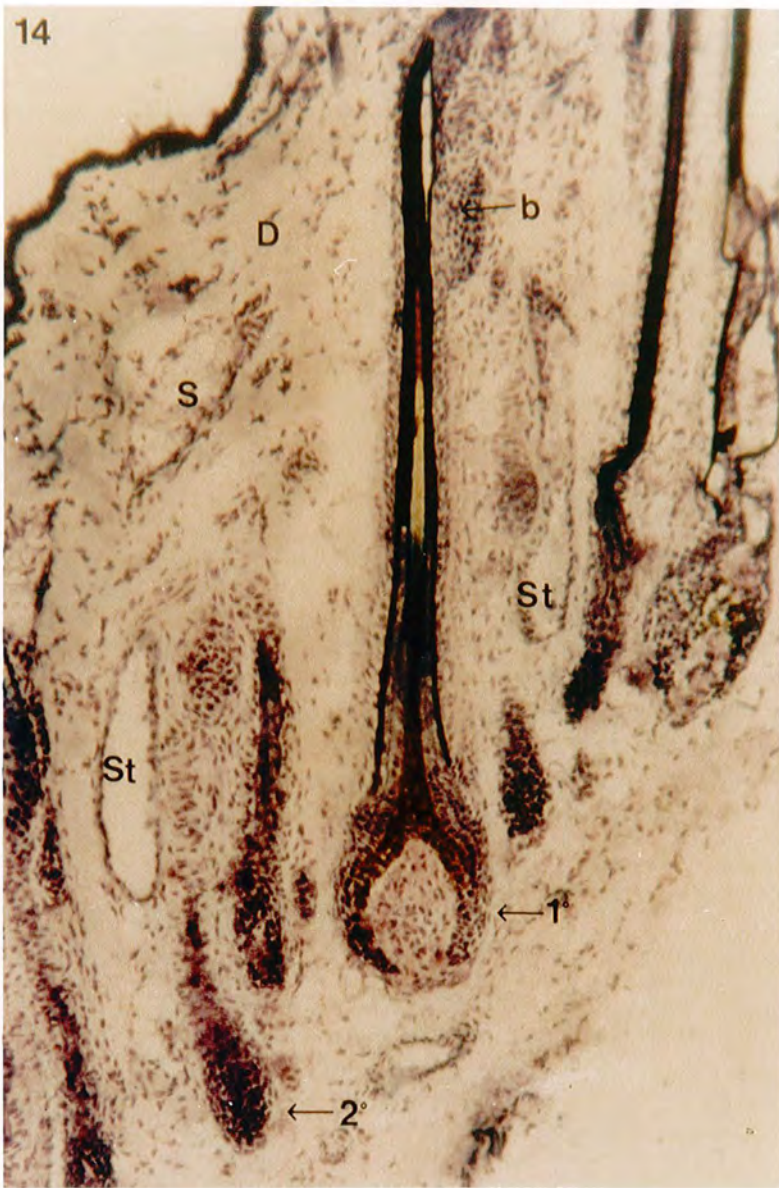
High power micrograph of end bulb of polecat anagen sinus follicle. Capillary (**Ca**) feeding the dermal papilla is strongly positive for anti-smooth muscle  $\alpha$ -actin. (Magn x153).



## Figure 12

Low power micrograph of the anagen sinus follicle of the meerkat. Dermal papilla (**dp**) is apparent, with the epidermal matrix morphology typical of an anagen follicle. Tissue above the epidermal matrix (**M**) is composed of the outer root sheath (**ors**). (**ds**) dermal sheath. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x113).





#### Figure 14

Low power light micrograph of a cryostat section through the pelage of the stoat. Two follicle type are apparent, large primary or guard follicles (1°), adjacent to which are secondary follicles (2°). The dermis (D) is thick with an abundance of sebaceous glands (S) and sweat glands (St) Bulge regions (b) are present in the upper isthmus of primary follicles. Section was stained with toluidine blue. (Magn. x170).

#### Figure 15

Immunofluorescent labelling of stoat pelage with the monoclonal antibody to smooth muscle  $\alpha$ -actin. faint labelling is visible within the dermal sheath (ds) layer of the pelage. (Magn. x350).

#### Figure 16a

High power micrograph of a grey squirrel anagen follicle end bulb. Dermal papilla (dp) is prominent, as are the layers of the epidermal matrix (M). Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x165).

#### Figure 16b

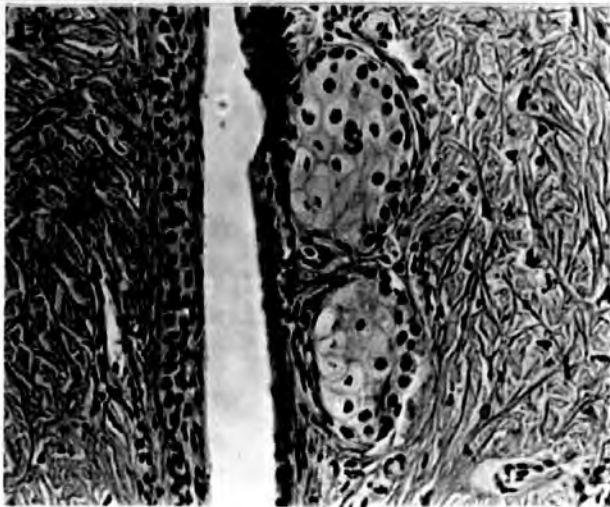
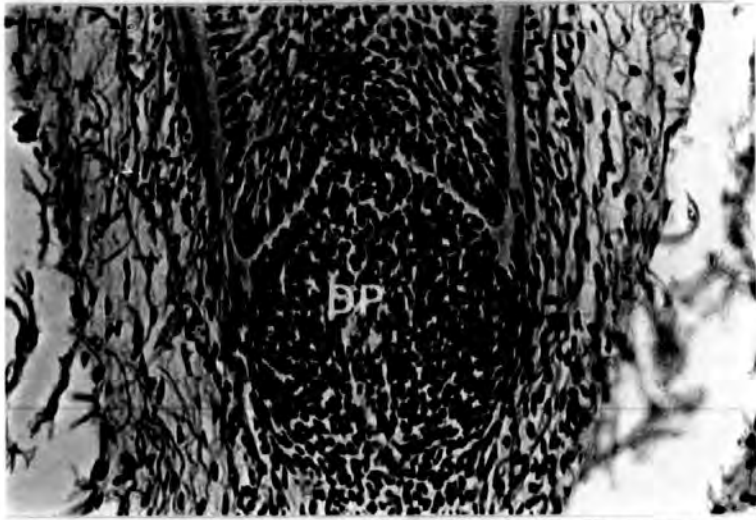
Low power micrograph of a grey squirrel anagen follicle. Dermal sheath cells (ds) in the proximal region have invaded the epidermal tissue of the follicle. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x82).

#### Figure 16c

High power micrograph of the invading dermal sheath (ds) cells in the proximal region of the grey squirrel sinus follicle. (gm) glassy membrane, (BL) basal lamina. (epi) epidermis. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x165).

#### Figure 16d

High power micrograph of an induced follicle in the proximal region of the grey squirrel sinus follicle. Dermal papilla (dp) and new epidermal matrix are apparent. (ors) outer root sheath, (gm) glassy membrane and (f) fibre. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x165).



## Discussion

### 2.3.6.1 Hair follicle anatomy

The range of literature on the hair follicle of wild mammalianis vast and often diverse, but it appears that the majority of it can be divided into three classes of interest. Those animals of the fur and wool trade, the aquatic mammals of the Pinnepedia, and the Marsupials. A primary role of hair is that of thermoregulation but in looking at the sinus and pelage anatomy, interesting characteristics become apparent that may relate to other physiological and ecological functions. The primary role of sinus follicles is a sensory one and much has been done on the innervation and tactile performance of these follicles (Vincent, 1913, Melargno and Montagna, 1953, Patrizi and Munger, 1966, Stephens et al., 1973, Dykes , 1975, Renouf, 1979, Hyvarinen, 1989, Loo and Halata, 1991, Mosconi and Rice, 1993 ). In all the species we have studied, innervation is very prominent, with a large nerve passing through the collagen capsule barrier and supplying the follicle. Innervation is greatest in the region of the ring sinus and ringwulst, primarily so that there is no loss of sensory function during early anagen, when the resting telogen fibre is still present and the new fibre is too small to emerge from the follicle. This type of innervation is a common characteristic of sinus follicles (Vincent, 1913, Melargno and Montagna, 1953, Patrizi and Munger, 1966, Hyvarinen, 1989, Marotte et al., 1992, Mosconi and Rice, 1993). . What is also apparent is that the glassy membrane is thickest in the region opposing the ringwulst. Hyvarinen (1989) in his study of Ringed seal vibrissae postulated that the glassy membrane in the ring sinus may have a comparable role to the basilar membrane of the cochlea in sound detection, although in the species I have looked at there was no evidence for sound detection, it may be that the same feature supports an increased sensitivity. Furthermore the glassy membrane was thickest in the mink, the most aquatic species studied. Alternatively to this the thinnest glassy membrane opposing the ringwulst was that of the meerkat, an arid living species (Haltenorth and Diller, 1977).

Mink, Polecat and Meerkat are all opportunistic predators of comparable size (Mink, head and body = 35-40cm (Corbert and Ovendon (1980), Polecat, head and body = 32-44cm (Corbert and Ovendon (1980) and Meerkat, head and body = 25-31cm (Haltenorth and Diller, 1977) and yet from the histological evidence the size of vibrissa gradually decreases respectively (Figs 4, 8, 13). In a paper by Korhonen et al (1983) they imply that the polecat was not as good a predator as the mink, this being reflected in diet as well as climatic range. However, what this also reflects is a greater prey diversity and hence a lesser evolutionary pressure to a more predatory role. This sinus follicle size reduction would therefore be even more amplified in the Meerkat whose prey diversity is great, eating insects; snails; scorpions; lizards; snakes; small mammals and birds; roots; fruits and tubers (Haltenorth and Diller, 1977). This reduction in a predatory lifestyle was compensated for by an increase in an opportunistic one. Therefore it would not be unusual to expect a reduction in sensory function to parallel this change. In a review by Halata (1990), it states that the amount and variety of nerve terminals within a follicle is size dependant, with the largest follicles having the greatest variety and number. Therefore sinus follicles are more innervated than vellus hairs, as should be expected. However it is not clear whether the size difference of sinus follicles between species also reflects this relationship.

What was also interesting was the preponderance of sinus follicles found to be in telogen. This contrasts with the rat or mouse, where telogen is a brief event in the hair cycle, and at any given time a high proportion of follicles are in anagen. Judging by the data we have, it appears that vibrissa follicles from the mink and meerkat had a greater tendency to remain in the telogen state. Pelage moult in wild mammals is strictly regulated by seasonal change and has been widely documented (Rothschild, 1942, Wright, 1942, Hart, 1956, Ling, 1970, Johnson, 1976, Martinet et al., 1984, Blandford, 1987, Korhonen, 1988) but what is often omitted is how vibrissae might cycle with seasonal change. Therefore the dominance of the telogen state might just reflect the season (late autumn-winter) in which we obtained the samples. In telogen

follicles of the meerkat, mink and polecat the glassy membrane was thickened over much of its length, this contrasted with the grey squirrel, rats and mice, where thickening of glassy membrane remained in specific areas. These differences might also be a reflection of their telogen states.

As with all sinus follicles, sebaceous glands were present in the upper isthmus of the shaft. The largest glands were associated with the mink and polecat. Both had structures in the proximal third of the follicle. However, in addition to this the polecat had another small pair of glands at the opening of the hair fibre. The particular role of these additional glands is unknown. However, historically the polecat was known as the foul-mart and even its latin name, *Mustela putorius*, means bad smelling musk bearer (Blandford, 1987), therefore the abundance of glandular material in the sinus and pelage, is likely to be connected to this.

In one grey squirrel anagen sinus follicle, a secondary anagen fibre was induced in the mid proximo-distal region of the follicle. As the grey squirrel is a rodent, it would seem logical that the sinus follicles cycled in a similar manner to that of other rodents. Throughout most of the anagen cycle in the rat vibrissae, a second resting follicle and fibre is present in the follicle. Therefore, it could be that the second anagen follicle present in the grey squirrel anagen sinus follicle, was the result of a re-induction of the resting fibre from the dermal sheath, or alternatively, the primary fibre may have been damaged and torn further up into the follicle, with a section of epidermal matrix and dermal papilla still connected. With these two components attached a second follicle could be induced, furthermore the originally damaged follicle would re-induce fibre production on another follicle. From the histological evidence, it is impossible to say how a second smaller follicle was produced, however what is interesting is that it shows that sinus follicles are capable of producing a secondary follicle within the collagen capsule.

Consistent with Korhonen et al., (1988) the mink histology sections showed a greater abundance of pelage follicles than the polecat but what was also interesting was that the mink dermis was far greater in thickness than the polecat, and therefore

follicles were longer and deeper. This reflects the more northerly range of *M. vison* (Korhonen et al., 1983). However unlike Rust et al (1965) ,who stated that the pelage consisted of 9 bundles to a guard follicle, with 24 secondary follicles in each bundle, we found that a single primary mink follicle could be linked to 3-4 bundles of secondary follicles with each bundle containing between 15-24 follicles. Dolnick (1959) states that there are 3 secondary bundles, with each bundle containing 6-10 follicles. However, the mink used in her study were only 3 months old and therefore more follicles may develop in the adult pelt or in the winter pelage. What was interesting was that Dolnick (1959) found no evidence that the secondary follicular bundles formed from the sides of established follicles. It was stated that individual follicles developed from a single hair bud. Hair fibres protruded from a single epidermal orifice that formed via degeneration of their individual skin openings. This development is contrary to common theory of pelage development (Hardy and Lyne, 1956).

In both the mink and polecat at least some of the pelage follicles penetrated the dermis, with their end bulbs protruding into the fat layer. In contrast with this, the pelage of the stoat was enclosed within the dermis, and the amount of follicular material was much reduced. In the stoat sections the guard follicles were not as closely associated with small bundles of secondary follicles, but as the exact position of the specimens were unknown, it was unclear whether this was consistent over the whole integument. The stoat has a similar geographic distribution to that of the mink (Corbert and Ovendon, 1980) so it was interesting that follicular density and arrangement was so contrasting. It is not clear from these sections why this should be the case.

### **2.3.6.2 Smooth muscle alpha actin labelling**

Smooth muscle  $\alpha$ -actin was recently shown to be highly expressed in the hair follicle dermal sheath (Skalli et al., 1986, Jahoda et al. 1991, Urabe et al. 1992, Reynolds et al., 1993) and it was noted that the expression changed within the hair cycle. Smooth muscle cells were known to be involved in slow contractile tissues, nevertheless

although smooth muscle actin expression correlates with different stages in the cycle no functional hypothesis has been put forward. The hair follicle is one of the most proliferative regions in the body Malkinson and Keane, (1978), therefore there must be a great deal of control to prevent random extension of the fibre. If the dermal sheath were to function like smooth muscle cells in capillaries, one would have a means of controlling the upward development of the projecting fibre. This can therefore be considered in detail with respect to the above evidence. In the anagen follicle of both pelage and vibrissae there was a high expression of smooth muscle  $\alpha$ -actin in the bottom third of the dermal sheath, generally in the region of follicular constriction above the end bulb. The smooth muscle  $\alpha$ -actin positive dermal sheath may be 'encaging' the epidermal layers providing a physical pressure to ensure a strong straight fibre. Expression would not have to occur further up as the basic fibre is already keratinized and fixed in its position. If the follicle were not directed in this fashion then it is possible that proliferation and fibre production could occur in any direction and invade non hair bearing dermis. The expression in the pelage is different, with the DS expressing smooth muscle  $\alpha$ -actin around the base of the end bulb. If the above hypothesis is correct why doesn't the pelage have similar expression? The vibrissa is enclosed within a dense collagen capsule that would prevent further down growth, however pelage follicles do not have this dense collagen layer and therefore the smooth muscle  $\alpha$ -actin might be required to provide that support. In telogen the smooth muscle  $\alpha$ -actin expression was greatly reduced in all follicles. As proliferation has stopped there should be little or no requirement to produce smooth muscle  $\alpha$ -actin but on resumption of anagen one would expect greater expression to allow down growth and fibre production.

Smooth muscle  $\alpha$ -actin expression in the telogen mink vibrissae follicle was around the constriction in the outer root sheath above the papilla. This may provide an insight into locating the stem cells. If the germinative stem cell population moves upwards with the overlying epidermis, the smooth muscle  $\alpha$ -actin may act in constricting the upward flow of the ORS cells and force the specialised GE cells to

remain close to the dermal papilla. The GE cells would then be ideally placed for the start of the next anagen cycle.

Although the function of smooth muscle  $\alpha$ -actin within a follicle can only be hypothesized upon, it is obvious from the data that as the cycle of the hair follicle changes, so to does the abundance of smooth muscle  $\alpha$ -actin. Therefore, there is a definite relationship between follicle activity and the presence or absence of smooth muscle  $\alpha$ -actin that suggests some functional role.

**Chapter 3**  
**Cell Culture**  
**Introduction**

### **3.1 Fibroblasts**

In the Dictionary of biology, Abercrombie et al (1951) described the fibroblast as a " cell of irregular, branching shape found distributed throughout vertebrate connective tissue". The primary function of fibroblasts was believed to be to maintain the extracellular matrix, and in particular to produce collagen (Hay, 1981, 1991). Fibroblast-like precursor cells are part of the differentiation process of many cell types, for example; chondrocytes; skeletal muscle cells; adipocytes; smooth muscle cells; and myofibroblasts (Yaffe, 1969, Green, 1979, Archer et al., 1982, Sappino et al., 1990, Stockdale, 1992). In this context, the word fibroblast is more a description of cell morphology than a specific cell type, particularly in culture.

Although chondrocytes, skeletal muscle cells, adipocytes, smooth muscle cells and skin fibroblasts have different functions *in vivo*, their function and morphology can be altered by a number of external factors. Coon (1966) was able to clone chick cartilage cells *in vitro* , however later research (Von der Mark et al., 1977, Archer et al., 1982) showed that cytodifferentiation of cartilage cells (chondrocytes) was a density dependent phenomenon. Therefore cartilage cells could be de-differentiated, into fibroblast-like cells, and differentiated back into cartilage cells purely by altering cell density. Other studies have also shown that cartilage cell phenotypes could be altered with extracellular matrix molecules. Early work by West et al., (1979) inhibited *in vitro* cartilage differentiation using fibronectin, whereas Mackie et al., (1987) enhanced cartilage differentiation with tenascin. Therefore, cell fate could be altered by cell to cell and cell to extracellular matrix interactions.

Choi et al., (1990) identified that MyoD, a helix-loop-helix DNA binding protein, could generate myogenic cells from dermal fibroblasts, chondroblasts and

smooth muscle cells independently of surrounding cell adhesion molecules, collagens and proteoglycans. Therefore, although these results present a contradiction, they also indicate the close developmental links between these fibroblastic cell types.

Even though early studies by Parker (1932) identified nine races of fibroblast, skin fibroblasts were generally thought of as a homogenous cell type within the dermis. Harper and Grove (1979) were later able to show that skin fibroblasts from different depths of the dermis had differences in proliferative function. Furthermore, Konterman and Bayreuther (1979) indicated that as rat fibroblasts aged *in vitro*, they could be identified as differentiating along particular cell lineages. From these previous studies, it becomes apparent that there are more differences amongst fibroblast-like populations. Bayreuther et al., (1988) provided morphological and biochemical evidence that human fibroblasts differentiate along a stem cell like lineage, and this group has tried to identify differentiation markers to this fibroblast stem cell population (Francz et al., 1989).

### **3.2 Follicular dermal fibroblasts**

By utilizing the microdissection system first employed by Cohen (1961), Jahoda and Oliver (1981) were able to isolate and culture a specialized fibroblast population of cells from the rat vibrissa papilla. Using the same techniques, Messenger (1984) isolated the human hair follicle equivalent. An interesting property of these human DP cell lines is that they possess androgen receptors, with the greatest number of receptors in the cells derived from the most androgen sensitive areas, eg beard and pubic hair. (Randall et al., 1991, 1994). The difference in receptors between the different DP cell lines implicate this as a good model for the study of androgen action on the dermal papilla *in vivo*.

Dermal papilla cells have also been cultured from a number of different species such as sheep ( Withers et al., 1986), rabbit and mouse (Reynolds, 1989). The rat vibrissa cells, although morphologically similar to their pelage follicle counterparts,

have very distinct behavioural properties *in vivo* and *in vitro*. Jahoda and Oliver (1984) showed that these cells were less proliferative than skin fibroblasts in culture and that they adopted a clumped phenotype *in vitro*.

Since then other dermal follicle components have been isolated. Horne (1987) describes the isolation and culturing of the connective sheath cells surrounding the follicle, known as dermal sheath (DS). Although these cells are connected to the DP, and resemble DP cells in culture, their aggregative behaviour is not as pronounced (Horne, 1987). Furthermore, their *in vivo* inductive properties are also subtly different. Freshly isolated dermal sheath could induce fibre production in amputated follicles only after the production of a new papilla (Horne and Jahoda, 1992), and in contrast with dermal papilla cells, cultured dermal sheath cells lose their inductive capabilities in the same bioassay (Horne, 1986).

Wounding of the skin is a regular occurrence, and it is here that a further skin fibroblast cell type, the myofibroblast, is identified (Gabbiani et al., 1971). This cell type was first identified in the granulation tissue of the wound site and found to be morphologically a cross between a smooth muscle cell and a skin fibroblast.

Therefore the skin dermis, rather than being a homogeneous tissue full of identical fibroblasts and collagen, is a complex tissue with a wide variety of subtly different mesenchymally derived cells.

In this chapter, morphological studies were carried out on a variety of cells derived from the skin of different animal species, and morphological changes were noted throughout the cells in early explant culture *in vitro*. In general DP, DS and Fi cells, derived from skin samples of mink, polecat, stoat and grey squirrel, were compared.

Differentiation markers to a number of cell types within the dermis have been isolated, (Lasek et al., 1979, Debus et al., 1983, Skalli et al., 1987), however these are few compared to the proposed complexity of system (Bayreuther et al., 1988).

Dermal papilla cells are also currently under investigation, and markers for them *in vitro* are being considered. Tobin et al. (1991) proposed that intranuclear

rodlets in human DP cell lines could be used as a marker, however these structures have not been identified in other animal species. Myofibroblasts have been shown to express the actin isoform smooth muscle  $\alpha$ -actin during wound healing (Darby et al., 1989), and Skalli et al., (1986) also noted its presence in the hair follicle. Jahoda et al., (1991) continued this research, and discovered that while smooth muscle  $\alpha$ -actin was restricted to the dermal sheath cells of the follicle *in vivo*, DP and DS cells both stained positively with smooth muscle  $\alpha$ -actin *in vitro*. Furthermore, when skin fibroblasts were found not to possess this actin isoform, it was proposed that this molecule could act as a marker for hair follicle derived dermal cell types *in vitro*. In an expansion of this concept grey squirrel DP and DS cells were also tested for smooth muscle  $\alpha$ -actin labelling.

The rat dermal papilla cell aggregation phenomenon (Jahoda and Oliver, 1984) is further characterised in this chapter with a morphological study. Cell proliferation was also monitored with respect to formation of the aggregates. Furthermore, within certain DP cultures large tissue type specific structures were identified, and their ultrastructure investigated. During the course of the project, a population of DS cells also altered, spontaneously displaying greater proliferation and changes in morphology.

## **Materials and methods**

### **3.3.1.1 Tissue Isolation and Cell culture**

Inbred PVG rats (Durham University) of either sex , and various ages, were used to set up initial primary cultures of dermal papilla, dermal sheath and skin fibroblasts.

### **3.3.1.2 Rat Dermal papilla cell culture**

Dermal papillae (DP) were dissected from the rat vibrissa follicles using the method described by Jahoda and Oliver (1981). Briefly, the mystacial pad was cut open, the skin inverted, and the end bulb region, of isolated sinus follicles removed. Fine forceps were then used to invert the collagen capsule of the end bulb and expose the papilla and epidermal matrix. The matrix component was then removed, and any epidermal tissue still present on the papilla was teased off. The papilla could be then removed using fine forceps and transferred to a culture vessel. Dissected tissue was cultured initially in 20% foetal bovine serum (Seralab) and Eagles minimal essential medium (E-MEM) with Glutamax-I, Earles salts and 25mM Hepes (Gibco). This medium was supplemented with antibiotics: kanamycin (50µg/ml), fungizone (1.25µg/ml), and penicillin/streptomycin (50µg/ml), (Gibco). Cell cultures were initiated in 35mm dishes (Falcon) and were continued in these vessels after the first passage. On the second passage the cells were transferred to 25cm<sup>2</sup> flasks (Falcon) and on subsequent passages 75cm<sup>2</sup> flasks. After the first passage the concentration of foetal bovine serum in the medium was reduced to 10% foetal bovine serum.

### **3.3.1.3 Rat Dermal sheath cell culture**

Dermal sheath (DS) tissue was isolated from vibrissae follicles as described by Reynolds (1989). During the extraction of DP cells, when the end bulb is dissected,

and the whole structure inverted using fine forceps, the papilla is now external to the end bulb and can be removed, as described in section (3.3.1.2). The DS remains attached to the inner face of the collagen capsule after removal of the DP, and could then be teased from it, and cultured as an explant, as described for DP cells (3.3.1.2). Extracted dermal sheath tissue was initially cultured in 20% FBS/ E-MEM with the antibiotics fungizone (1.25µg/ml), penicillin/streptomycin (50µg/ml) and kanamycin (50µg/ml) in 35mm culture vessels. On subsequent passages the cells were transferred to 25cm<sup>2</sup> and later 75cm<sup>2</sup> flasks with medium containing 10% FBS/Eagles-MEM and the above concentrations of antibiotics.

#### **3.3.1.4 Rat Skin fibroblast cell culture**

Skin from the flank of the animal was utilised as a source of skin fibroblasts. External hair was removed from the donor area of tissue by shaving, and fat layers were scraped so that the dermal side of the skin was in complete contact with the base of a 35mm culture dish (Falcon). Skin explants (size, 5x5mm) were dissected and allowed to adhere to the culture dishes by partially drying the tissue. The skin sample was then covered with 20% FBS + E-MEM and cultured as for DP cells (section 3.3.1.2). Epidermal cells and fibroblasts would both grow out from the explant, however fibroblasts would quickly outgrow the epidermal cells and cover the majority of the culture vessel. Epidermal cells were lost after the first passage. In subsequent passages the cells were maintained in 10% FBS/E-MEM.

#### **3.3.1.5 Cell culture from mink, polecat and grey squirrel**

DP, DS and Fi were isolated from the tissues in the methods described in sections (3.3.1.2-.4). Primary cultures were initiated in 35mm culture vessels. If cell density was enough, cells would be passaged and cultured as summarized for rat DP cells (section 3.3.1.2).

### **3.3.2.1 Transformed DS cell material**

Cultures of DS cells with spontaneously altered morphology and enhanced proliferation were named transformed DS cells (tDS). Transformed DS cells were cultured as follows. Cells were maintained in Eagles-MEM supplemented with 10% FCS and antibiotics, fungizone, kanamycin and penicillin/streptomycin, with the amino acid glutamine.

### **3.3.2.2 Growth Curve and cell sizing of tDS cells**

For growth studies the cells used were at passage 8. Initial plating density was  $5 \times 10^4$  cells/ml in 35mm culture dishes, and culture medium was changed every day. Cell counts were carried out on days 1, 2, 3, 4, 5 and 7 using an haemocytometer. Three replicate cultures were counted on each day, and the mean was calculated from ten counts from each 35mm culture dish. The mean cell size was calculated using an electronic counter (Coulter counter), as described in the manual provided. The percentage of cumulative weight was plotted against the range of diameter settings on the coulter counter, and a normal distribution was obtained with the peak being the mean size of the cells.

### **3.3.3.1 DP aggregate formation *in vitro*.**

DP cells were plated into a 25cm<sup>2</sup> flask. On the base of the culture vessel a random square was marked. The cells were monitored and fed at regular intervals. Data was recorded with photographs and annotated diagrams from inside the marked square.

Cells were monitored from subconfluency to the clumped state first described by Jahoda and Oliver (1984).

### **3.3.3.2 Growth curve and Brdu labelling of DP cells *in vitro*.**

Dermal papilla (DP) cells were plated out on 35mm culture dishes at an initial density of  $2 \times 10^4$  cells. Cell counts were taken at intervals using haemocytometer. Ten counts were taken from each culture dish, with three culture dishes used on the each count day.

Between confluency and aggregating phase, DP cell cultures were fixed for 5-Bromo-2'-deoxy-uridine labelling (Brdu) using a detection kit (Boehringer Mannheim Biochemica). Cells were labelled for 60 minutes in 10mmol/l Brdu in 10% FBS. Cultures were then fixed in 70% ethanol in 50mmol/l glycine buffer for a minimum of 20 minutes at  $-20^{\circ}\text{C}$ . After several washes in phosphate buffered saline the cells were then incubated at  $37^{\circ}\text{C}$  for 30 minutes with anti-Brdu working solution (1 in 10 dilution with incubation buffer). The second antibody, anti mouse Ig fluorescein, was added and incubated as described in section (2.2.4) after several washes, again in PBS. The Brdu fluorescent labelling was conserved by mounting the cells in citifluor (Agaraid). Labelling was viewed under epi-illumination using a fluorescent microscope (Carl Zeiss Axiovert). The number of labelled cells were counted in five fields at x50. Three replicates were counted for each day that labelling was carried out. Labelling was also performed on three DP cell phenotypes; confluence (Confl), aggregating (AG) and clumped (AD) using the behavioural state of the cells as the criteria for staging, rather than a time course.

### 3.3.3.3 Cell population doubling times

Mean population doubling time (PDT) of a cells in culture was calculated as described by Kovac & Fleishmajer (1974). The equation used is described below.

$$\text{PDT} = \frac{\text{hours of growth}}{\text{number of divisions}}$$

where the number of divisions =  $(\log_{10}N_1 - \log_{10}N_0) / \log_{10}2$

$N_0$  = Initial number of cells

$N_1$  = Cell number at a subsequent time

### 3.3.4.1 Transmission electron microscopy of *in vitro* DP cells

Transmission electron microscopy procedure was as described in the following sections.

### 3.3.4.2 Fixation and resin embedding

Cells were fixed, as described by Karnovsky (1965), in 2% paraformaldehyde, 2.4% glutaraldehyde and 0.1M sodium cacodylate for 1.5hrs at 4°C. Cells were then post fixed in a 1% osmium tetroxide solution buffered with 0.2M sodium cacodylate for 30 mins at room temperature. The tissue was then dehydrated through an increasing series of ethanol to absolute. Cells were then infiltrated with resin for 30 mins with 1:1 100% alcohol/araldite followed by pure araldite for a further 30 mins. Araldite was

composed of 10 mls araldite CY212, 10mls D.D.S.A, 1ml dibutyl phthalate and 0.5ml D.M.P. Araldite was replaced for a third time and allowed to harden at 60°C. Areas of interest were isolated from the main block and re-embedded in araldite in smaller embedding wells, thus allowing easier sectioning. Semi thin sections of approximately 1µm were cut on a Reichert OMU3 microtome and stained with toluidine blue for light microscopy. Ultra thin sections were floated onto grids, stained with uranyl acetate, washed in dH<sub>2</sub>O and stained again with lead citrate (Reynold, 1963). Stained ultra thin sections were viewed under a Philips 400T transmission electron microscope.

#### **3.3.4.3 Immunofluorescent labelling of tDS cells and Grey Squirrel DP and DS cells with monoclonal antibody to smooth muscle alpha actin**

Transformed dermal sheath (tDS) cells were grown on glass coverslips in 35mm culture dishes. Once the desired stage of confluency was reached, the cells were washed in several changes of PBS and fixed in absolute alcohol for 20 minutes at -20°C. Cells were then given three 5 minute washes in PBS. A 1 in 10 dilution of smooth muscle  $\alpha$ -actin mouse monoclonal antibody (Gabbiani, Geneva) with PBS was then applied to the cells for 30 mins at room temperature. After three 5 min washes in PBS, a 1 in 9 dilution of anti-mouse rhodamine (IgG) in PBS was used as the second antibody. The cells were then incubated in the dark for 30 mins at room temperature. After a final triplicate washing in PBS, the cell covered coverslips were mounted onto a glass slide with citifluor (Agaraid), and viewed with an axiovert microscope (Carl Zeiss) with epi-illumination. On some glass coverslips, either the 1st or 2nd antibody was removed, and replaced with PBS as controls.

Grey squirrel DP and DS cells were also grown on coverslips and labelled with the anti-smooth muscle  $\alpha$ -actin (Skalli et al, 1986) as described for tDS cells in this section.

#### **3.3.4.4 Immunofluorescent labelling of tDS cells with a polyclonal antibody to Transforming Growth Factor $\beta$**

Transformed DS cells were also screened for the presence of transforming growth factor  $\beta$  using a panspecific TGF- $\beta$  antibody raised from recombinant human TGF- $\beta$ 1, porcine TGF- $\beta$ 1.2, pTGF- $\beta$ 2 and recombinant amphibian TGF- $\beta$ 5 (British Biotechnology Ltd). The method used was described in section (3.3.4.3). 1mg/ml of anti TGF- $\beta$  was used in a 1:200 dilution with phosphate buffered serum, this was visualized using anti rabbit fluorescein linked IgG (1:40 dilution with PBS) and citifluor under epi-illumination using an axiovert microscope (Carl Zeiss). Controls involved the removal of the 1st or 2nd antibody and replaced with PBS.

## Results

### 3.3.5.1 Grey squirrel *in vitro* vibrissae DP and DS cell morphology

Dermal papilla (DP) and dermal sheath (DS) cells were found to grow in a similar manner to that described for previously mentioned species. (Reynolds, 1989; Jahoda and Oliver, 1981). The DP cells grew from the isolated papilla tissue, and extended from the core in four distinct streams (Fig 18a). Cell streaming was more reminiscent of that illustrated by rat skin fibroblasts. The original papilla had flattened, with smaller cells nearer the core, but on the periphery, the cells were more streamlined (Fig 18b). The overall morphology of the cells, were similar to that described for the rat and human papilla cells (Jahoda and Oliver, 1981; Messenger, 1984). The cells were thin, with a stellate appearance. Cell lamellipodia were often seen at the front of the streaming edge (Fig. 18c). Cells isolated from the main body of the outgrowth were larger and more flattened (Fig 18d). Cells isolated from the dermal sheath, also displayed streaming behaviour away from the central core of transplanted tissue (Fig 18e). As with the DP cells, the DS cells had a fibroblastic appearance (Fig. 18f).

In later passaged DP cell cultures, one interesting feature observed, was cells that exhibited as many as 6 or 7 nuclei (Fig 18g). Furthermore, the nuclei often had distinct spatial arrangements, the commonest of which was a roseate pattern (Fig 18h). Some of the nuclei appeared to be joined in a chain as if budding off from other nuclei (Fig 18i). These multinucleate cells were also larger than the surrounding mononucleated cells (Fig. 18j).

### 3.3.5.2 Immunofluorescent labelling of grey squirrel DP and DS cells with the monoclonal antibody to smooth muscle alpha actin

Both grey squirrel DP and DS cells stained positively for smooth muscle  $\alpha$ -actin (Fig 19 a,b). Smooth muscle  $\alpha$ -actin was visible throughout the cells in distinct patterned

arrangements of filaments, however some cells had focal points of labelling with smooth muscle  $\alpha$ -actin filaments joining them (Fig 19c). This labelling is consistent with that observed by Jahoda et al (1991) on the equivalent rat cells. Approximately, 70-80% of *in vitro* DP or DS cells, in any one microscopic field, were positively labelled for smooth muscle  $\alpha$ -actin. Furthermore, smooth muscle  $\alpha$ -actin labelling occurred in retracting filaments that protruded from the cytoplasm (Fig 19d).

### **3.3.6.1 Stoat vibrissae DP and DS morphology *in vitro***

Dermal papilla and dermal sheath cultures from stoat follicles displayed early outgrowth that contrasted with that described above for grey squirrel cells (section 3.3.5.1). In both the DP and DS cultures, the transplanted tissue was not able to completely break down, instead the cells remained as distinct clumps within the culture dish (Fig 20a). In the DP cultures, the cells presented various morphologies. Cells nearer the original explant were densely packed and smaller (Fig 20a), but cells on the periphery were more flattened and static in appearance (Fig 20b). The latter suggestion was supported by the lack of apparent lamellipodial regions on these cells (Gail and Boon, 1971). Unlike the grey squirrel cells, there was no directed streaming of cells into the culture dish. The stoat DS cells were similar to those of the DP, however the cells nearer the explant were not as densely packed, with a more orderly morphology (Fig 20c). Single cells were distinguishable immediately adjacent to the explant. Cells at the edge of the culture were large with a broad cytoplasmic array, however cells near the explant were small and compact (Fig 20d).

Epithelial cells and dermal fibroblasts emanated from stoat skin explants (Fig 20e). Epithelia remained in close proximity to the explants, adopting a typical pavement like character (Fig 20f), whereas fibroblast cells rapidly colonized the remainder of the vessels (Fig 20e). Stoat skin fibroblast morphology was unlike that described for DP and DS cells. The cytoplasm protruded from a large central nucleus,

in thin spike like processes (Fig 20g), rather than the broad, flattened morphology of stoat DP and DS cells.

#### **3.3.7.1 Mink vibrissae DP and skin fibroblast (Fi) morphology *in vitro*.**

Mink DP morphology was similar in morphology to rat skin fibroblasts, with cells arranged in a bipolar fashion. Cell patterning was not as obvious as that illustrated for fetal fibroblasts (Elsdale and Bard, 1972), even though their morphologies were not dissimilar (Fig 21a). When confluent, mink DP cells were densely packed, with no obvious overlapping of cells (Fig 21b). In comparison with the mink DP cells, the mink fibroblasts had a distinct *in vitro* pattern. The fibroblast cells were spindly, with a neuronal appearance (Fig 21c). Furthermore, these cells looked unhealthy, with many vacuoles present around the nucleus (Fig 21d). Fibroblast cells did not exhibit lamellipodia and long tail like structures indicative of cell movement.

#### **3.3.8.1 Polecat DS and Fi morphology**

Polecat DS cells were broad and flat (Fig 22a), with a similar morphology to that of stoat DS cells. A number of the polecat DS cells appeared necrotic in the culturing conditions employed, with large vacuoles in the cytoplasm. The structure of these DS cells was often irregular with no distinct polarity. Follicular dermal cells were quite distinct between mink and polecat, but in contrast to this, their fibroblasts were similar (Fig. 22b). Polecat fibroblasts had a spindly morphology, with dendritic cytoplasmic processes. Pockets of vacuoles were positioned near the nucleus (Fig 22c).

### **3.3.9.1 Transformed DS cell morphology**

The more proliferative tDS cells demonstrated subtle changes in their morphology in comparison with the typical cultured rat DS cells. The tDS cells in their confluent state were smaller and more reminiscent of rat skin fibroblasts (Fig 23a). When subconfluent, the tDS cells were distinctly thinner and less flattened in appearance. When passaged some of these cells altered their phenotype further. The cells became spindly, with a distinctly stellate arrangement (Fig 23b). Aggregative behaviour was also witnessed within these stellate tDS cells, but this was not as obvious as that shown by rat dermal papilla cells in culture.(Fig 23c). This aggregation also occurred among groups of cells when the culture was subconfluent. Transformed DS stellate morphology was reminiscent of myoblasts. However, tDS cells were not multi-nucleate and did not construct large interlacing, fusiform cells. Under the same magnification the dermal sheath cells were larger with a broader cytoplasm.

### **3.3.9.2 Transformed DS growth curve and cell sizing**

On day 1 tDS cells had entered the growing phase, with cell numbers beginning to reach the plateau stage at day 7. Mean cell density at day 7 was 240 000 (Fig 24a). Transformed DS cell population doubling time (PDT) from day1 to day3 was 33.7 hrs (Table 1). Transformed DS cell sizing, using a coulter counter, produced a normal distribution of size (Fig 24b). The mean range of cell diameters was 17.06-21.5  $\mu\text{m}$ .

**Table 2** Population doubling time of rat transformed dermal sheath, dermal sheath, dermal papilla and skin fibroblast cells during 48 hours of growth in culture.

| Cell type | Hours of growth | Population doubling time (hrs) | Reference                |
|-----------|-----------------|--------------------------------|--------------------------|
| tDS       | 48              | 33.7                           |                          |
| DS        | 48              | 39.4                           | Reynolds(1989)           |
| DP        | 48              | 39.1                           | Jahoda and Oliver (1984) |
| Fi        | 48              | 23.4                           | Jahoda and Oliver (1984) |

### 3.3.9.3 Immunofluorescent labelling of tDS cells with monoclonal antibody to smooth muscle alpha actin

Transformed DS cells stained positive for smooth muscle  $\alpha$ -actin, however the intensity of labelling was not always uniform throughout the cytoplasm(Fig 25a,b).

Approximately 90% of cells were positive for smooth muscle  $\alpha$ -actin, however only 30% had a high intensity of stain with the remaining cells at low intensity. Smooth muscle  $\alpha$ -actin fibres were arranged throughout the cytoplasm in a similar arrangement to that seen in rat DS cells (Fig 25c). Interacting groups of cells would therefore display a mixture of smooth muscle  $\alpha$ -actin intensity (Fig 25d,e).

### 3.3.9.4 Immunofluorescent labelling of tDS cells with a polyclonal antibody to Transforming Growth Factor $\beta$

tDS cells were labelled with a polyclonal antibody that was panspecific for a number of TGF $\beta$  family members; recombinant human TGF- $\beta$ 1, porcine TGF- $\beta$ 1.2, pTGF- $\beta$ 2

and recombinant amphibian TGF- $\beta$ 5 (British Bio-technology ltd). Overall cell labelling was diffuse and faint, however labelling was concentrated around the nucleus (Fig 26). Controls had no immunofluorescent marking. No positive label was present outside the cells, all labelling was restricted to the cytoplasm.

#### **3.4.1.1 Rat DP *in vitro* aggregation process**

In the time course study of *in vitro* dermal papilla cell behaviour, day 1 subconfluent cells (SC) were distinctly fibroblastic, and evenly spaced with a mixture of broad flattened and bipolar morphologies (Fig 27a). By Day 8, cells were confluent, with possible signs of clump formation (Fig 27b). At day 13, cells were aggregating and channeling into a distinctive patterning. Within the photographed field, two future sites of DP clumps were visible (Fig 27c). On Day 16; clump formation was well advanced, cells were pulling apart and being drawn into aggregates (Fig 27d). Cells in this stage were recorded as fully aggregating (AG).

Later on, the two aggregates seen forming on day 16 continued to be clearly visible, however, by day 28, the cells were fully clumped. This clumped stage is the final step in the DP cell aggregation process. Once aggregates were formed, the majority of cells were in, or associated with these structures. The base of the culture vessel was beginning to be visible between clumps (Fig 27e). Dermal papilla cells from this stage were recorded as being clumped (CL). It should be noted, however, that these timings are not strict and altered in different DP cell lines, although the general progression of events was consistent. Once in the clumped state the cultures did not alter in appearance for long periods unless deleterious environmental conditions came about.

### **3.4.1.2 Rat DP growth curve**

Phenotypic changes in cultured dermal papilla cells were monitored against a growth curve. The general trend of the growth curve was consistent with Jahoda and Oliver (1984). The fastest growing phase was between day 7 and day 13 with a plateau beginning at day 17. Changes in cell behaviour were as follows, day0-day7 subconfluency, day7-day 13 confluency and day 13 onwards aggregation phenomenon (Fig 28).

### **3.4.1.3 Cell proliferation during rat DP aggregate formation**

Cell proliferation was monitored over the time of aggregate formation using Brdu labelling. The number of marked cells were counted from the first days of confluency until aggregate formation, the time of highest activity (i.e day 7-17). The overall trend was a decrease in cell proliferation, this being consistent with the growth curve described above (Fig 29a). Aggregation was first noted on day 10, therefore between the early and later stages of aggregate formation there was a decrease in cell proliferation. Proliferation was also monitored on a phenotypic basis, with counts made of dividing cells when cultures were confluent, aggregating and clumped. Cells were confluent from day 7-12, aggregating from day 12-26 and clumped from day 26 onwards (Fig 29b). The greatest decrease in proliferation occurred from confluent to aggregating cells. This was consistent with figure (Fig 28). From aggregating to clumped phenotypes there was a further decrease in proliferation. Bright field micrographs of aggregating cells showed clump formation (Fig 29c), however Brdu labelled cells were diffuse across the culture (Fig 29d). Proliferating cells were not present in distinct foci.

#### **3.4.1.4 Super aggregated (SAD) rat DP cell structures**

Super aggregate structures would occur in approximately 1 out of 4 lines of long term DP cell cultures. Structures appeared if cells were maintained in a highly confluent state without passaging. Super aggregates formed by the cells detaching from the culture vessel and the resultant forces pulling the cells into densely aggregated structures (Fig 30a). Adjacent to the large clumps, free areas of culture dish or a fine monolayers of cells were visible (Fig 30a). Clumps formed were often large 3 dimensional structures, standing upright from the culture vessel. Neighbouring the large clumps were densely constructed arms of tissue supporting the centrally arranged aggregate (Fig 30a,b). Cells were also visibly stretched between the central aggregate and the detached zone (fig. 30b). The size of clumped configurations was not uniform, and morphological differences were also apparent. Within the smaller aggregated structures were large amounts of extracellular matrix material between a relatively small number of cells (Fig 30c,d). In the larger aggregates, this was also the case, however there was also an encasing layer of DP cells that surrounded the relatively few central cells extracellular material (Fig 30e).

#### **3.4.1.5 Transmission electron microscopy of SAD DP structures**

Cells within the super aggregates had a variety of morphologies, structures and states of health. In general, cells at the surface of the aggregate were arranged in layers, however, cells on the inner edge of these strata showed signs of deterioration (Fig 31a). Inside the outer layers of cells were large amounts of extracellular matrix (ECM). Cells within the ECM were smaller and more rounded, not thin and flattened like the peripheral cells (Fig 31b,c). Many cells contained large granular lysosomes (Fig 31d,e), which gave the DP cells a mast cell like appearance (Combs et al., 1965). These granular lysosomes were also noted previously by Reynolds (1989), who discovered them in germinative epidermal/dermal papilla cell recombinations. Central cells were

generally more necrotic than those of the periphery (Fig 31f). The extracellular matrix was homogeneous in nature with no visible fibrous collagen (Fig 31g). Filamentous material was discernible within cells of the aggregates (Fig 31h) and cells of the non clumped material (Fig 31i). Cells between super aggregates were multilayered, thin and flattened, like the cells on the periphery of the clumps (Fig 31j). What appeared to be apoptotic cells, were also present within the aggregates (Fig 31k). Apoptotic cells showed a condensing of chromatin in the nucleus and a reduction in size of the cytoplasm. Cells were also seen endocytosing these apoptotic nuclei (Fig 31L). A few DP cells within these SAD clumps also contained many dense vesicles (Fig 31c,d,e), but it was not clear whether these were secretory vesicles or apoptotic bodies from an endocytosed chromatin dense nucleus.

**Figure 20a**

Phase contrast micrograph of stoat dermal papilla cells. Cell morphology is varied, with cells nearest the explant being small and compact. (Magn. x68)

**Figure 20 b**

Stoat dermal papilla cells on the periphery of the culture are larger and flatter than those cells nearest the explant. There is no apparent cell streaming. (Magn. x68, phase contrast)

**Figure 20c**

Stoat dermal sheath cells nearer the explant are small but with a more orderly morphology than that of the stoat dermal papilla cells. (Magn. x68, phase contrast)

### **Figure 18g**

Phase contrast micrograph of grey squirrel dermal papilla cells. Cells exhibited unusual nuclear morphology, with 6 to 7 nuclei present in a single cell. Cells were fixed and nuclei visualised with geimsa stain. (Magn. x250)

### **Figure 18h**

Phase contrast micrograph of grey squirrel dermal papilla cells. Multiple nuclei are arranged in a roseate pattern. Cells are fixed and visualised with geimsa stain. (Magn. x250)

### **Figure 18i**

Phase contrast micrograph of grey squirrel dermal papilla cells. In some cells, nuclei appeared to be budding off from one another. Cells were fixed and nuclei visualised with geimsa stain. (Magn. x250).

### **Figure 18j**

Phase contrast micrograph of grey squirrel dermal papilla cells. Multi-nucleate cells were larger than the surrounding mononucleate cells. Cells were fixed and nuclei visualised with geimsa stain. (Magn. x85).

### **Figure 19 a,b**

Immunofluorescent histochemistry of grey squirrel dermal papilla (a) and dermal sheath cells (b) when labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. Both cell types show arranged smooth muscle  $\alpha$ -actin filaments. (Magn. x290)

### **Figure 19 c,d**

Immunofluorescent histochemistry of grey squirrel dermal papilla and dermal sheath cells when labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. A grey squirrel dermal papilla cell (c) shows smooth muscle  $\alpha$ -actin filaments arranged in distinct focal points. A grey squirrel dermal sheath cell (d) with smooth muscle  $\alpha$ -actin filaments present in the retracting cytoskeleton of the cell. (Magn. x290)

### **Figure 17a**

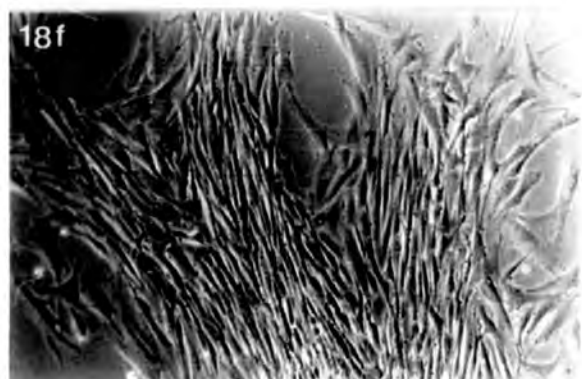
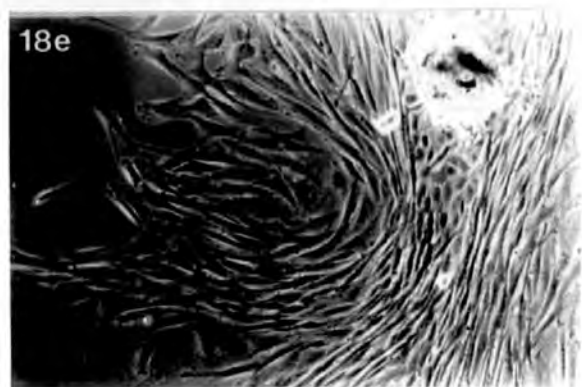
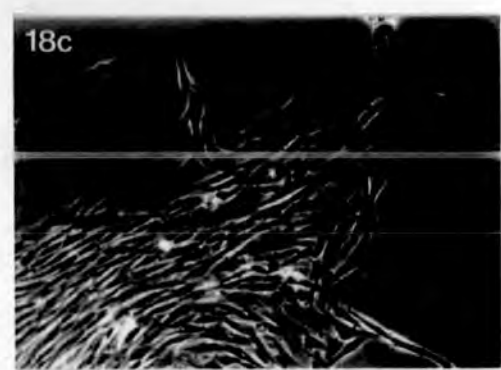
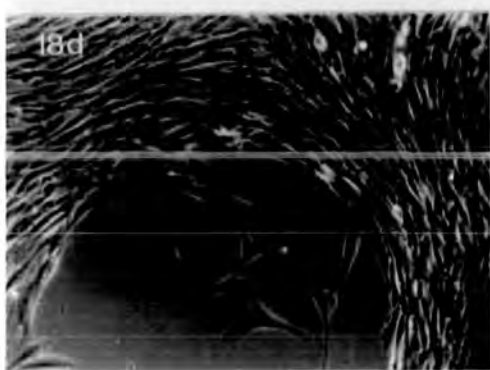
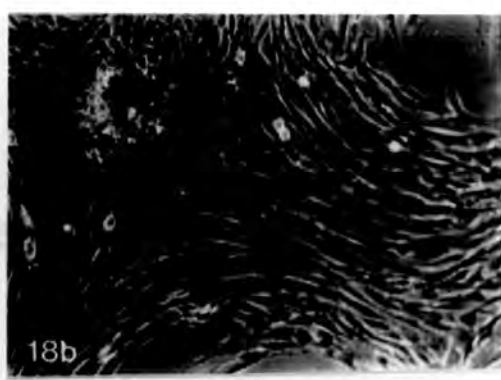
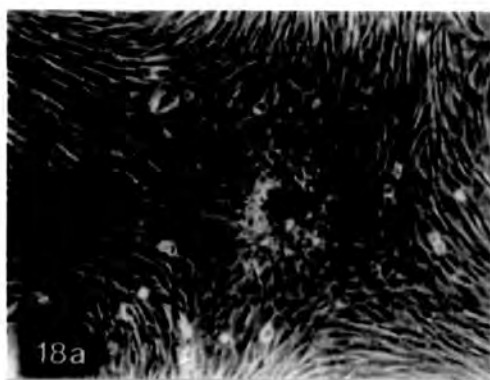
Micrograph of a grey squirrel telogen sinus follicle. The epidermis consists of outer root sheath (**ors**) cells. With dermal papilla (**dp**) reduced in size compared to the anagen follicle. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x170).

### **Figure 17b**

High power micrograph of the end bulb of the grey squirrel telogen sinus follicle. The dermal papilla (**DP**) is reduced, with the epidermis consisting of outer root sheath (**ORS**) cells. (**ds**) dermal sheath. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x350).

### **Figure 17c**

Micrograph of the sebaceous gland (**S**) of the grey squirrel sinus follicle. The gland is small and round, with many large vacuolar cells within it. Section was stained with Alcian blue, Weigert's Haemotoxylin and Curtis Ponceau. (Magn. x350).

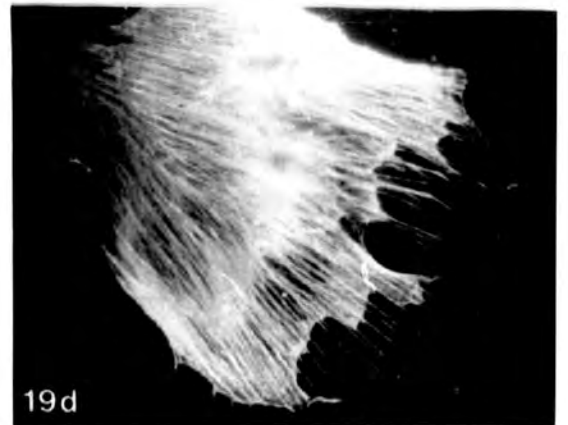
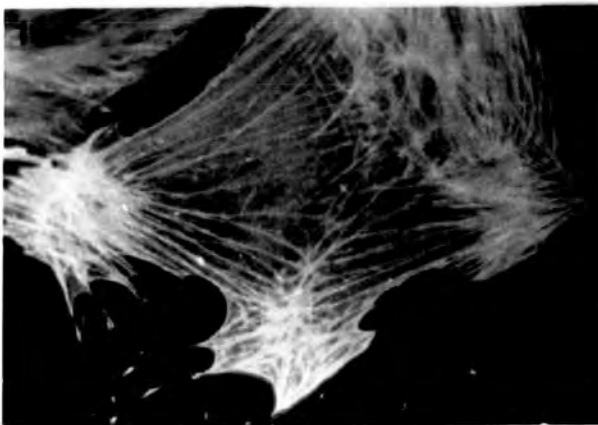
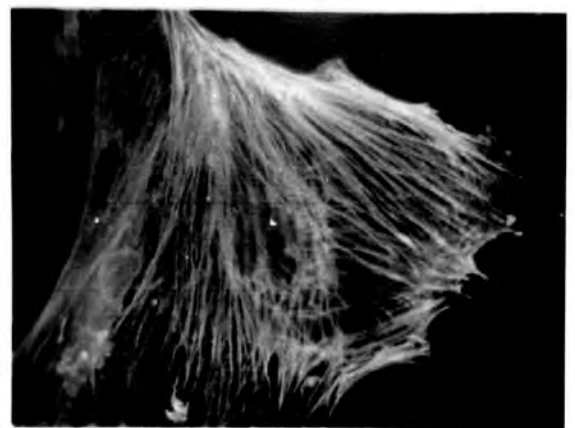
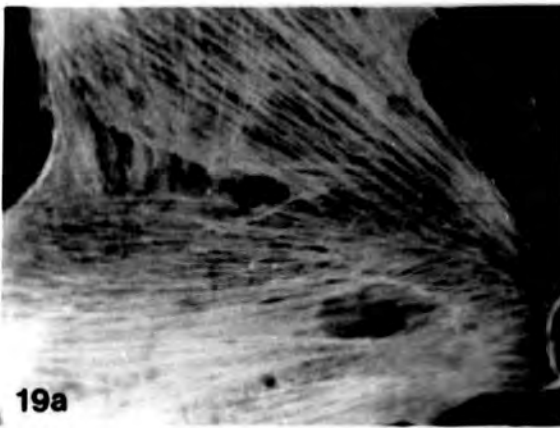
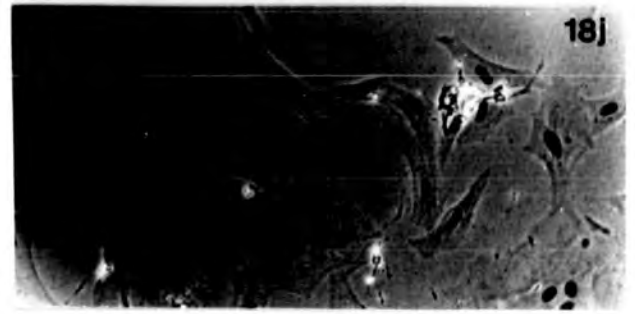
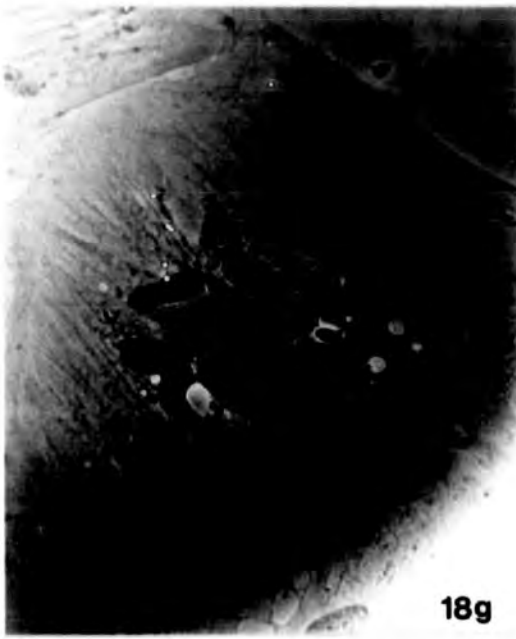


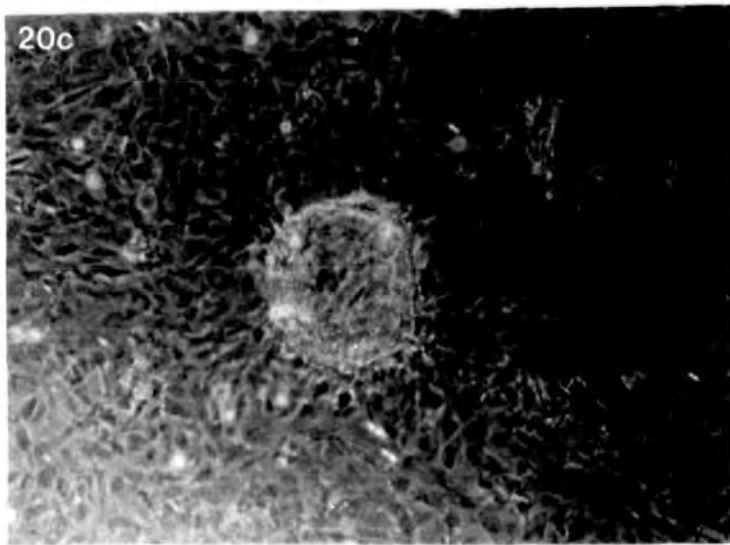
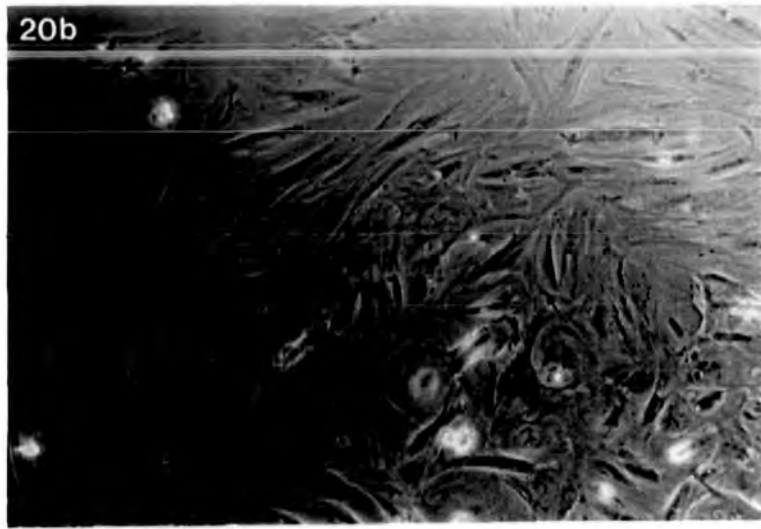
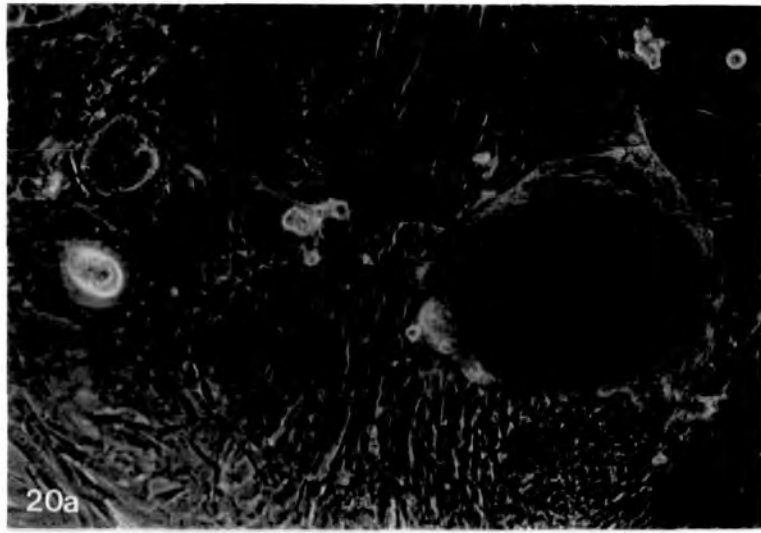
### **Figure 18 a,b,c,d**

Phase contrast micrographs of grey squirrel dermal papilla cells in culture. Cells are present streaming from the central core of explanted tissue. a) cells near the explant are smaller than the more streamlined cells on the periphery, b) & c) cell lamellipodia is often seen on the edge of the stream of migrating cells. Cells isolated from the streaming regions are flatter and larger (d). (Magn. x60)

### **Figure 18 e,f**

Grey squirrel dermal sheath cells are visible streaming from the central explant. Cell morphology is reminiscent of skin fibroblasts. (Magn. x60, phase contrast)





### **Figure 20d**

Phase contrast micrograph of stoat dermal sheath cells. Cells on the periphery of the culture are larger and flatter. There is no apparent cell streaming. (Magn. x68)

### **Figure 20e**

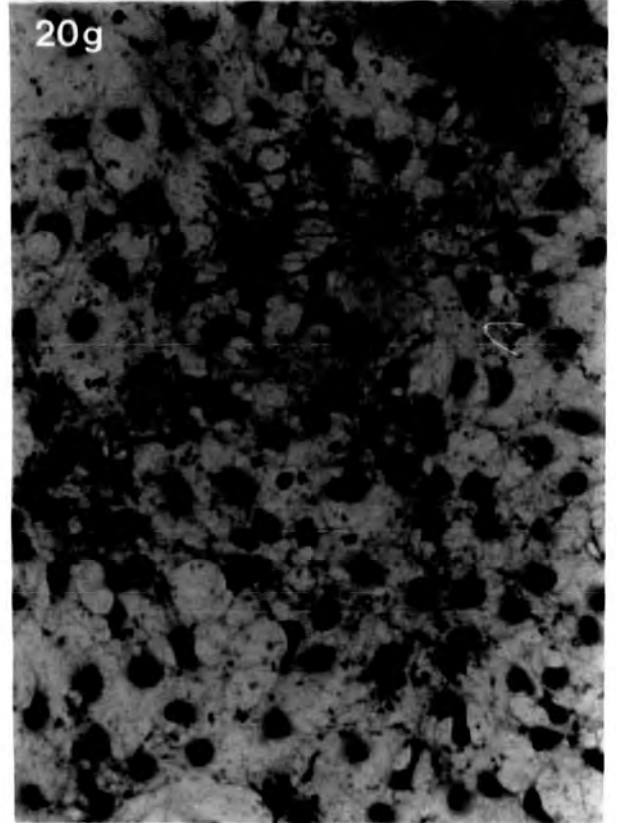
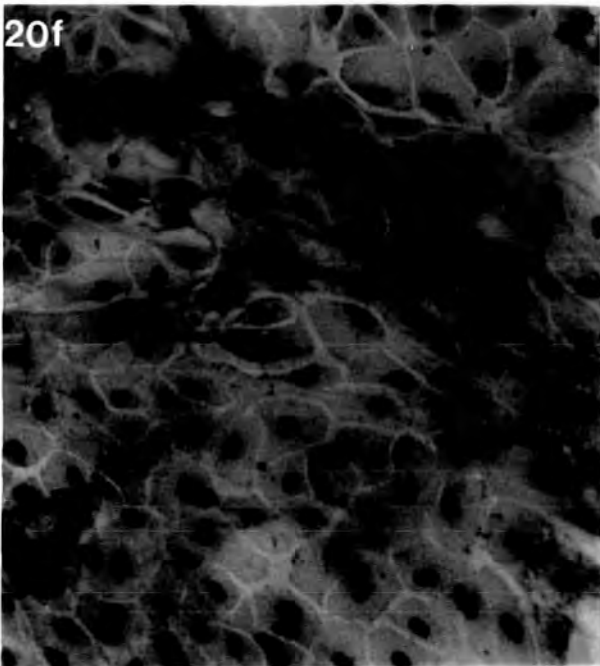
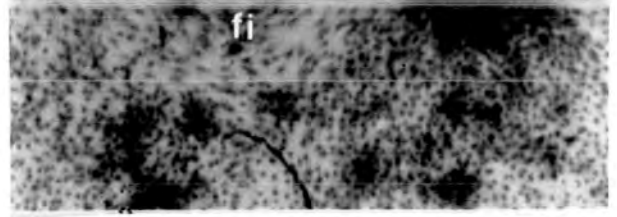
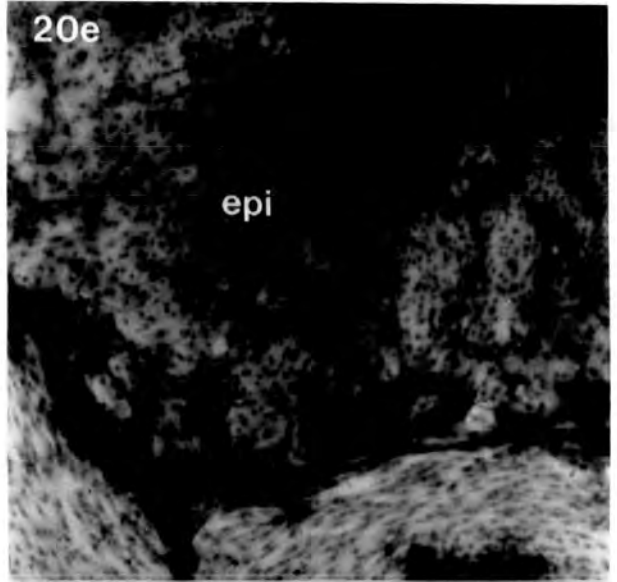
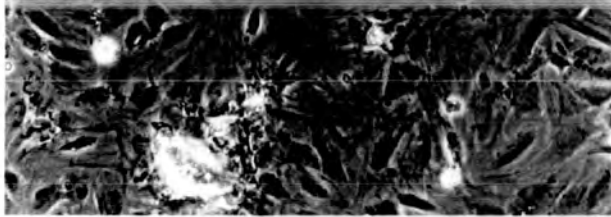
Phase contrast of stoat skin explant. Epidermal cells (**epi**) are visible adjacent to the explant whereas fibroblasts (**fi**) have colonised the remainder of the dish. The tissue was fixed and visualised with geimsa stain. (Magn. x68)

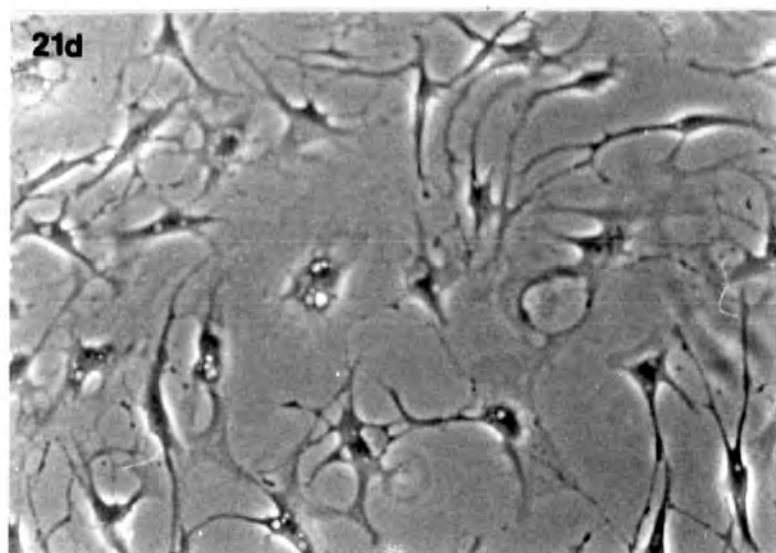
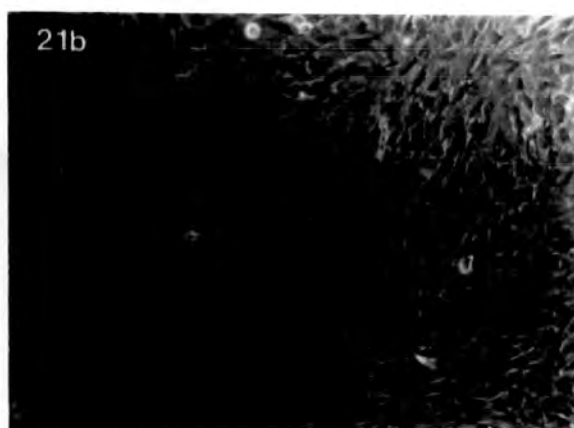
### **Figure 20f**

Stoat epidermal cells adopted a typical pavement like arrangement. Cells were fixed and visualised with geimsa stain. (Magn x170, phase contrast)

### **Figure 20g**

Phase contrast of stoat fibroblast cells. Cell morphology is spindly with cytoplasm protruding from the nucleus in thin spike like processes. Cells were fixed and visualised with geimsa stain. (Magn x170)





### **Figure 21a**

Phase contrast of mink dermal papilla cells. Cell morphology was bipolar and reminiscent of skin fibroblasts. (Magn x68)

### **Figure 21b**

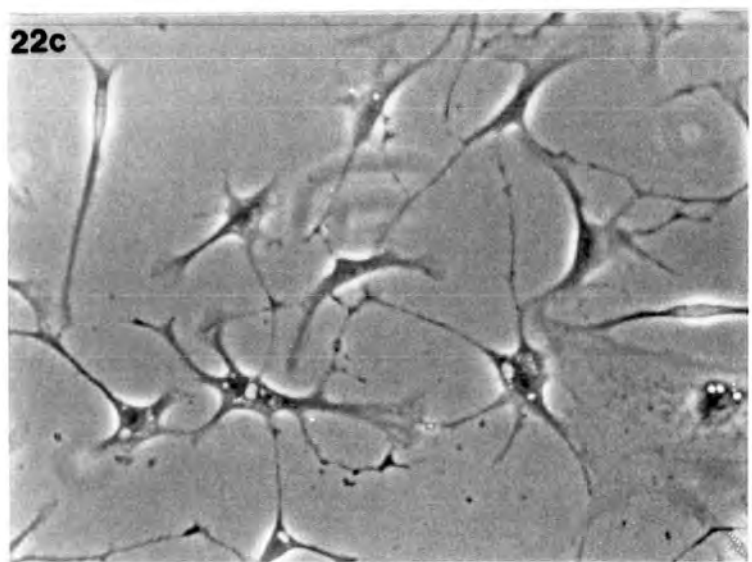
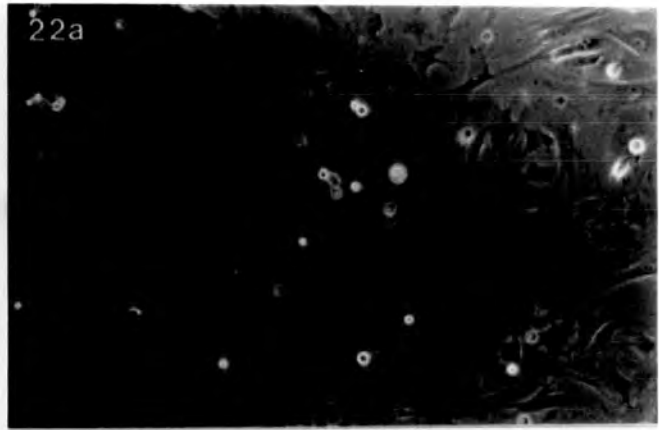
Phase contrast of confluent mink dermal papilla cells. Cells are densely packed but with little cell overlapping and cell crawling. (Magn x68)

### **Figure 21c**

Mink skin fibroblast cell morphology is spindly with a neuronal appearance. (Magn x68, phase contrast)

### **Figure 21d**

Phase contrast of mink skin fibroblast cells. A few cells appear unhealthy due to abundance of vacuoles arranged around the nucleus. (Magn. x100)



**Figure 22a**

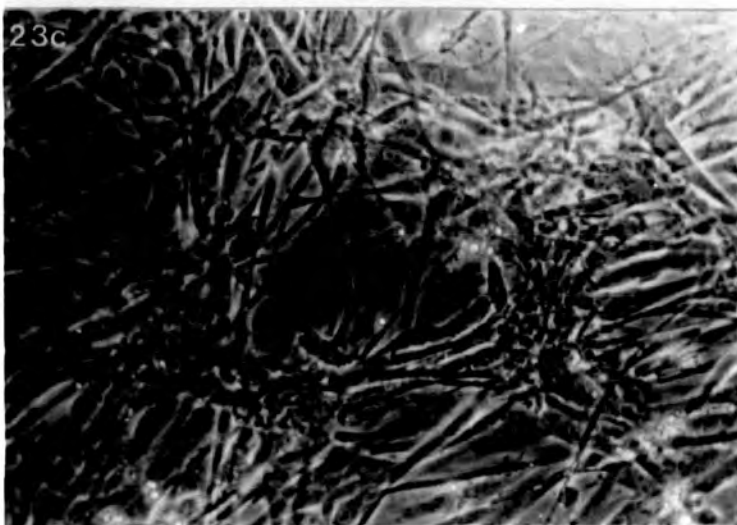
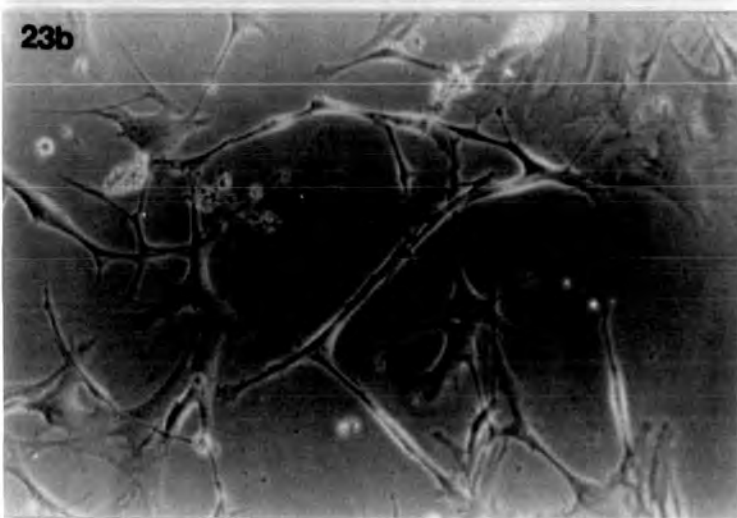
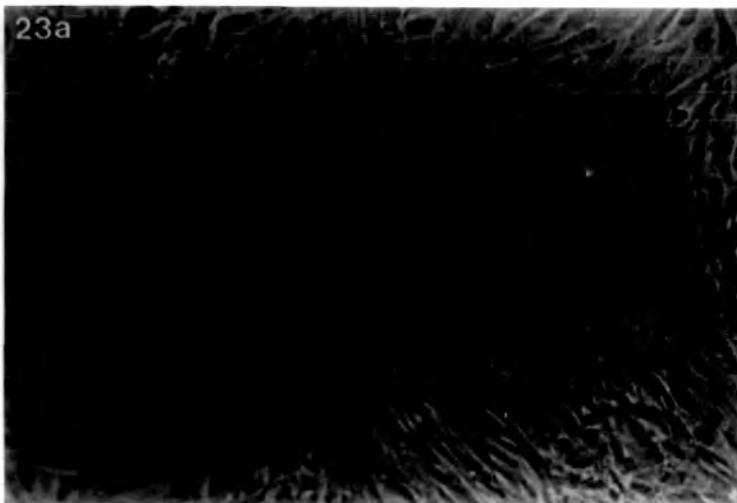
Phase contrast of polecat dermal sheath cells. Cell morphology is broad and flat. A few cells exhibiting vacuoles near the nucleus. (Magn. x68)

**Figure 22b**

Polecat skin fibroblast cells are spindly with visible dendritic processes . (Magn. x68, phase contrast)

**Figure 22c**

Phase contrast of polecat skin fibroblast cells. Pockets of vacuoles within the cytoplasm are visible and positioned near the nucleus. (Magn. x100)



**Figure 23a**

Phase contrast of transformed rat dermal sheath cells. When confluent, cells are small and reminiscent of skin fibroblasts. (Magn. x68)

**Figure 23b**

Transformed rat dermal sheath cell morphology has altered, becoming spindly and stellate in shape. (Magn. x110, phase contrast)

**Figure 23c**

Transformed rat dermal sheath cells showing simple aggregate phenomenon. Individual cell morphology is spindly and stellate like. (Magn. x110, phase contrast)

Fig. 24a

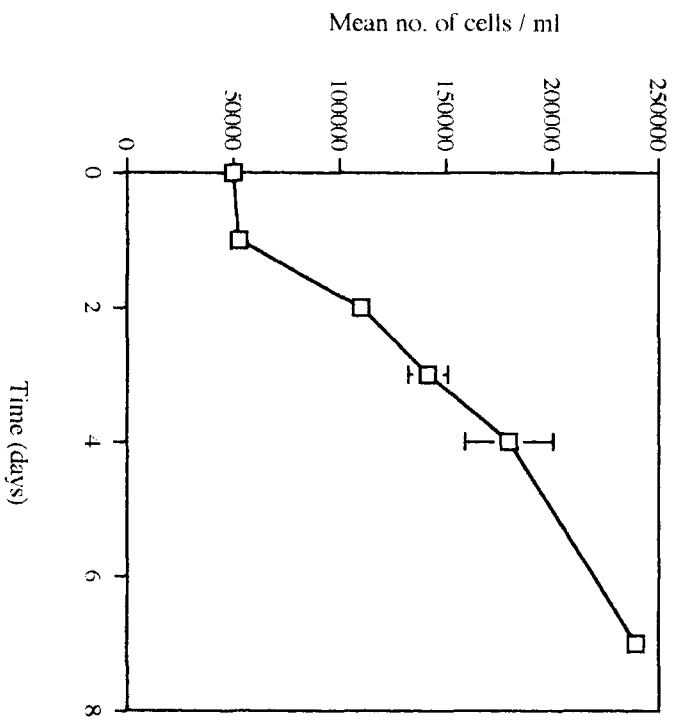
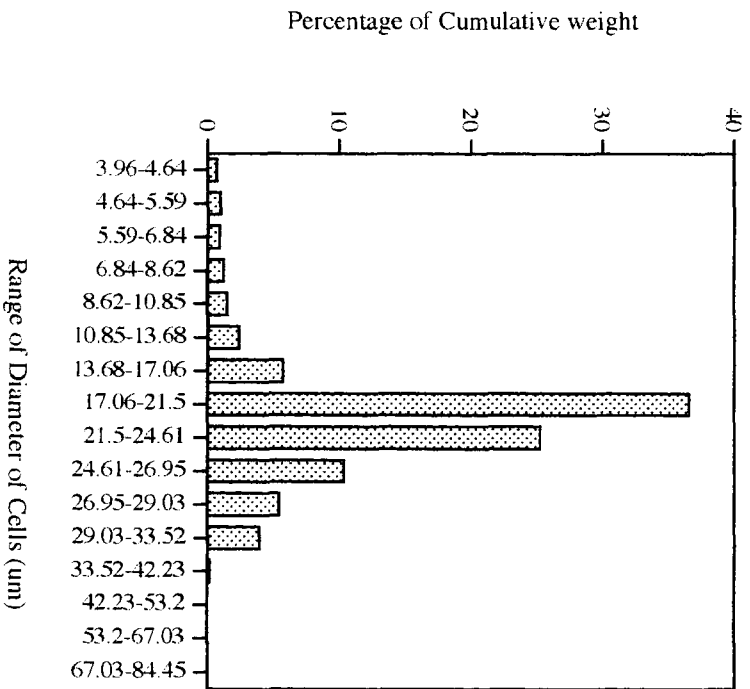


Fig. 24b

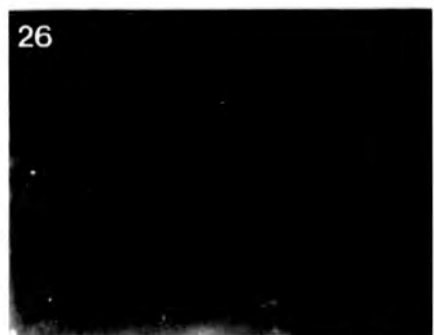
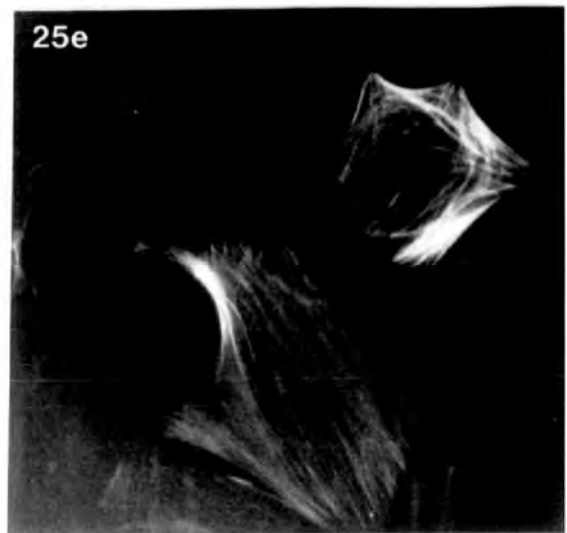
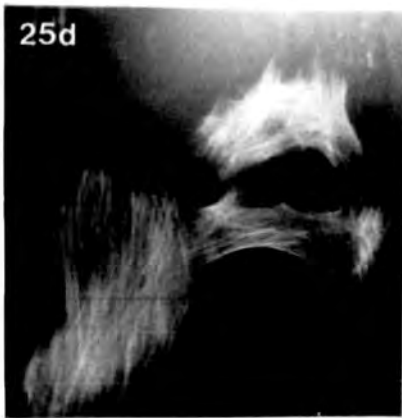
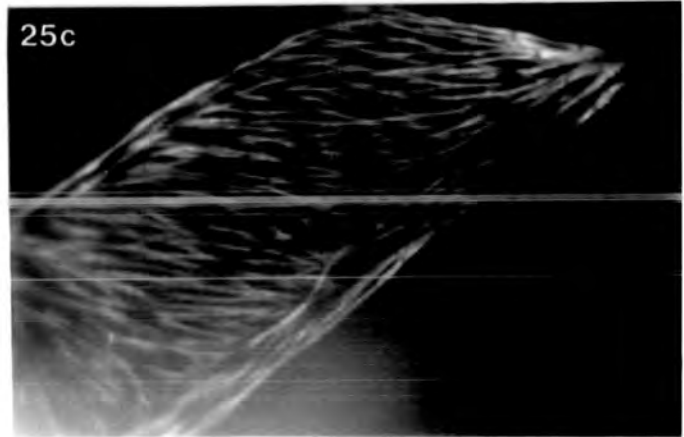
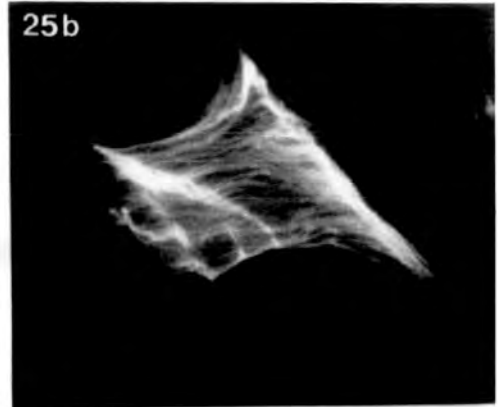
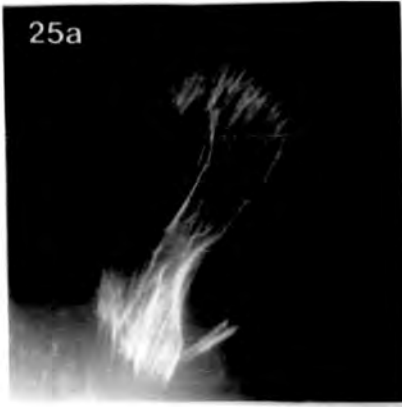


**Figure 24a**

Growth curve of rat transformed dermal sheath cells in culture.

**Figure 24b**

Size distribution of rat transformed dermal sheath cells. The majority of cells have a diameter range between 17.06-21.5 $\mu$ m.



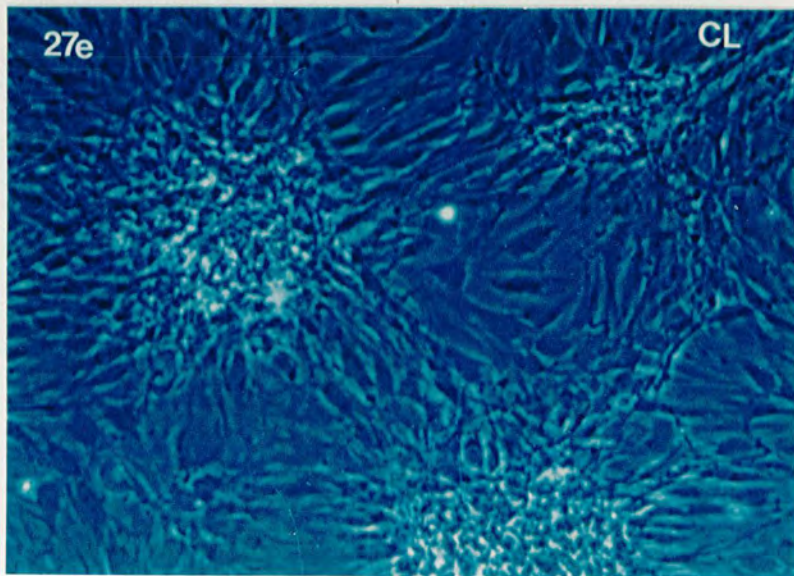
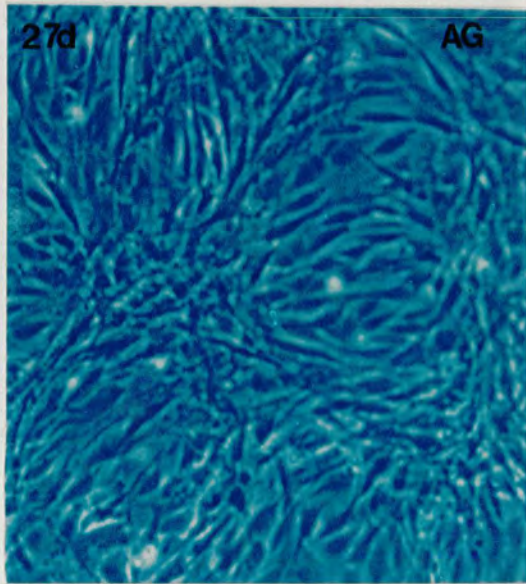
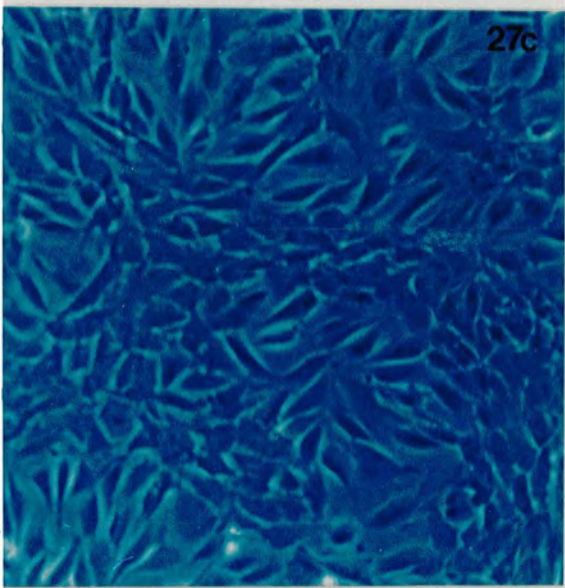
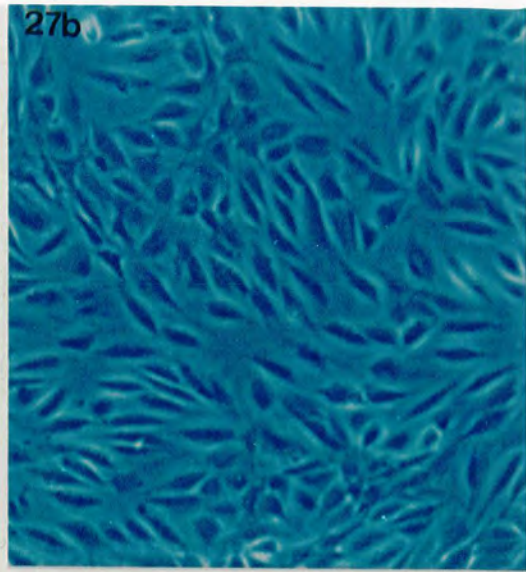
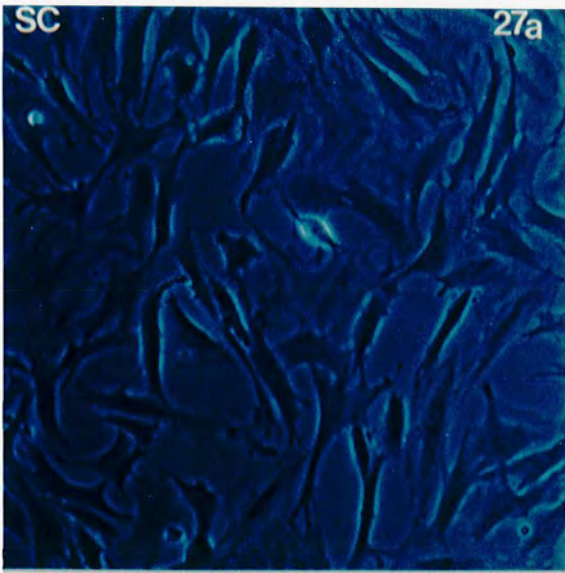


Fig. 28

Graph of dermal papilla cell growth in culture. The changes in cell morphology being noted with time. (SC) subconfluent, (Confl) confluent and (AG) aggregating dermal papilla cell morphology, as described in section (3.4.1.1).

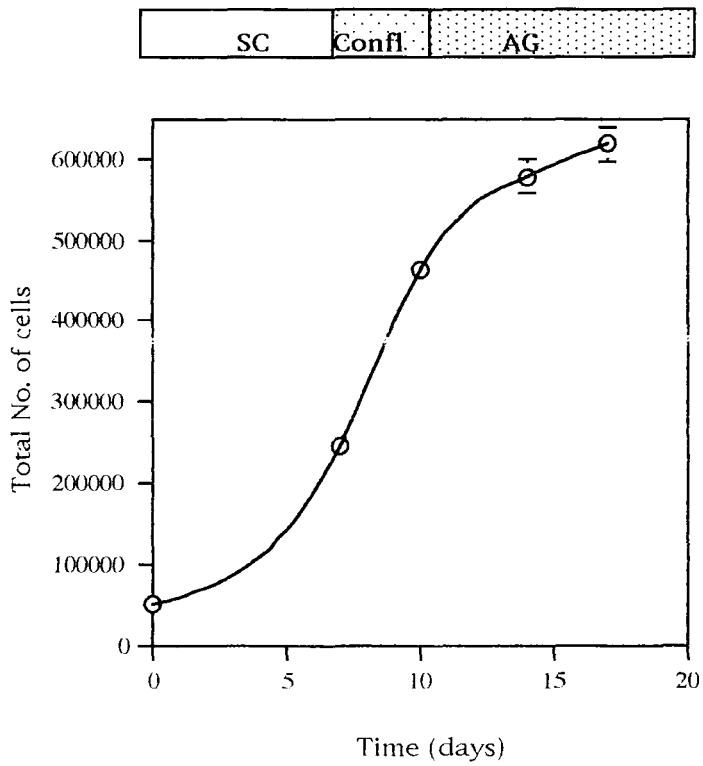


Fig. 29a

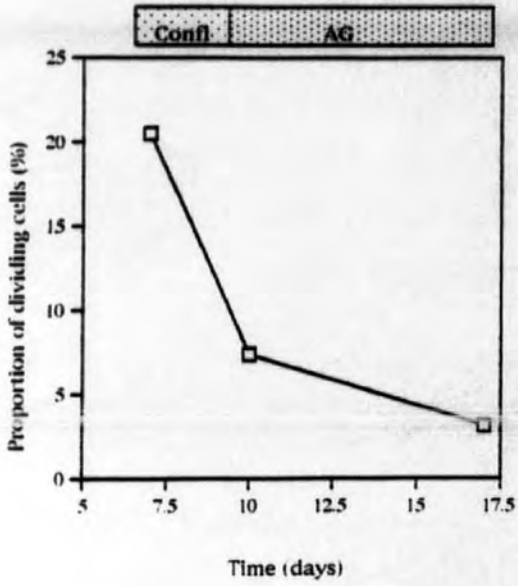
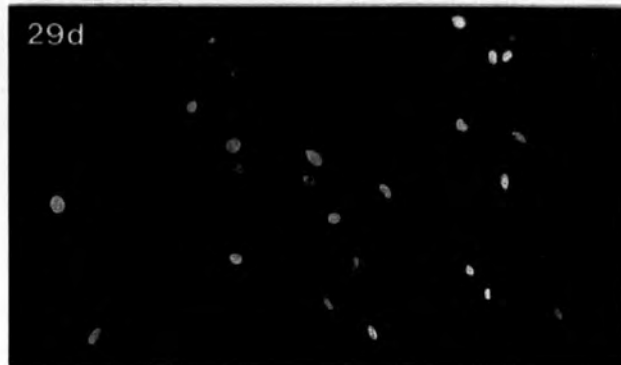
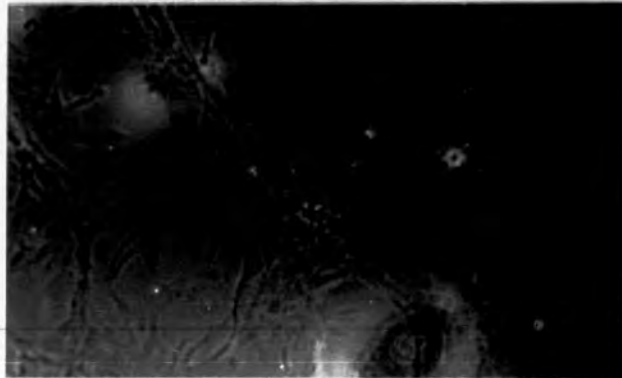
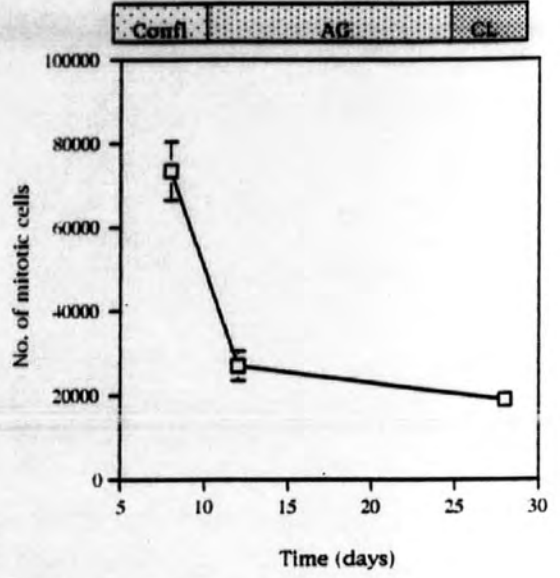
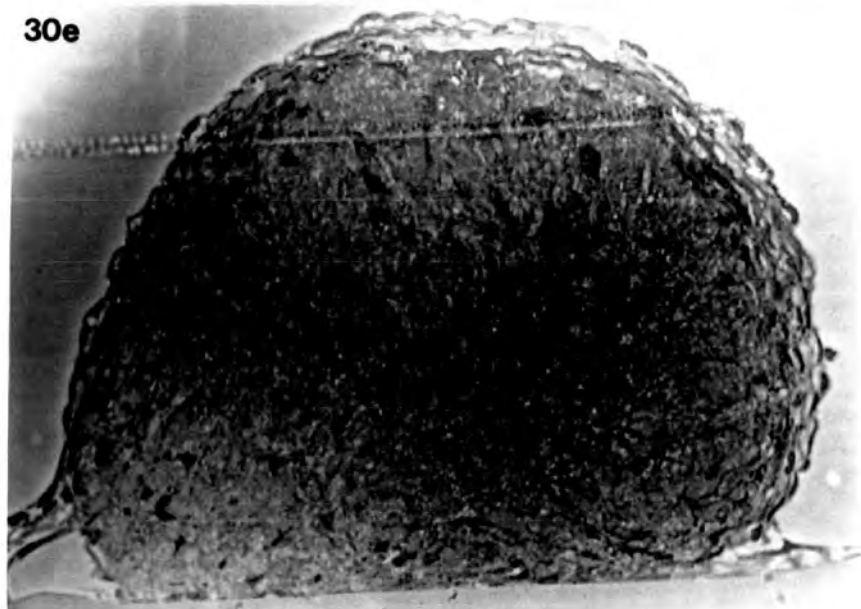
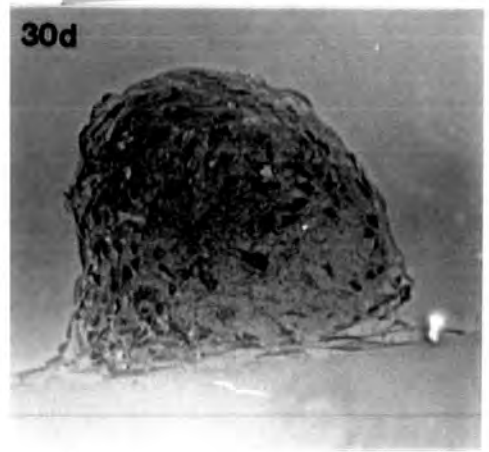
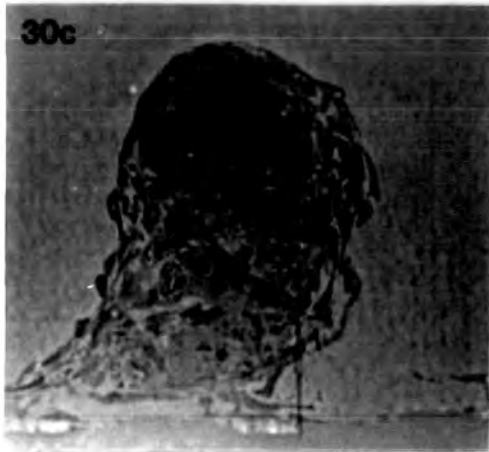
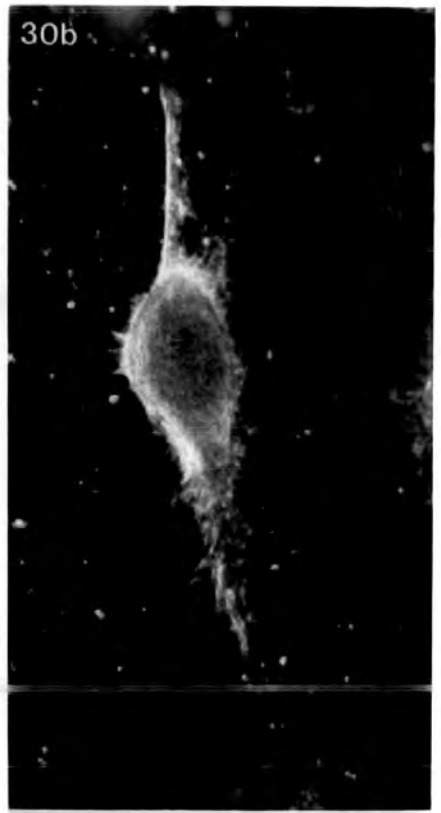
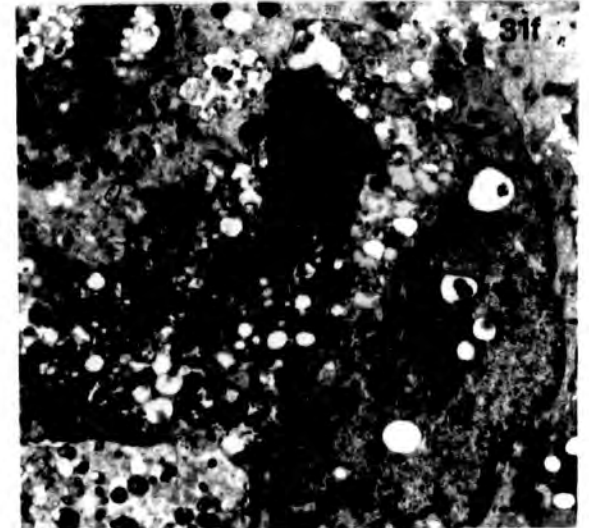
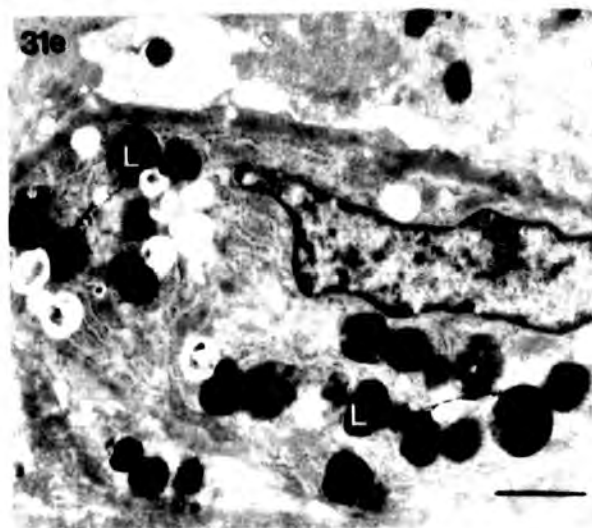
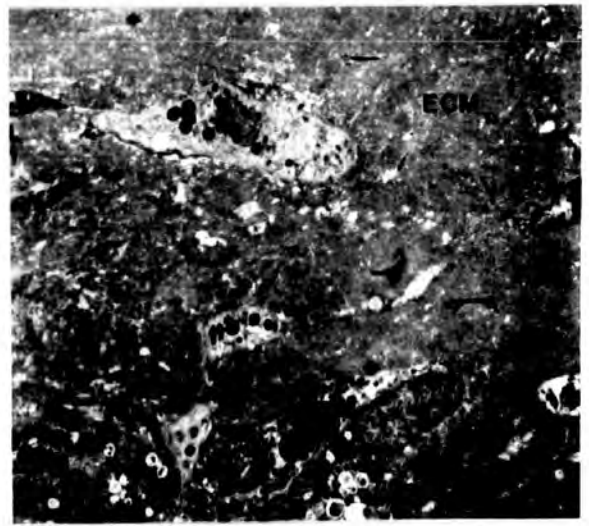
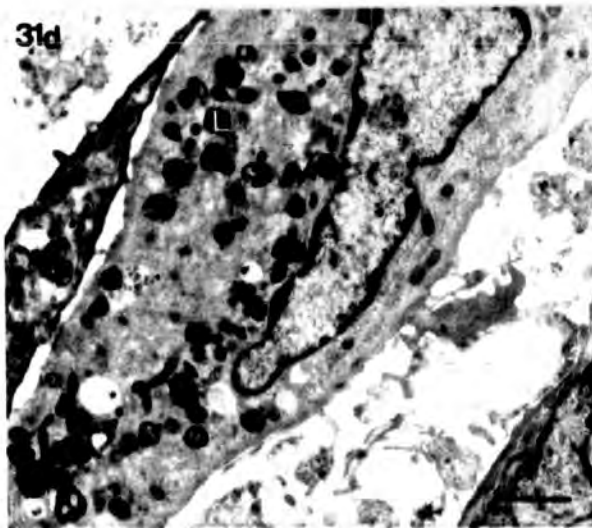
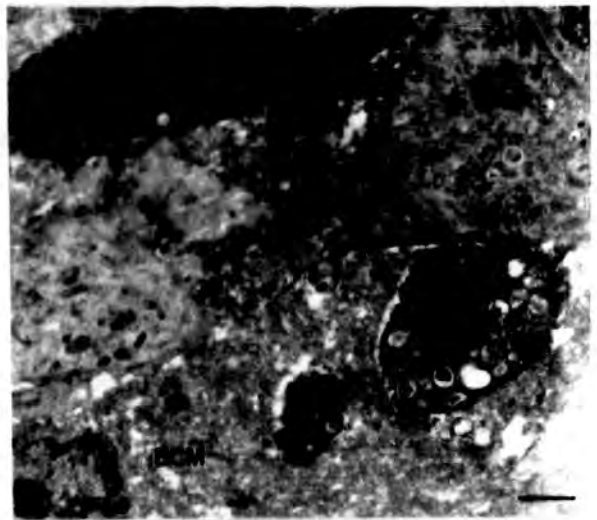
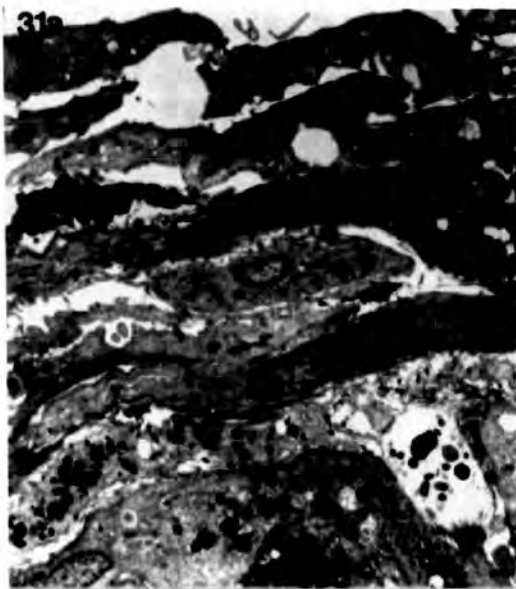
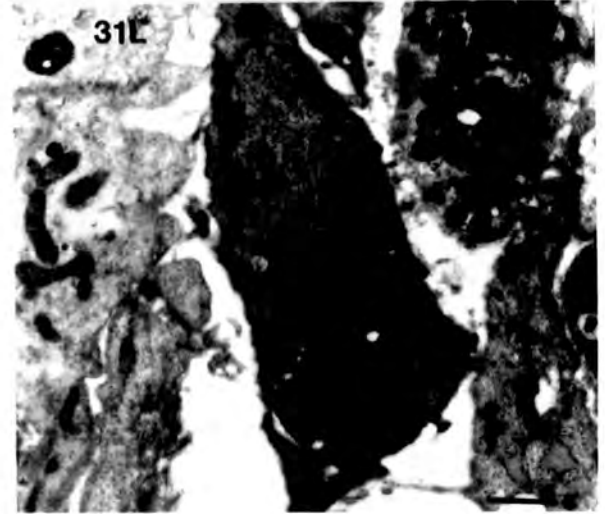
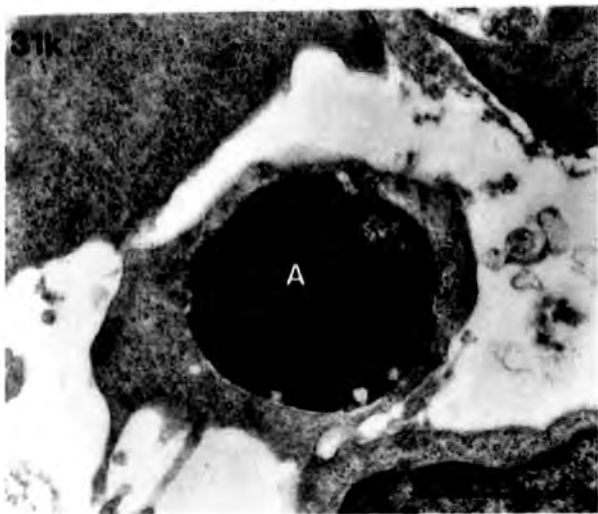
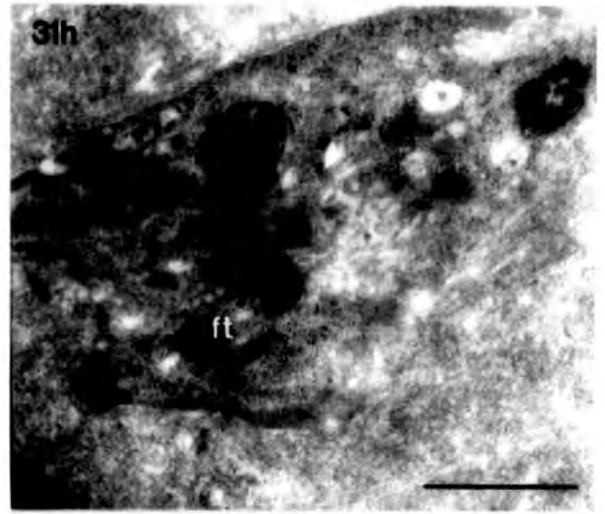
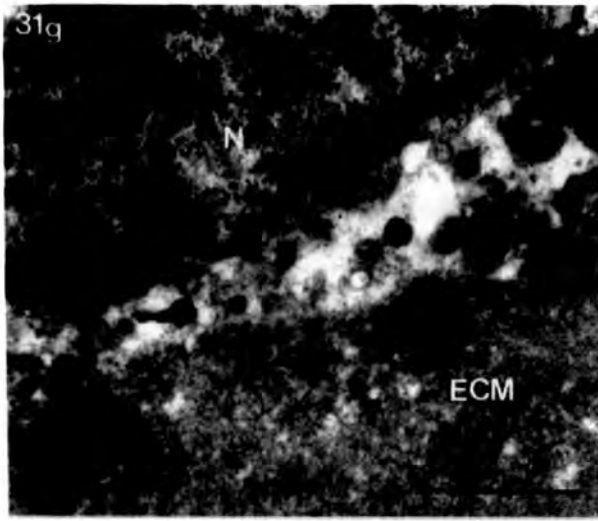


Fig. 29b









### **Figure 31a**

Transmission electron micrograph of super aggregated dermal papilla cells. Cells at the surface of the aggregate are generally arranged in layers. Cells on the inner edge of the layers are beginning to become necrotic. (Magn. x3600, bar = 1 $\mu$ m)

### **Figure 31 b,c**

Transmission electron micrographs showing dermal papilla cells within the extracellular core are small and rounded. (b) Magn. x3600 & (c) Magn. x8000; bar = 1 $\mu$ m. (ECM, extracellular matrix)

### **Figure 31 d,e**

Transmission electron micrograph of super aggregated dermal papilla cells. Large granular lysozomes are present in many of the cells within the structure. (d) Magn. x10000 & (e) Magn. x 13000; Bar = 1 $\mu$ m. (L, lysozome)

### **Figure 31 f**

Transmission electron micrograph of necrotic dermal papilla cells within the centre of a super aggregate. Magn. x6000; Bar = 1 $\mu$ m.

### **Figure 31 g**

Transmission electron micrograph of super aggregated dermal papilla cells. Homogenous extracellular matrix (ECM) is adjacent to a dermal papilla cell. (N) nucleus. Magn. x 22000; Bar = 1 $\mu$ m.

### **Figure 31 h**

Transmission electron micrograph of a super aggregated dermal papilla cell showing filamentous material within the cytoplasm. Magn. x 22000; Bar = 1 $\mu$ m. (ft, filaments)

### **Figure 31 i**

Transmission electron micrograph of dermal papilla cells between the super aggregated regions. Filamentous material is apparent within the cytoplasm of the dermal papilla cell. Magn. x 10000; Bar = 1 $\mu$ m. (ft, filaments)

### **Figure 31 j**

Dermal papilla cells between the super aggregated structures are multilayered, similar to those on the periphery of an aggregated structure. Transmission electron micrograph. Magn. x 4600; Bar = 1 $\mu$ m.

### **Figure 31k**

A dermal papilla cell within the super aggregate, illustrating the signs of an apoptotic cell, a condensed nucleus with much reduced cytoplasm, is shown in this transmission electron micrograph. Magn. x 22000; Bar = 1 $\mu$ m. (A, apoptotic cell)

### **Figure 31 l**

Transmission electron micrograph of a super aggregated dermal papilla cell endocytosing a large apoptotic nuclei. Magn. x 10000; Bar = 1 $\mu$ m.

**Figure 30a**

Low power micrograph of super aggregated dermal papilla (SAD) cells. A large 3 dimensional structure is visible protruding from the culture vessel. A monolayer of cells can be seen adjacent to the structure. (Magn. x20)

**Figure 30b**

Low power micrograph of super aggregated dermal papilla (SAD) cells. The aggregated structure is supported by densely constructed arms of tissue. (Magn. x20)

**Figure 30 c,d,e**

Semi thin sections through a variety of resin embedded 3 dimensional super aggregated dermal papilla cells. Cells within these aggregates are often separated by large volumes of extracellular material. Sections are stained with toluidine blue. (Magn x110).

### **Figure 29a**

Dermal papilla cell proliferation was monitored over time, with the cell phenotype being noted. Proportion of dividing cells were plotted against time. Cells were labelled with brdu. (**Confl**) confluent, (**AG**) aggregating.

### **Figure 29b**

Dermal papilla cell proliferation was monitored during changes in dermal papilla cell *in vitro* phenotypes. The number of mitotic cells were plotted against time. Cells were labelled with brdu. (**Confl**) confluent, (**AG**) aggregating and (**CL**) clumped phenotypes.

### **Figure 29c**

Phase contrast micrograph of clumped dermal papilla cells. (Magn. x70)

### **Figure 29d**

Immunofluorescent micrograph of clumped dermal papilla cells after being labelled with brdu and its respective antibody. Dividing cells are present throughout the micrograph and are not associated in distinct foci with the clumped dermal papilla cells. (Magn. x70)

**Figure 27 a,b,c,d,e**

Phase contrast micrographs of rat dermal papilla cell *in vitro* aggregation process. **a)** (SC) subconfluent cells; cells are evenly spaced being broad and flat with a bipolar morphology, **b)** cells are confluent with possible signs of aggregate formation, **c)** early stages of aggregation occurring, **d)** aggregate formation is advanced, aggregates are visible. This phase is termed aggregating (AG) and **e)** (CL) clumped, the cells are visible in tight clumps. This being the final phenotype of the aggregation process. (Magn. x105).

**Figure 25a,b**

Immunofluorescent histochemistry of transformed dermal sheath cells when labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin . Cytoskeletal smooth muscle  $\alpha$ -actin is arranged in parallel filaments. (Magn. x380)

**Figure 25c**

Immunofluorescent histochemistry of dermal sheath cells when labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. Cytoskeletal labelling is similar to that of the transformed dermal sheath cells. (Magn. x 290)

**Figure 25d,e**

Immunofluorescent histochemistry of transformed dermal sheath cells when labelled with the monoclonal antibody to smooth muscle  $\alpha$ -actin. Interacting groups of transformed dermal sheath cells exhibited different intensities of smooth muscle  $\alpha$ -actin labelling. (Magn. x380)

**Figure 26**

Immunofluorescent histochemistry of transformed dermal sheath cells when labelled with a panspecific polyclonal antibody to transforming growth factor- $\beta$ . Labelling is diffuse throughout the cytoplasm, with a slight concentration around the nucleus. (Magn. x70)

## Discussion

### 3.5.1.1 Hair follicle dermal cells *in vitro*

In the past two decades, the isolation and characterisation of cells *in vitro* has greatly progressed. Today cell culture is used as an experimental system in a wide variety of fields, from intracellular biochemistry to the behavioural expression of individual cells and tissues. However, a major problem is that highly differentiated cells often alter their characteristics in the artificial environment of the culture vessel. Dermal papilla cells are one of a minority of cell types that apparently maintain many of their *in vivo* characteristics, this having been discussed in chapter 1. By isolating dermal papilla cells from a variety of different animal tissues, it was hoped that common links could be established that would help in further characterisation of this interesting cell population.

Due to the limited availability of tissue, not all follicular components were able to be cultured from all the species obtained. Tissue was only obtained in a non-sterile condition and samples were prone to contamination. In all cases the initial cultures were not dissimilar to those described by Jahoda and Oliver (1981). The observation that early on, primary explants would often become centrally necrotic even though cells on the periphery were healthy and proliferating is common in explant culture, and may just reflect inadequacies in the culture medium or changes in the extracellular environment. In vibrissae, both the DS and DP are highly vascularized (Scott, 1955), so when these are removed and placed in culture, the central cells of the dermal papilla are no longer being directly supplied with metabolites and therefore may get starved of nutrients. Central necrosis may occur as a result of this. Nevertheless, from the cells which did survive, this was the first time that these cells had been grown in culture.

Aggregate formation of rat vibrissa DP cells (Jahoda and Oliver, 1984 and section 3.4.1.1) is a phenomenon rarely seen in other cell types, although equivalent morphologies can be induced by the action of additional extracellular factors (Harris et al., 1984, de Wever, 1990). However, cultured DP cells from a variety of different

animal species, including human, do not always exhibit the aggregated phenomenon. In the previous results, this variability appears to be extended within the species examined. Reynolds (1989), showed that aggregative behaviour was specific to DP cells derived from the vibrissae, when compared to their pelage follicle counterparts. However, the DP cells isolated from the sinus follicles of the animals described in the previous chapter, showed no aggregative behaviour. Could this lack of this clumping behaviour reflect the stage of the hair cycle from which the original DP was isolated. Mink sinus follicles at the time of dissection were mostly in the telogen stage of the cycle, and from the histology (Fig 4b) the dermal papilla was virtually indiscernable from the dermal sheath and dermal stalk cells. Therefore, this lack of aggregative behaviour might be as a result of the papillae being in telogen.

Jahoda et al (1991) proposed that smooth muscle  $\alpha$ -actin could be used as a marker for DP and DS cells in culture. In support of this both DP and DS cells from the grey squirrel were both positive for smooth muscle  $\alpha$ -actin.

Grey squirrel DP cells had multinuclear bodies, which were often arranged in a roseate pattern. Bi-nucleate DP cells have been previously reported (Reynolds, 1989; Jahoda and Oliver, 1981), however 6 to 7 nuclei were discovered in the grey squirrel cells (Fig 18g-j). Multiple nuclei are present in myogenic cells (Yaffe, 1969), but these nuclei are generally arranged in a line, before fusing to form myotubes (Wakelam, 1985). The grey squirrel cells were arranged in a roseate pattern. Polynuclear grey squirrel cells were larger than neighbouring mononuclear cells, and therefore it may be that these cells have formed by fusion. However, in one cell it appeared that these smaller nuclei may be dividing from the main nucleus (Fig. 18i), therefore creating a multinucleate cell, especially if after nuclear division, cytokinesis does not occur. An extreme of this is the multinuclear bodies occurring in the *Drosophila* embryo, where the embryonic cell undergoes 13 rounds of nuclear division without cell division, producing a single cell with 6000 nuclei.

In contrast to the grey squirrel cells, the stoat DP and DS cells did not spread rapidly into the culture vessel. Both the stoat DS and DP cells had compacted and

rounder morphology nearer the explant, whereas those at the edge had a more flattened appearance. This is not unusual as the cells lose their attachment from the main explant and attach to the plastic. Mink and polecat cells were dissimilar, with mink DP cells being reminiscent of rat skin fibroblasts, whereas the polecat DS were broad, flat and irregularly shaped, with no obvious polarity. This is in contrast with the fibroblasts from each population, both of which were very spindly and neuronal in shape. Although the cells appeared stable, it is unclear how healthy they were because of the large vacuoles adjacent to the nucleus. Vacuoles adjacent to nuclei, are generally considered as a sign of necrosis (Wylie et al., 1980). However, another possibility is that they could be storing lipids. Fat storage cells or adipocytes (Green, 1979) are comprised mainly of a large vacuole, with negligible cytoplasm. Adipocytes are thought to be derived from a fibroblast precursor, and indeed 3T3 fibroblasts have been made to differentiate into the fat cells by the action of foetal bovine serum (Green, 1979). Therefore, what may be occurring with the mink and polecat fibroblasts, is that they are in the intermediate stages between the fibroblast precursor and mature adipocyte. In both animals there is an extensive fat layer subjacent to the dermis (fig 6a & 11), and when the fibroblasts are cultured, the skin biopsy grows dermis side down on the culture vessel. If these precursor cells could be identified, it would be logical that they would be nearest the fat layer with a possible inward differentiation process. Therefore, it may be that by culturing these cells in this manner, one may be preferentially favouring growth from this precursor stock. Alternatively, if 3T3 fibroblasts, a highly transformed cell line, could be stimulated into fat cell differentiation by FBS, then it may be that the mink and fibroblast populations may also be sensitive to the action of FBS.

Francz et al., (1991) described that like fibroblasts, dermal papilla cells in culture could be thought of as a stem cell system, with cells showing increasing signs of differentiation during their time in culture. If this is the case, then differences in cell morphology in the primary explant may be due to this progressive differentiation.

Morphological differences between hair follicle derived dermal cells and skin fibroblasts have been previously reported for rat (Jahoda and Oliver, 1984) and human (Messenger et al, 1986). Present studies of cultured cells from different species support these results, in that they give further evidence that the dermis, rather than consisting of a homogeneous fibroblast composition, is composed of a heterogeneous number of cells. However, it is unclear how this heterogeneity might be created. Bayreuther et al., (1988) points towards a stem cell type differentiation within the dermal fibroblasts and follicularly derived DP cells (Francz et al., 1991), however these concepts are largely derived from *in vitro* work.

Dermal papilla and dermal sheath differentiation of *in vivo* sinus follicles is more complicated. Both come from the dermal condensation present at the start of follicle development. However, there is circumstantial evidence that they are derived from neural crest cells. Lumsden (1987, 1988), showed that the dental papilla were neural crest derivatives. Furthermore, dermal condensation events prior to dental papilla formation are morphologically very similar to that of sinus follicle formation, with a large number of identically expressed molecules (Chiquet-Ehrismann et al., 1986, Heine et al., 1987, Thesleff et al., 1987, Thesleff et al., 1988, Gonzalez et al. 1990, Lyons et al., 1990, Trautman et al., 1991, Vaahtokari et al., 1991, Vainio and Thesleff, 1992 and Kaplan and Holbrook, 1994). It was shown by Oliver (1973), that oral epithelium could form hair follicles when recombined with a dermal papilla, and moreover, Reynolds (1989) was able to stimulate dentine forming structure when recombined with hair epidermal germinative cells and tooth adult dental papilla. When considering all these similarities in morphology, expression and inductive capabilities, there is the real possibility that the vibrissa DP and DS could be neural crest derivatives.

Evidence for a differentiation system within dermal sheath cells has also been discussed by Reynolds et al., (1993). Dermal sheath cells derived from different proximo-distal regions of the rat hair follicle were found to have different amounts of smooth muscle  $\alpha$ -actin expression. Bjorkerud (1991) described smooth muscle  $\alpha$ -actin

as a differentiation specific filamentous marker. Therefore, it may be that the upper DS are less differentiated because they have a reduced expression of smooth muscle  $\alpha$ -actin.

### 3.5.1.2 Transformed DS cells

Morphologically, and to a degree behaviourally, tDS cells are distinct from isolated DS. However, it is of particular interest that these *in vitro* tDS cells express smooth muscle  $\alpha$ -actin, even with reduced expression. Reynolds et al.,(1993) noted that cultured DS from different levels in the follicle had various amounts of smooth muscle  $\alpha$ -actin expression. Therefore, it is possible that isolated DS from follicles came from a variety of regions in the proximo-distal arrangement of the follicle. When tDS cells were first observed, they were seen as large proliferative pockets within the normal DS cells layer. This culture was then passaged and the resulting cultures classed as tDS cells. Therefore, rather than a single tDS cell line, early on there would have been a heterogeneous population of the proliferative tDS cells and DS cells. Due to the increased proliferation of the tDS cells, subsequent passages would yield a relatively homogeneous tDS cell line, nevertheless, these cells should not really be classed as single cell line.

Transformed DS population doubling time (PDT) was significantly less than both DP and DS cells, however this increased rate proliferation was not greater than that shown by skin fibroblasts. Transformed DS population doubling time was five hours faster than that of DS cells (39.4 hrs) over the same time period (Reynolds, 1989). DP cell population doubling time, shown by Jahoda and Oliver (1984), was also consistently slower (39.1 hrs), however skin fibroblast PDT was 23.4hrs and therefore considerably faster than tDS, DS and DP cells alike. Once again, the fact that these experiments were performed at a relatively low passage number means that the population may have comprised of a mixture of fast and slow growing cells. Cloning of these tDS cells, would be of great interest as one could then isolate a possible immortal

cell line of smooth muscle  $\alpha$ -actin positive cells. Future experiments would be to isolate an immortal population of smooth muscle  $\alpha$ -actin positive cells by cloning (Freshney, 1983). Dermal sheath cell implants with germinative cells behave more like DP cells and stimulate follicle production (Reynolds, 1989). If the tDS cells are a highly proliferative DS cell line, then it would be of great interest to see if they had inductive properties. Immortal rat DP cells with fibre producing qualities have been isolated by (Filsell et al., 1994), but as yet an immortal or transformed DS cell line has not been isolated.

### **3.5.1.3 DP *in vitro* aggregation**

Aggregation phenomenon in DP cells was first described by Jahoda and Oliver (1984). Furthermore, Messenger et al. (1986) later reproduced this work with human DP cells, and found that although they aggregated, this clumping was not as marked as that shown by rat vibrissa DP cells. In the species examined we found that contrary to these discoveries, there was no obvious aggregation. However, Messenger et al. (1986) stated that aggregation was more pronounced in later cultures of human DP cells. In the species examined, passage numbers were always low, and therefore cells may not have had the time to aggregate. Jahoda and Oliver (1984) briefly discussed how these aggregates were formed, however by closer analysis it was possible to divide the aggregation steps into distinct categories from subconfluent to clumped. This technique worked well when cells were in the middle of a particular phenotypic phase, however interpretation of phenotypes at the transition from one phase to another were harder to distinguish. This was further compounded by the fact that the aggregation process did not occur simultaneously in every culture dish, but generally appeared as a wave effect, with some areas aggregating whilst others were still confluent. Nevertheless, it was still possible to categorise each phenotypic stage in a particular DP cell line. Therefore, it was feasible to investigate each stage in the formation of an aggregated vessel.

Aggregates similar to DP cell aggregation *in vitro* have not previously been identified, nevertheless an interesting parallel has been discovered between DP cells and smooth muscle cells. De Wever et al.,(1990) showed that calf aortic smooth muscle cells retracted and formed aggregates in culture on induction with serotonin, a vasodilator or constrictor of blood vessels. This contraction could also be stimulated by FBS. These aggregates are morphologically similar to the aggregates formed by DP cells and furthermore, both cell types are positive for the contractile actin isoform smooth muscle  $\alpha$ -actin (Jahoda et al. 1991, Vandekerchove and Weber, 1979, Skalli et al., 1987) Moreover, recent unpublished data from our group indicates an increased smooth muscle  $\alpha$ -actin labelling within the DP *in vitro* aggregates. Therefore, there is circumstantial evidence that contraction of cells may be involved in some aspects of aggregation phenomenon. However, de Wever et al., (1990) was capable of producing these aggregates within a couple of hours, whereas DP aggregation, in a more concentrated FBS environment, took several days to form. Further possible mechanisms of aggregate formation will be discussed later.

#### **3.5.1.4 DP cell proliferation and growth curve**

Focal proliferation, as a method of creating mesenchymal condensations, was discounted by Bard (1990), primarily because temporal control was too slow to account for the condensation effect. Earlier work by Wessells and Roesner (1965) showed that this was true of *in vivo* hair follicle dermal condensation. *In vitro* dermal papilla proliferation was found to decrease during the aggregation process, with a low maintenance of cell division in the fully clumped state. This also compliments the findings of Summerbell and Wolpert (1972), who showed that the mitotic index was inversely proportional to cell density within the condensing mesenchyme of the limb. When one looks at the micrograph of Brdu labelling (Fig. 29b), the number of dividing cells is diffuse across the whole field of the cells and not in distinct focal zones coinciding with the future sites of aggregation.

### 3.5.1.5 Super aggregated DP cells (SAD)

Organ culture is commonly used to investigate the events in development. Feather, hair, tooth and kidney being good examples of this. However, the role of cell culture in forming developmental tissue or organs has only just begun. *In vitro* epidermal cells have been used for a long time now to restore damaged tissue and reduce scarring by transplantation, and *in vitro* DP cells have been able to induce follicle formation in a follicular sites. However, in both these cases, the functionality of the cells is restored only when brought back into contact with the *in vivo* system.

Therefore, is it possible for cells in culture to maintain their *in vivo* properties? In a series of experiments, Reynolds and Jahoda (1994) were able to break down the hair follicle into all its major components, culture the respective cells and replace them in a similar spatial distribution as that found in the *in vivo* follicle. From this complex recombination, a *de novo* follicle was produced. This is probably the most advanced example of cell culture organ formation. Furthermore, it shows that cell culture techniques, can often reflect events *in vivo*.

One of the most currently studied of these *in vitro* models is the micromass culture of chondrocytes (Ahrens et al., 1977). In this case, dissociated mesenchymal cells from a developing wing bud are able to form a condensed mesenchyme, that is morphologically similar to the *in vivo* system, and furthermore, can produce cartilage specific collagen (Dessau et al., 1980). Moreover, by manipulating this model with growth factors, direct insights into chondrogenesis have been discovered (Sakai & Langille, 1992, Mackie et al, 1987, Frenz et al, 1994, Leonard et al, 1991, Zanetti & Solursh, 1984, Jiang et al, 1993)

Large organotypic structures have been noted in recombination cultures of dermal papilla and germinative epidermal cells, from rat and human derived tissues (Reynolds, 1989; Reynolds and Jahoda, 1993). These form as 3D structures that often

protrude from the culture vessel, with the epidermal cells being engulfed within the DP cells.

In the SAD cells described above, similar tissue specific structures were produced, however, on closer examination their internal anatomy was very different. Overcrowding in culture vessels usually results in detachment and necrosis of fibroblast-like cells, however in long term DP cultures, cells adopted a dormant-like state, remaining in their aggregated phenotype. In many of these cultures large three dimensional tissue specific structures were produced. In some cases, monolayers or empty areas of culture vessel were adjacent to these structures. It is therefore apparent that the DP cells have become detached from the culture vessel and pulled up into these structures. However, these structures did not occur overnight but were formed gradually. Organotypic structures remained in culture over long periods with no external signs of necrosis. On closer examination, the structures had layers of DP cells enclosing a "soup" of ECM and rounded DP cells. Contrary to their external appearance, cells within the super aggregates had a variety of cell morphologies and health. Cells on the periphery were thin and flattened, as if still attached to the base of the culture vessel. Many of the cells within these super aggregates had large granular lysosomes. This granular arrangement was reminiscent of mast cells (Combs et al., 1965). Furthermore, these cell structures had previously been noted in *in vitro* epidermal germinative/DP cells recombinations from rat and human tissue (Reynolds, 1989; Reynolds et al., 1993) Dying cells were generally apoptotic, with large condensing nuclei and reduced cytoplasm (Wyllie et al., 1980). This contradicts events in the hair follicle, because the only tissue type that continually expresses bcl-2, a known regulatory gene of apoptosis (Hockenberry et al., 1990), is the dermal papilla (Stenn et al., 1994).

A number of cells within the super aggregates also had a mast cell like appearance (Combs et al., 1965). Several studies (Robbins et al., 1970 ;Lipetz & Cristofalo, 1972; Brandes et al., 1972) have indicated that cell ageing in culture is paralleled with a vast increase in the number of lysosomes within the cytoplasm.

Moreover, the amount of golgi apparatus and cytoplasmic fibrils increases, and nuclei often becoming lobed. Therefore, the appearance of these lytic vesicles, and slight lobing of the nucleus in the *in vitro* super aggregated DP cells may be indicative of the age of these cells in culture. However, a greater abundance of golgi apparatus and cytoplasmic fibrils was not obvious and therefore it remains unclear whether this excess of lytic material is a result of the cells physical age.

In figure (31 l), a dermal papilla cell was engulfing the condensed nucleus from a previously apoptotic cell. Therefore, these 'mast' like DP cells may in fact contain apoptotic bodies from other dying cells. With this in house control of unhealthy cells, it is not surprising that necrosis was restricted to a few cells. Moreover, it appears that rather than a random production of these tissue specific structures, the cells were controlling their formation and maintaining them. The internal composition of these structures was also of interest, cells were often embedded in a homogeneous extracellular matrix (ECM). *In vitro* DP cells are capable of a wide range of ECM production, (Choung et al., 1991, Taylor et al., 1992) however, without *in situ* hybridization, immunofluorescent histochemistry or immunogold labelling, it remains unclear what this ECM might be.

Certain aspects of super aggregated dermal papilla cell morphology is reminiscent of the *in vivo* dermal papilla. During anagen, granulocytes are not present in the DP, however, as the follicle enters catagen and telogen, autolysis of papilla cells occurs, with a reduction in cytoplasm and synthesis of granular lysosomes (Young , 1980). During anagen the DP cells are GAG rich, and hence contain active golgi zones, but in telogen GAG production is terminated, with a corresponding decline in golgi bodies (Young & Oliver, 1976). Golgi bodies in the micrographs of SAD cells were not obvious. Therefore, there are correlations between the telogen papilla and these super aggregated structures, however their morphology contradicts their *in vitro* activity. It is known that DP cells in culture express an abundance of molecules, in particular glycosaminoglycans and proteoglycans (Taylor et al., 1992). Nevertheless, these molecules are largely absent from the *in vivo* telogen papilla. The lack of

obvious golgi bodies is also unusual, as these organelles are fundamental to GAG production.

Looking at these structures in the *in vitro* context, rather than trying to draw conclusions between the *in vitro* and *in vivo* tissues, it is apparent that there are a number of interesting points. First, the fact that once these super aggregates are formed they remain in a relatively healthy state with only a few signs of necrotic tissue, and second, that these structures occur spontaneously in what is a monoculture of cells, when previously these structure have only occurred in epidermal/dermal recombinations. Furthermore, within these aggregates there are a variety of cell morphologies, extracellular molecules and a number of lytic or apoptotic cells. Therefore, it appears that rather than a random event in the culture vessel these structures are able to form, regulate and maintain themselves in a controlled manner.

**Chapter 4**  
**Cell Motility**  
**Introduction**

**4.1.1.1 *In vitro* DP cell migration as a method of aggregate formation**

Cellular condensations are crucial morphogenetic events in embryonic development. A major interest in this phenomena, relates to pattern formation and spatial/temporal distributions of structures, however much still remains to be understood about the creation of individual cell condensations or aggregations. In the latter context, the initiation of bone, somite and skin appendage condensations, involves many similar features.

Bard (1990) describes four possible mechanisms by which mesenchymal condensates might form *in vivo*:- focal proliferation of cells; a loss of extracellular matrix around cells; increases in adhesion between cells; and a physical movement or migration of cells to a specific foci. Most probably, a combination of these events occurs, but any one aspect may play a greater role in a particular condensate formation.

Concerning appendage formation, thymidine labelling studies suggest that differential mitosis is involved in producing feather dermal condensations (Wessells, 1965), whereas Wessells and Roesner (1965) consider that local proliferation does not appear to be a factor in hair primordia condensation. Bard (1990) believes the latter to be the case for all condensation events, as "local proliferation is too slow to account for formation".

Focal migration has largely been neglected, although one group has postulated that it is involved in chondrogenesis and feather formation (Ede and Agerbak, 1968, Ede et al, 1971). Others suggest that migration is unlikely to be important since precondensation cells are relatively close together (Davidson 1978), and therefore distances are too small to necessitate focal movement.

*In vivo* investigations of dermal condensation point towards focal adhesion (Choung et al, 1991) and changes in the extracellular matrix as being important mechanisms of condensation (Chiquet-Ehrismann et al.1986; Vainio and Thesleff, 1992; Thesleff et al 1990; Trautman et al , 1991, Vainio et al, 1991; Adams and Watt, 1993). Other studies suggest that growth factors may also play a significant role (Heine et al., 1987; Lehnert and Akhurst, 1988; Gonzalez et al., 1990; Jones et al., 1991; Leonard et al.,1991; Millan et al.,1991; Hirai et al., 1992; Blessing et al., 1993).

Direct investigation of the molecular basis for appendage condensations has been hampered by the relative absence of *in vitro* models. One established system (Harris, Stopak and Warner, 1984) shows skin fibroblasts capable of creating cell aggregations via the influence of extracellular matrix and tractional forces. Our approach has been to use hair follicle cells, since a direct lineage can be demonstrated from the early mesenchymal condensation to the adult dermal papilla, situated at the base of the adult follicle (Wessells and Roesner, 1965).

Since adult rat whisker follicle dermal papilla cells form distinct aggregates in culture in the absence of epidermal cells, or the addition of artificial extracellular matrix (Jahoda and Oliver,1981,1984), it has been postulated that this behaviour mimics *in vivo* condensations. This idea is supported by the fact that cultured papilla cells, when harvested and re-implanted into skin, spontaneously reform condensations Jahoda et al.(1993). Moreover, once formed these papilla like structures are stable, and do not disperse, over the lifetime of the animal. In the previous chapter the aggregative behaviour of papilla cells was described, and the mitotic activity during aggregate formation was measured. Proliferation within the aggregates was shown not to be the driving force in *in vitro* aggregate formation (section 3.4.1.3). In the course of this chapter the phenomenon of dermal papilla cell aggregation *in vitro* is examined experimentally.

Migration assays were performed on cultured dermal papilla (DP) cells to test whether a motile activity might be involved in aggregate formation? DP cell motility was examined at different stages during the establishment of aggregates, and

furthermore, conditioned medium was retrieved from each of the different phenotypic stages described in section (3.4.1.1). This medium was then assayed against the DP cells and other related cells.

Evidence from the pilot experiments (Appendix 1) indicated that a) cells from different stages of the aggregation process had different motile abilities and b) that conditioned medium derived from each of the different aggregation phenotypes (3.4.1.1) stimulated different responses from dermal papilla cells. Further migration assays were carried out after the findings of these preliminary experiments to investigate the motile mechanisms behind this aggregation process.

From the preliminary experiments (Appendix 1), the greatest motility was stimulated from medium conditioned by aggregating dermal papilla cells (AG), (Fig. 27d). Fractionation of the aggregating dermal papilla conditioned medium, using microconcentrators, was applied as a method to isolate the molecular weight ranges in which the greatest motile activity occurred. Aggregating dermal papilla cell conditioned medium was also serially diluted and tested in the chemotactic chambers to ascertain the sensitivity of DP cells to the motility factor. Further investigations involved locally derived dermal cells, such as dermal sheath and skin fibroblast cells to assess the specificity of the influence produced by the dermal papilla cells.

#### **4.1.1.2 *In vitro* DP cell response to growth factors**

Motility within cells has been well documented *in vitro*, and in many cases attributed to a number of growth factors (Deuel et al., 1982, Seppa et al., 1982, Senior et al., 1983, Senior et al., 1986, Postlethwaite et al., 1987, Stoker et al., 1987,). In a few cases this growth factor induced motility has been attributed to specific *in vivo* functions, for example in relation to the role of platelet derived growth factors (PDGFs) in wound repair (Fingerle et al., 1989, Pierce et al., 1991). In chapter 1, a

review of molecules involved in mesenchymal and dermal condensation was carried out, from the literature it became apparent that the molecules TGF $\beta$  and bFGF were either expressed during condensation or were able to stimulate the condensation process. As motility has been suggested as a mechanism of condensation formation, growth factors were assayed in chemotactic chambers with dermal papilla cells to see what kind of response occurred. Furthermore, in this current study, the growth factors TGF  $\beta$ , bFGF and aFGF were assayed alongside conditioned medium from DP cells, as a comparative study of migration, and as a start to the process of identifying possible motility candidates.

#### **4.1.1.3 Transforming Growth factor $\beta$**

TGF  $\beta$  was first isolated as a molecule that could induce normal rat kidney (NRK) fibroblasts to grow and form colonies of cells in soft agar in the presence of epidermal growth factor (Roberts et al., 1981 ). Since then it has been discovered in many cell types, having a variety of different functions. After the initial purification and characterisation it was found to be a 25 KD homodimeric protein (Frolik et al., 1983, Assoian et al., 1983, Roberts et al., 1983). Since the first isolation it has been discovered that there are multiple forms of TGF  $\beta$ ; TGF $\beta$ <sub>1</sub>; TGF  $\beta$ <sub>2</sub>; TGF  $\beta$ <sub>3</sub>; each with subtly different expression domains within the developing embryo.

TGF  $\beta$  was first discovered in the developing hair follicle by Heine et al., (1987) using a polyclonal antibody to TGF  $\beta$ <sub>1</sub>. Its distribution was limited to a transient expression in the papilla, before the appearance of the hair germ, which increased in intensity as the follicle developed. However, TGF  $\beta$ <sub>1</sub> expression terminated in the adult follicle. This type of expression was also present during tooth formation, in the region of the dental papilla (Heine et al., 1987). The consistent expression of TGF  $\beta$  molecules in the condensing dermis of two epithelial/mesenchymal interactions implies a function for this molecule in the early development of skin appendages.

What is unclear is how TGF  $\beta$  molecules function in this role? TGF $\beta$ s has been shown to be multi functional (Sporn & Roberts, 1988), however, one function of TGF $\beta$  is that it is chemotactic to monocytes (Wahl et al., 1987) and human fibroblasts (Postlethwaite et al., 1987). Therefore, would *in vitro* dermal papilla cells respond in a chemotactic fashion to TGF  $\beta$ ?

#### 4.1.1.4 Fibroblast growth factors

Fibroblast growth factor (FGF) was first isolated from purified brain and pituitary extract (Gospodarowicz, 1974,1975) and given its name because of its mitogenic effects on fibroblasts. Later it was discovered that two forms existed, acidic FGF (aFGF) from the brain (Thomas et al., 1984 ) and basic FGF from the pituitary (Bohlen et al., 1984 )

Basic FGF was first described as a 146 amino acid single chain protein with a molecular weight of 16.5 KD (Esch et al., 1985, Simpson et al., 1987). Likewise, aFGF was characterized by classical protein sequencing and found to be 15.5KD containing 140 amino acids (Gimenez-Gallego et al, 1985). Acidic FGF and basic FGF are homologous with 55% sequence identity with each other at the protein level.

Both aFGF and bFGF have been noted in the developing hair follicle. During mesenchymal condensation, bFGF is present in the basal lamina and supra-basal layers of the pre-germ epidermis and hair germ (Gonzalez et al., 1990; Du Cross et al., 1993), and aFGF had a similar distribution (du Cros et al., 1993). Although the position of aFGF and bFGF at the epithelial/mesenchymal interface implies a distinct function, it still remains unclear how these molecules interact with the cells and the extracellular matrix during the development of the follicle. Senior et al., (1986) showed that bFGF acted as a chemoattractant for fibroblasts and astroglial cells. As bFGF and aFGF are expressed at the time of condensation formation of the hair germ, the question of whether these molecules have a chemoattractive effect on *in vitro* DP cells was addressed?

## **Materials and Methods**

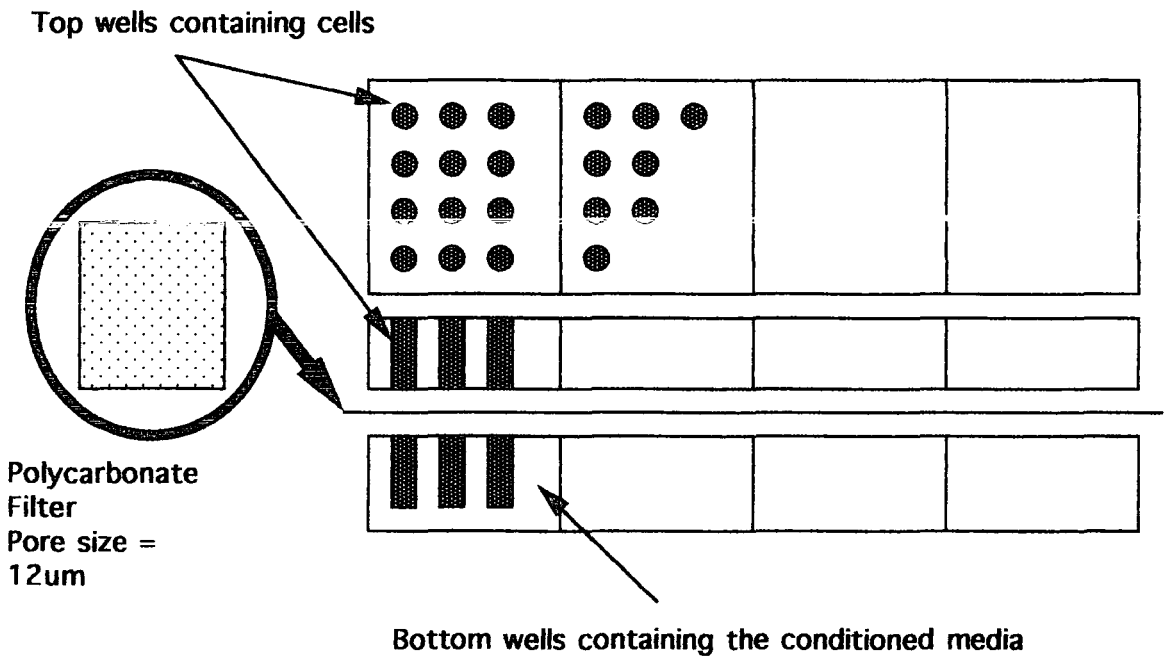
### **4.2.1.1 Conditioned Medium**

To prepare conditioned medium, cultures (maintained in 25cm<sup>2</sup> flasks) were washed three times in MEM, including a short incubation (10 minutes at 37°C, 5% CO<sub>2</sub>) . Cells were then incubated (37°C, 5% CO<sub>2</sub>) in 4 mls of MEM and antibiotics for 16hrs. The medium was then removed and centrifuged (5 mins, 2000rpm) to remove any cellular debris . The supernatant was removed and frozen (-80°C) or used immediately. Conditioned medium was always retrieved from the three dermal papilla cell phenotypic stages (subconfluent, aggregating and clumped) 2 days after a fresh renewal of the growth media.

### **4.2.1.2 Migration assay**

Assayed cells were exposed to test media using a 48 well modified Boydens chemotactic chamber (Nucleopore) (fig 32a). Cells were harvested as above and resuspended in 2.5mls of MEM containing 10% FBS (Gibco). The same batch of serum was used for all migration experiments. Particular care was taken to obtain a single cell suspension, using automatic pipette tips and fine disposable pasteur pipettes. Cells were counted using an haemocytometer, and appropriate numbers of cells were carefully pipetted into the top wells of the chamber using an automatic pipette, the test medium having already been prepared in the bottom compartments. Cells and test medium were separated by a 12µm pored polycarbonate membrane (Nucleopore). The chamber was then incubated at 37°C, 5% CO<sub>2</sub> for 4 hrs. After incubation the membrane was removed and fixed in 70% ethanol. Cells that had not migrated (those still on the upper surface) were scraped off, prior to a brief washing in distilled water. The remaining cells were then stained with geimsa stain (Sigma) according to the method of Freshney (1983). The membrane and cells were then mounted with DPX

**Fig 32a** A diagrammatic representation of the modified Boyden's chamber. Response cells were placed in the top wells with the response medium in the bottom wells, cells and medium were separated by a 12um pore size polycarbonate filter.



(BDH), under a coverslip (6.5 x 3.5 cm), on a microscope slide . Cell nuclei were counted using a Nikon binocular microscope (x10 objective.) and a grid eyepiece graticule. The area covered by the graticule was approximately  $\frac{1}{5}$  of the total migration area. The graticule was positioned at random within the field and all the cells counted within the area of the graticule. This was repeated and the mean number of cells calculated. Numbers were then extrapolated to get an estimate of the total number of migrated cells. If cell migration was very low then x4 objective was used and the whole field was counted.

#### **4.2.1.3 Relative motilities of different DP and Fi phenotypes to 10% FBS**

The relative motilities of dermal papilla cells at different stages of the aggregation process was tested by placing equal numbers of cells from culture dishes displaying the three aforementioned phenotypes (subconfluent ,SC; aggregating, AG; and clumped, CL DP cells) in the upper wells of the chamber and exposing them to 10% FBS in MEM, in the bottom wells. Confluent skin fibroblasts were used as a comparison. In these experiments  $1 \times 10^4$  cells were added to each 43 $\mu$ l well. Of the 48 wells ,12 wells were dedicated to each cell type. The layout of the experimental chamber is shown in appendix (2a). This experiment was repeated.

#### **4.2.1.4 The response of dermal papilla cells upon exposure to conditioned media from the different dermal papilla phenotypes.**

Motile influences produced by conditioned media from the different dermal papilla phenotypes were examined (Figure 27a,d,e). In successive experiments each phenotypic dermal papilla cell stage was tested against conditioned media from each of the different phases of aggregate formation and 10% FBS in MEM,so for example in one set of experiments, the response of subconfluent DP cells to SC, AG and CL cell media was assayed. Appendix (2b) illustrates the combination of cells in the top wells



with the conditioned media types in the bottom wells. In all cases, the number of cells used was  $1 \times 10^4$  per 43 $\mu$ l well. Each chamber consisted of 48 wells, with 12 wells per test media. Experiments were repeated in triplicate with some variation on the initial seeding density.

#### **4.2.1.5 Pre-aggregating DP cell migration**

An intermediate grouping of pre-aggregating DP cells, that is cells that were beginning to interact and pile up on each other (Fig. 27b,c), were assayed against the three DP conditioned medium types (SC, AG and CL CM) and 10% FBS in MEM, as described above. Twelve wells were dedicated to each response medium (appendix 2c), and  $1 \times 10^4$  cells were used per well. Chambers were incubated as described earlier.

#### **4.2.1.6 Molecular Weight separation of AG DP CM**

AG DP conditioned medium (CM) was concentrated into various molecular weight ranges by using two similar methods.

1) AG DP CM was concentrated into  $> 10\text{KD}$ ,  $< 10\text{KD}$  and  $> 30\text{KD}$ ,  $< 30\text{KD}$  molecular weights using Centricon-10 and Centricon-30 microconcentrators (Amicon) respectively. 1ml of crude AG DP CM was placed in the top compartment of each microconcentrator, which was then spun down at 6000rpm for 30 mins. Medium remaining above the filter was  $> 10\text{KD}$  and  $> 30\text{KD}$  depending on which centricon was used and the corresponding medium in the filtrate cup was  $< 10\text{KD}$  and  $< 30\text{KD}$  respectively. The medium was then re-diluted with MEM to regain the correct salt balance.

To concentrate the AG DP CM into a more accurate molecular weight range microsep microconcentrators (Filtron) were used.

2) AG DP CM was concentrated into > 1KD, > 3KD, > 5KD, > 10KD, > 30KD, > 50KD, > 100KD by the following method. Crude AG DP CM was placed into the top compartment as before. Each microsep concentrator was then spun at 7500 rpm for 1hr. As each step was completed, the non concentrated medium, that is, the medium that passed through the filter, was then applied to the next microconcentrator. Hence non-concentrated media from the 100KD microconcentrator was applied to the 50KD microconcentraor. Therefore creating a concentrate of > 50KD and less then 100KD. This was continued down through the range of microconcentrators to yield the aforementioned molecular weight cut offs in the medium. Conditioned media from the different protocols were then used for migration assays in the 48 well chemotactic chambers.

Expt 1) Subconfluent DP cells were presented with AG DP CM, 10% FBS and concentrated AG DP CM > 10KD and < 10KD (appendix 3a). Each chamber had twelve replicates for each of the test media, with  $1.2 \times 10^4$  cells put in each 43 $\mu$ l upper well. Chambers were incubated for 4hrs at 5% CO<sub>2</sub>, 37°C. Membranes were then removed, fixed and counted as described previously .

Expt 2) In a similar experiment, SC DP cells were presented with AG DP CM, 10% FBS and concentrated AG DP CM > 30KD and < 30KD (appendix 3b). Each 43 $\mu$ l well contained  $1.9 \times 10^4$  cells, and 12 wells were tested with each media sample. Incubation, membrane fixation, staining and counting was as described in section (4.2.1.2)

Expt 3) SC DP cells were presented with AG DP CM, AG DP CM > 10KD, AG DP CM > 3KD and AG DP CM > 1KD (appendix 3c). Cells were used at a density of  $1.2 \times 10^4$  in each 43 $\mu$ l well of the chamber, and 12 wells were tested with each media

sample. Incubation, membrane fixation, staining and counting was as described in section (4.2.1.2)

Expt 4) SC DP cells were presented with AG DP CM, AG DP CM > 100KD, AG DP CM > 50KD and AG DP CM > 30KD (appendix 3d). Assays were performed as described above (Expt 3). Incubation, membrane fixation, staining and counting was as described in section (4.2.1.2)

#### **4.2.1.7 DP cell migration in reduced or absent FBS**

In the basic assay protocol used for most experiments, cells were put in the upper wells of the test chambers in medium that contained 10% FBS. To test the effect of this foetal bovine serum on migration, AG DP cells were put into upper test wells in the presence of decreasing concentrations of FBS, and serum free MEM (appendix 4a). Cells were trypsinized as described previously. The action of the trypsin was then neutralized by resuspension in 10% FBS; the cells were spun down, and the 10% FBS poured off. Cells were then resuspended in MEM and centrifuged again. The final traces of 10% FBS / MEM were poured off and replaced with serum free MEM. Cells were counted and then diluted in equal volumes of 20%, 10%, 5% FBS and MEM to yield working dilutions of 10% FBS, 5% FBS, 1% FBS and MEM. Cells contained in each of these media were then aliquoted into 12 wells, at a final density of 8000 cells per well. Cells were assayed against AG DP conditioned medium, contained in the bottom wells under conditions as previously described. (section 4.2.1.2)

#### **4.2.1.8 Dilution effect of AG DP conditioned medium**

AG DP cells were assayed against serially diluted AG conditioned medium (appendix 4b). Conditioned medium was isolated as described previously, and was taken as

undiluted, represented as 100%. The 100% AG DP CM was then diluted with MEM, to provide dilutions of 50%, 10% and 1%. Twelve well replicates were dedicated to each dilution of conditioned medium, with 16 000 cells used per upper well. These response cells were dispersed in serum free MEM, to provide maximum opportunity of observing a motility response from the test conditioned medium. All other test conditions and subsequent procedures, were as previously described.

#### **4.2.2.1 Relative motilities of SC and Confl Fibroblast cells to 10% FCS**

The relative motilities of skin fibroblasts were tested by placing equal numbers of cells harvested from culture dishes in the subconfluent and confluent state in the upper wells of the chamber, and exposing them to 10% FBS in the bottom well. Skin fibroblasts were obtained as described in section (3.3.1.4). Confluent skin fibroblasts were maintained at confluency for 4 weeks prior to the experiment, therefore they represented the fibroblast equivalent of clumped DP cells. Ten thousand cells were added to each 43 $\mu$ l well, and 24 wells were dedicated to each of the subconfluent and confluent cell types. The layout of the experimental chamber is shown in (appendix 5a). Test conditions and analysis were as described previously in section (4.2.1.2).

#### **4.2.2.2 Skin fibroblast motility in response to conditioned medium from the three dermal papilla phenotypes**

Skin fibroblasts were used in a comparative set of experiments designed to test their responsiveness to the DP cell conditioned media. Subconfluent skin fibroblasts were used as response cells and tested against SC, AG and CL dermal papilla conditioned medium, and 10% FCS as a control. Cell density was  $2 \times 10^4$  per 43 $\mu$ l well. The experimental combinations can be seen in appendix (5b). Test conditions and subsequent analysis was standard.

#### **4.2.3.1 Cell migration to growth factors**

The effects of growth factors on dermal papilla cell motility was tested in the following series of experiments. Fibroblasts were also used as response cells for comparison.

#### **4.2.3.2 Cell response to Transforming Growth Factor beta**

Equal numbers of subconfluent DP cells and subconfluent skin fibroblasts were assayed with human transforming growth factor  $\beta_1$  (TGF $\beta_1$ ) ( R & D Systems) at concentrations of 1, 0.1 and 0.01 ng/ml in MEM, and 10% FBS. Cells were maintained in 10% FBS. The basic experimental protocol being described earlier (section 4.2.1.2). Half of the upper level of the chemotactic chamber contained the DP cells, with the other half holding the Fi population. Both cell types were dispersed into the test wells in 10% FBS in MEM, at a density of 3000 cells per well. Each concentration of TGF $\beta$  was present in 12 bottom wells, 6 below the DP cells and 6 underneath the fibroblasts. The exact layout of the different concentrations of growth factors is shown in appendix (6a). The chamber was incubated as before, and after incubation the cell membrane was mounted, stained and counted as described in section (4.2.1.2).

#### **4.2.3.3 Cell response to basic Fibroblast Growth Factor**

In a similar experiment to section (4.2.3.2) SC DP and SC Fi cells were tested against 5, 0.5, 0.05, 0.005 ng/ml of bovine bFGF ( R & D Systems) in MEM. The procedure and layout of the experiment was the same as that described in section (4.2.3.2) (appendix 6b), except that 6500 cells of each type were used in each top well. The chamber was incubated as in section (4.2.1.2). A repeat of this assay was carried out,

but in it the lowest concentration of bFGF (0.005 ng/ml) was removed, and replaced with 10% FBS in MEM.

#### **4.2.3.4 Cell response to acidic fibroblast Growth Factor**

In duplicate experiments, subconfluent dermal papilla and fibroblast cells were presented with bovine acidic FGF ( R & D Systems). Both cell types were tested against concentrations of 5, 0.5 and 0.05ng/ml aFGF with 10% FBS in MEM as a control. Cell densities in each top well were 3400 for the first experiment, and 3100 for the repeat. Experimental procedures were as described in section (4.2.1.2). A diagram of the experimental layout is in appendix (6c)

#### **4.2.3.5 DP cell migration to TGF $\beta$ and bFGF when response cells were tested in the presence of MEM**

As a consequence of earlier findings, in this experiment, when response cells were dispersed into the top wells, they were in MEM without FBS. Migration of DP cells to various concentrations of TGF $\beta$  was assayed as described above (section 4.2.3.2). Pre-aggregating DP cells were presented with AG DP CM and 1, 0.1 and 0.01 ng/ml of TGF $\beta$  in MEM. Similar to the previous experiment (section 4.2.3.3) DP cells were presented with the following concentrations of bFGF in MEM, 5ng/ml, 0.5ng/ml and 0.05ng/ml.

#### **4.2.4.1 DS and tDS migration to DP conditioned medium**

Subconfluent dermal sheath and tDS cells were assayed against conditioned media from each of the different phases of DP cell aggregate formation, and 10% FBS as a control. Appendix 7a illustrates the combination of cells in the top wells, with the conditioned media types in the bottom wells. The number of cells used per 43 $\mu$ l well

was variable (see table 9). In each of the 48 well chambers, 12 wells each contained one of the four test media. In other experiments DS cells were tested under the same test conditions as those of the DS described above. The experiments were repeated.

## Results

### 4.3.1.1 Migration of *in vitro* DP cells from the different phenotypic stages

Each of the three papilla cell phenotypes responded to 10% FBS in a distinct but consistent manner (Table 2). In the example shown graphically (fig. 33a), DP cells from the aggregating phase were significantly more motile than subconfluent DP and clumped DP cells (1.5-15 fold). No significant differences were found between the latter two phenotypes. In repeat experiments aggregating cells were 7 to 30 times more motile than CL cells which always exhibited a very low motility in comparison to other dermal cell phenotypes. In one repeat experiment the SC dermal papilla cells showed significantly greater motility than the clumped dermal papilla cells (fig. 33b). However, AG dermal papilla cell migration was always significantly greater than the other two dermal papilla phenotypes. Confluent skin fibroblasts used as a comparison to dermal papilla cell migration, were significantly more motile than SC and CL DP cells.

**Table 3** Migration of *in vitro* subconfluent, aggregating and clumped DP cells in response to 10% FBS

| cell density | test media | Number of migrated cells |       |       |          |
|--------------|------------|--------------------------|-------|-------|----------|
|              |            | SC DP                    | AG DP | CL DP | Confl Fi |
| 10 000       | 10% FBS    | 4 ± 1                    | 61±12 | 2±1   | 33±6     |
| 10 000       | 10% FBS    | 18±2                     | 27±4  | 4±2   | 89±15    |

### 4.3.2.1 Migration of DP cells in response to conditioned medium

### 4.3.2.2 Subconfluent DP cells:

Consistent with the above findings (section 4.3.1.1), migration of subconfluent dermal papilla cells was generally at an overall low level. However, when assayed to conditioned medium from the different phenotypes, migration was always significantly greater in the presence of conditioned medium from aggregating cells compared with the other medium (fig. 34a,b,c). In all replicates, subconfluent DP cells demonstrated the same trend, with movement two to four fold greater towards AG DP CM.

**Table 4a** Migration of subconfluent dermal papilla cells in response to SC, AG and CL dermal papilla conditioned medium

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |          |         |          |
|--------------|-----------|--|----------|---------|----------|
|              |           | 10% FBS  | SC DP CM | AG DPCM | CL DP CM |
| 10000        | SC DP     | 2±1  | 7±2      | 42±10   | 4±2      |
| 7000         | SC DP     | 56±13  | 20±4     | 36±6    | 10±3     |
| 4000         | SC DP     | 18±1   | 16±1     | 88±2    | 33±2     |

#### 4.3.2.3 Aggregating DP cells:

Where cell numbers were equivalent, aggregating DP cells tested against the three conditioned media demonstrated a generally overall higher level of migration than SC cells, (consistent with Table 2). However, the aggregating conditioned medium again elicited the biggest motility response the responding AG cells(fig. 35 a,b,c). In repeat experiments, stimulation of aggregating cells by AG conditioned medium was consistently greater than with SC and CL medium.

**Table 4b** Migration of aggregating dermal papilla cells in response to SC, AG and CL dermal papilla conditioned medium

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |          |         |          |
|--------------|-----------|--|----------|---------|----------|
|              |           | 10% FBS  | SC DP CM | AG DPCM | CL DP CM |
| 10 000       | AG DP     | 11±4   | 39±8     | 108±16  | 57±10    |
| 40 000       | AG DP     | 41±5   | 4±1      | 105±5   | 39±11    |
| 40 000       | AG DP     | 25±3   | 33±3     | 135±9   | 32±5     |

#### 4.3.2.4 Clumped DP cells:

Again, consistent with previous findings, clumped dermal papilla cells, presented with the three variations of conditioned medium, all demonstrated very low levels of motility (fig. 36 a,b). No significant difference was discovered between any of the test conditions. The poor migratory capacity of these cells in the presence of any conditioned medium or the 10% FBS in MEM was a repeated phenomenon.

**Table 4c** Migration of clumped dermal papilla cells in response to SC, AG and CL dermal papilla conditioned medium

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |          |         |          |
|--------------|-----------|--|----------|---------|----------|
|              |           | 10% FBS  | SC DP CM | AG DPCM | CL DP CM |
| 10 000       | CL DP     | 2±1  | 0.4±0.2  | 4±1     | 4±1      |
| 14 000       | CL DP     | 2±1  | 0.3±0.2  | 1±0.5   | 2±1      |

(Fractions of cell numbers come about, since counts represent the mean number of total cells from twelve replicate wells)

#### 4.3.2.5 Pre-aggregating DP cells:

For pre-aggregating DP cells, that is, cells that were not fully aggregating by the criteria in section (3.4.1.1), general motility was greater than that shown previously for subconfluent cells. More interestingly, although direct comparison was not made, migration was consistently high and appeared to be even greater than that demonstrated

by the AG DP cells. As with all previous experiments, migration was always strongest towards the AG DP CM. Response of cells to SC DP CM and 10% FBS was comparable, while the response to CL conditioned medium was generally greater than to these two, but still significantly less than that shown by cells to AG DP CM (fig. 37a,b,c).

**Table 4d** Migration of pre aggregating dermal papilla cells in response to SC, AG and CL dermal papilla conditioned medium, in three replicate experiments.

|              |           | No. of cells moving across the membrane in response to the test medium |          |          |          |
|--------------|-----------|--|----------|----------|----------|
| Cell density | Cell type | 10% FBS  | SC DP CM | AG DP CM | CL DP CM |
| 10 000       | Pre-AG DP | 88±16  | 77±15    | 185±25   | 105±10   |
| 10 000       | Pre-AG DP | 47±8   | 26±3     | 184±38   | 109±17   |
| 10 000       | Pre-AG DP | 80±13  | 82±8     | 281±30   | 180±17   |

### 4.3.3.1 Molecular Weight separation of AG DP CM

Fractionation at 10KD: Results show the largest migration of pre aggregating papilla cells was to AG DP CM (table 5a,fig. 38a), however of the two fractions the highest motility was induced by CM > 10KD. The response to aggregating conditioned medium < 10KD, was no different to the response to 10% FBS in MEM (fig. 38a).

Fractionation at 30KD: Subconfluent DP migration was greatest towards the AG DP CM (Fig 38b, table 4b), however the next greatest stimulus was provided by the conditioned medium > 30KD, which in turn was greater than the response elicited by the 10% FBS. Conditioned medium < 30KD stimulated motility at a level that was not significantly different than that produced by 10% FBS in MEM.

Results with the second concentration system (Tables 5c, 5d) showed an overall reduction in migration compared with the above (Table 5a, 5b), but again migration was greatest to AG DP CM. However, the results show a subtle change in motility in favour of CM > 30KD but <50KD (Fig. 38c), with no significant difference between conditioned medium >10KD, >3KD and >1KD (Fig 38d), all produced a very low response

**Table 5a** Migration of pre aggregating dermal papilla cells in response to 10% FBS, AG DP CM, AG DP CM > 10KD and AG DP CM < 10KD.

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |          |           |           |
|--------------|-----------|--|----------|-----------|-----------|
|              |           | 10% FBS  | AG DP CM | CM > 10KD | CM < 10KD |
| 12000        | Pre-AG DP | 32±10  | 315±20   | 187±13    | 26±9      |

**Table 5b** Migration of pre aggregating dermal papilla cells in response to 10% FBS, AG DP CM, AG DP CM > 30KD and AG DP CM < 30KD.

|              |           | No. of cells moving across the membrane in response to the test medium |          |           |           |
|--------------|-----------|--|----------|-----------|-----------|
| Cell density | Cell type | 10% FBS  | AG DP CM | CM > 30KD | CM < 30KD |
| 19000        | Pre-AG DP | 122±33   | 910±84   | 369±37    | 126±13    |

**Table 5c** Migration of subconfluent dermal papilla cells in response to AG DP CM, AG DP CM > 10KD, AG DP CM > 3KD and AG DP CM > 1KD.

|              |           | No. of cells moving across the membrane in response to the test medium |           |          |          |
|--------------|-----------|--|-----------|----------|----------|
| Cell density | Cell type | AG DP CM   | CM > 10KD | CM > 3KD | CM > 1KD |
| 12000        | SC DP     | 50±5   | 9±4       | 7±2      | 11±4     |

**Table 5d** Migration of subconfluent dermal papilla cells in response to AG DP CM, AG DP CM > 100KD, AG DP CM > 50KD and AG DP CM > 30KD.

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |            |            |           |
|--------------|-----------|--|------------|------------|-----------|
|              |           | AG DP CM   | CM > 100KD | CM > 50 KD | CM > 30KD |
| 12000        | SC DP     | 29±4   | 3±1        | 6±1        | 14±4      |

#### **4.3.4.1 Effect of the presence of foetal bovine serum in with the test cells on AG DP cell response**

Consistent with previous findings overall migration of aggregating dermal papilla cells was at a high level, however there was a considerable difference in cell migration when the test cells were presented to the AG conditioned media in the presence of different concentrations of FBS. There was a gradual decrease in migration relative to an increase in FBS, with the largest migratory response from the cells that were in zero or low concentrations of FBS. Cells that were put into the upper well in MEM alone showed approximately three times more migration to the AG conditioned medium, than cells that were dispersed with MEM containing 10% FBS, the regular experimental protocol (Table 5a, fig. 39 a,b).

**Table 6a** Migration of aggregating dermal papilla cells, dispersed in MEM, 1%FBS in MEM, 5% FBS in MEM and 10% FBS in MEM, in response to AG dermal papilla conditioned medium.

|              |                      | Response Medium:-<br>Aggregating DP CM |
|--------------|----------------------|--|
| Cell density | Cell type and medium | Mean no. of migrated cells             |
| 8000         | AG DP in MEM         | 370±50                                 |
| 8000         | AG DP in 1% FBS      | 273±24                                 |
| 8000         | AG DP in 5% FBS      | 159±15                                 |
| 8000         | AG DP in 10% FBS     | 101±8                                  |
| 8000         | AG DP in MEM         | 199±18                                 |
| 8000         | AG DP in 1% FBS      | 195±26                                 |
| 8000         | AG DP in 5% FBS      | 158±14                                 |
| 8000         | AG DP in 10% FBS     | 66±6                                   |

#### 4.3.4.2 Dilution effect of AG DP conditioned medium

Increasing dilutions of AG DP CM resulted in a corresponding decrease in migration by AG DP cells. Cell movement was greatest to undiluted conditioned medium. There was a decrease in migration of nearly half with a 50% dilution, and a further decrease when the conditioned medium was diluted to 10%. There was no further decrease in effect between the 10% and 1% dilutions of conditioned medium (Table 5b, fig. 39c).

**Table 6b** Migration of aggregating dermal papilla cells in response to the following dilutions of AG dermal papilla conditioned medium; 100%; 50%, 10% and 1% AG DP CM.

|              |           | No. of cells moving across the membrane in response to the test medium |           |           |          |
|--------------|-----------|--|-----------|-----------|----------|
| Cell density | Cell type | 100% AG CM   | 50% AG CM | 10% AG CM | 1% AG CM |
| 16000        | AG DP     | 94±14  | 52±9      | 19±2      | 17±3     |

#### 4.3.4.3 Relative motility of SC and Confl Fi to 10% FBS

Both subconfluent and confluent fibroblasts responded well, with a high migration to 10% FBS. There was no major difference between the two phenotypes (fig 40a).

**Table 7a** Migration of *in vitro* subconfluent and confluent fibroblast cells in response to 10% FBS

|              |            | No. of cells moving across the membrane in response to the test medium |          |
|--------------|------------|--|----------|
| Cell density | Test media | SC Fi  | Confl Fi |
| 10 000       | 10% FBS    | 106±9  | 92±13    |

#### 4.3.4.4 Subconfluent Fi cells migration to DP CM

In subconfluent skin fibroblasts (SC Fi) assayed for a migratory response to conditioned medium from the three phenotypes of dermal papilla cells. Response was always greatest towards the AG DP conditioned medium (fig 40b,c). However, there was no significant difference between the movement of fibroblasts to AG DP conditioned medium and 10% FBS (means of  $667\pm49$  and  $516\pm103$ ) respectively, whereas aggregating DP cells showed an increased response to AG DP conditioned medium when compared to 10% FBS. (section 4.3.2.3). The response to SC and CL DP CM was somewhat lower (Table 6b), and therefore the general trend follows a similar pattern to that shown by dermal papilla cells.

**Table 7b** Migration of subconfluent fibroblast cells in response to SC, AG and CL dermal papilla conditioned medium

| Cell density | Cell type | No. of cells moving across the membrane in response to the test medium |            |             |            |
|--------------|-----------|--|------------|-------------|------------|
|              |           | 10% FBS  | SC DP CM   | AG DP CM    | CL DP CM   |
| 16 000       | SC Fi     | $516\pm103$  | $418\pm50$ | $667\pm49$  | $300\pm39$ |
| 17 000       | SC Fi     | $687\pm55$   | $266\pm25$ | $730\pm108$ | $301\pm46$ |

#### **4.3.5.1. Cell migration to characterized growth factors**

#### **4.3.5.2. Cell migration to TGF $\beta_1$**

Fibroblasts demonstrated generally higher levels of motility than the DP cells. DP cells maintained a uniform motility to all the concentrations of TGF $\beta_1$ . In one experiment, 10% FBS produced a slight increase (Fig. 41a), however in the other DP motility was not significantly different in response to TGF $\beta_1$  and 10% FBS. In parallel experiments Fi motility was consistently higher to 10% FBS than to any of the growth factor concentrations. The trends in response of both DP and Fi cells to migration to different levels of TGF $\beta_1$  were difficult to establish. For example, in fibroblast migration there were conflicting results. Migration in one replicate (fig. 41a) was greatest to the concentrations 0.1ng/ml and 0.01 ng/ml TGF $\beta_1$ , however motility in another (Fig. 41b) was highest at the 1ng/ml and 0.01ng/ml concentrations.

**Table 8a** Migration of subconfluent DP cells and skin fibroblasts in response to 1ng/ml TGFβ; 0.1ng/ml TGFβ; 0.01ng/ml TGFβ and 10% foetal bovine serum

| Cell Density      | Cell type | No. of cells moving across the membrane in response to the test medium |                          |                            |                              |
|-------------------|-----------|--|--------------------------|----------------------------|------------------------------|
|                   |           | 10% FBS  | 1ng/ml TGFβ <sub>1</sub> | 0.1ng/ml TGFβ <sub>1</sub> | 0.01 ng/ml TGFβ <sub>1</sub> |
| 9500<br>(fig 41a) | SC DP     | 109±15   | 74±20                    | 112±22                     | 98±17                        |
| 8300<br>(fig 41b) | SC DP     | 458±70   | 345±52                   | 360±55                     | 335±40                       |
| 9500<br>(fig 41a) | SC Fi     | 263±19   | 52±14                    | 139±23                     | 165±28                       |
| 8300<br>(fig 41b) | SC Fi     | 1103±197   | 349±33                   | 248±48                     | 378±56                       |

#### 4.3.5.3. Cell migration to bFGF

Consistent with TGFβ data, in both replicates (Fig 42 a,b) fibroblasts showed greater overall migration towards 10% FBS and bFGF concentrations than DP cells. In one experiment (Fig. 42a), DP migration peaked at 0.5ng/ml bFGF but this was significantly less than the migration to 10% FBS. In the same experiment, fibroblast migration also peaked at this concentration, however this was not significantly greater than migration to 0.05ng/ml bFGF. Once again there were inconsistencies between experiments, since in the second assay (fig. 42b) dermal papilla migration was greatest to 0.05ng/ml bFGF, but there was no such peak for fibroblast migration.

**Table 8b** Migration of subconfluent DP cells and skin fibroblasts in response to 5ng/ml bFGF; 0.5ng/ml bFGF; 0.05ng/ml bFGF; 0.005ng/ml bFGF and 10% foetal bovine serum

| Cell Density      | Cell type | No. of cells moving across the membrane in response to the test medium |             |               |                |                 |
|-------------------|-----------|--|-------------|---------------|----------------|-----------------|
|                   |           | 10% FBS  | 5ng/ml bFGF | 0.5ng/ml bFGF | 0.05ng/ml bFGF | 0.005ng/ml bFGF |
| 7300<br>(Fig 42a) | SC DP     | 110±17   | 24±3        | 67±9          | 21±3           |                 |
| 6500<br>(Fig 42b) | SC DP     |  | 43±18       | 41±9          | 202±40         | 38±9            |
| 7300<br>(Fig 42a) | SC Fi     | 436±48   | 162±30      | 224±29        | 197±34         |                 |
| 6500<br>(Fig 42b) | SC Fi     |  | 256±47      | 445±66        | 358±57         | 400±72          |

#### 4.3.5.4. Cell migration to aFGF

As in all previous assays, fibroblast migration was greater than DP cells, and for both cell types the largest response was to 10% FBS. In both experiments there was very little difference in DP cell motility in response to growth factors and 10% FBS (Fig. 43a,b). Only the 0.05ng/ml concentration in the first assay produced a significant lowering of cell movement. Fibroblast cells in the first assay showed a peak in motility at 0.5 ng/ml aFGF (Fig. 43a), however once again this was not repeated in the second assay (Fig. 43b).

**Table 8c** Migration of subconfluent DP cells and skin fibroblasts in response to 5ng/ml aFGF; 0.5ng/ml aFGF; 0.05ng/ml aFGF; and 10% foetal bovine serum

| Cell density      | Cell type | No. of cells moving across the membrane in response to the test medium |             |               |                |
|-------------------|-----------|--|-------------|---------------|----------------|
|                   |           | 10% FBS  | 5ng/ml aFGF | 0.5ng/ml aFGF | 0.05ng/ml aFGF |
| 3400<br>(Fig 43a) | SC DP     | 25±5   | 16±7        | 20±5          | 5±1            |
| 3100<br>(Fig 43b) | SC DP     | 11±3   | 7±4         | 7±2           | 7±3            |
| 3400<br>(Fig 43a) | SC Fi     | 152±35   | 57±19       | 140±18        | 67±15          |
| 3100<br>(Fig 43b) | SC Fi     | 97±21  | 43±25       | 29±21         | 25±7           |

### 4.3.6.1 DP cell migration in absence of 10% FBS to growth factors TGFβ<sub>1</sub> and bFGF

### 4.3.6.2 Pre-aggregating DP cell migration to TGFβ<sub>1</sub>

In the absence of serum in the top wells, dermal papilla migration was highest to AG DP CM and to 1ng/ml TGFβ<sub>1</sub>, with no significant difference between these two conditions. Migration was significantly lower at the 0.1 and 0.01 ng/ml concentrations of TGF β<sub>1</sub>. (fig. 44)

**Table 9a** Migration of pre-aggregating DP cells, dispersed in serum free medium, in response to 1ng/ml TGFβ; 0.1ng/ml TGFβ; 0.01ng/ml TGFβ and aggregating DP conditioned medium.

| Cell Density | Cell Type | No. of cells moving across the membrane in response to the test medium |                          |                            |                             |
|--------------|-----------|--|--------------------------|----------------------------|-----------------------------|
|              |           | AG DP CM   | 1ng/ml TGFβ <sub>1</sub> | 0.1ng/ml TGFβ <sub>1</sub> | 0.01ng/ml TGFβ <sub>1</sub> |
| 14000        | Pre-AG DP | 171±17   | 180±18                   | 112±14                     | 112±13                      |

### 4.3.6.3. Pre-aggregating DP cell migration to bFGF

DP migration was greatest to AG DP CM, consistent with previous findings. The level of response produced by the bFGF was lower at all concentrations, and indeed there was no significant difference in motility at any of the bFGF concentrations (fig 45).

**Table 9b** Migration of pre-aggregating DP cells, dispersed in serum free medium, in response to 5ng/ml bFGF; 0.5ng/ml bFGF; 0.05ng/ml bFGF and aggregating DP conditioned medium.

| Cell Density | Cell type | No. of cells moving across the membrane in response to the test medium |             |               |                |
|--------------|-----------|--|-------------|---------------|----------------|
|              |           | AG DP CM   | 5ng/ml bFGF | 0.5ng/ml bFGF | 0.05ng/ml bFGF |
| 14000        | Pre-AG DP | 110±10   | 67±7        | 78±8          | 77±12          |

#### 4.3.7.1 DS and tDS cells migration to DP conditioned medium

DS and tDS cells responded to DP conditioned medium, in a pattern similar to their DP counter parts, in that migration was largest to the AG DP CM. Dermal sheath cells showed variable results between experiments in their response to SC DP CM and when presented with 10% FBS. In the first assay (fig 46a) migration in the presence of 10% FBS was lower than that to AG DP CM, whereas migration in the second experiment (fig. 46b) was greatest to 10% FBS. In both assays was a reduction in migration to the CL DP conditioned medium from that seen with AG DP CM, but this was not as great as that shown previously by the DP cells. tDS cells produced similar results, but at a lower overall level of motility (fig. 46c,d). The response of tDS cells was greatest to AG DP conditioned medium, and migration to the SC DP and CL DP conditioned medium was reduced significantly in both assays. The tDS cells generally showed greater motility in response to conditioned medium than to 10% FBS.

**Table 10** Migration of subconfluent dermal sheath cells, and transformed dermal sheath cells in response to SC, AG and CL dermal papilla conditioned medium

| Cell Density | Cell type | No. of cells moving across the membrane in response to the test medium |          |          |          |
|--------------|-----------|--|----------|----------|----------|
|              |           | 10% FBS  | SC DP CM | AG DP CM | CL DP CM |
| 13000        | SC DS     | 530±63   | 791±52   | 1237±69  | 1079±72  |
| 9500         | SC DS     | 354±26   | 8±3      | 208±5    | 145±12   |
| 25000        | SC tDS    | 66±7   | 106±5    | 338±32   | 149±13   |
| 15000        | SC tDS    | 20±3   | 25±5     | 117±6    | 37±5     |

Fig. 33a

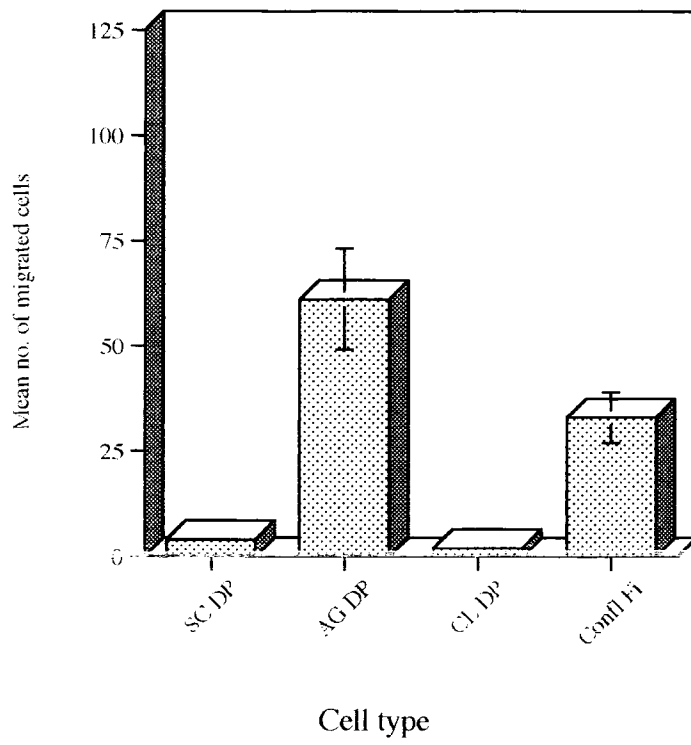


Fig. 33b

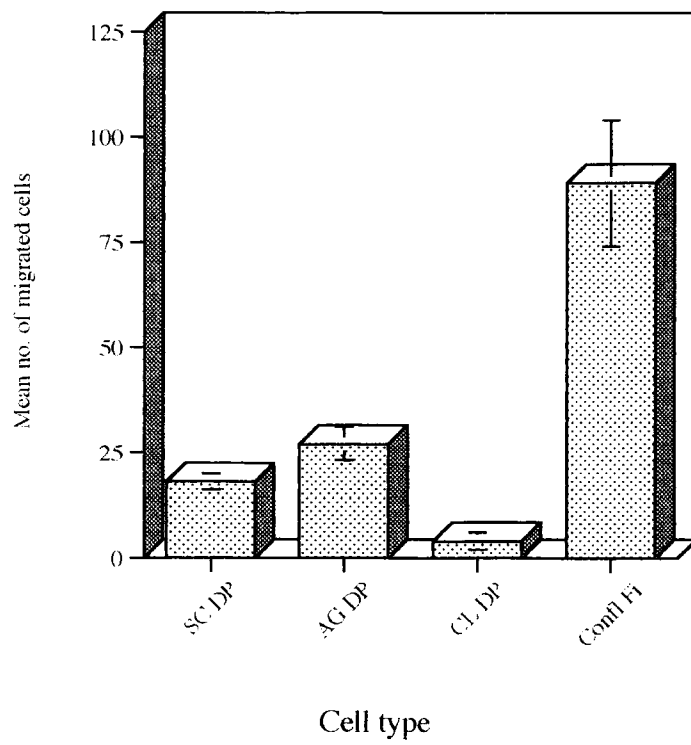


Fig. 34a

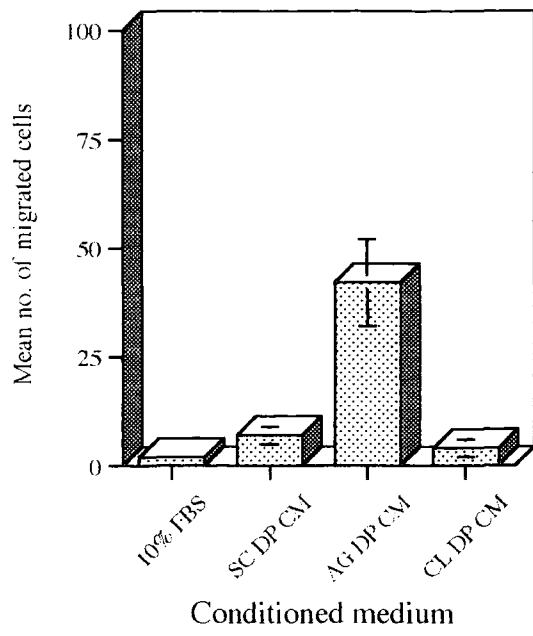


Fig. 34b

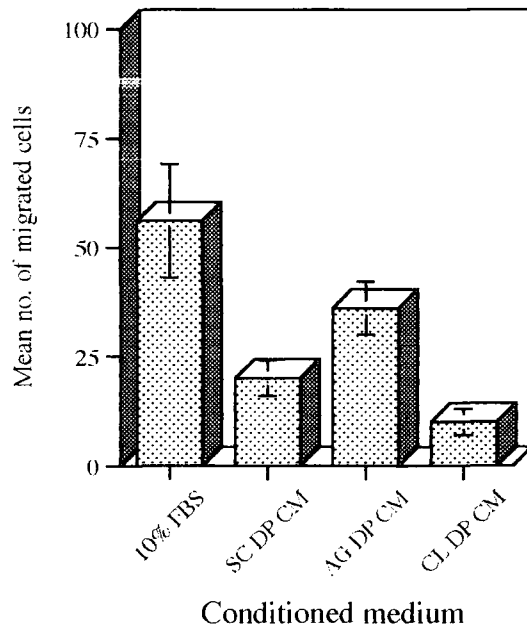


Fig. 34c

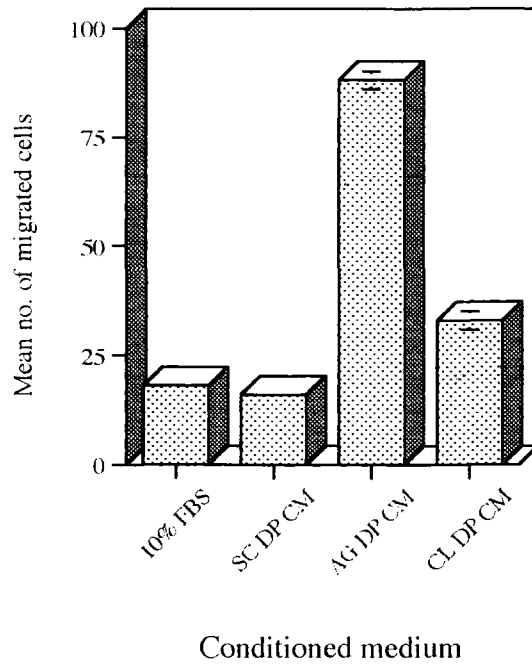


Fig. 35a

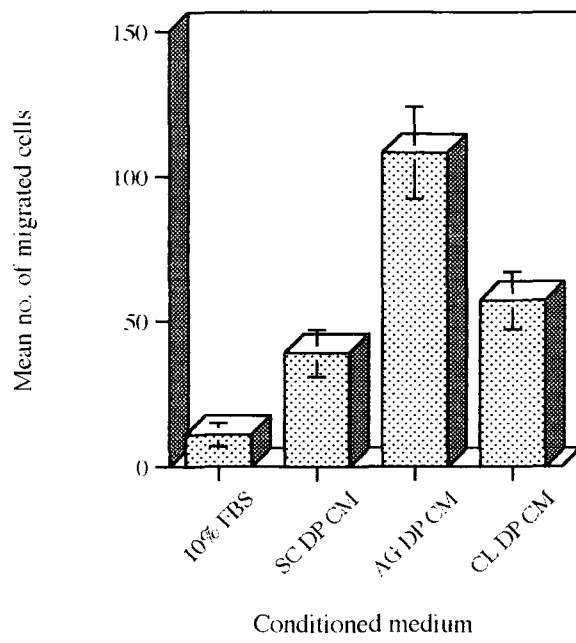


Fig. 35b

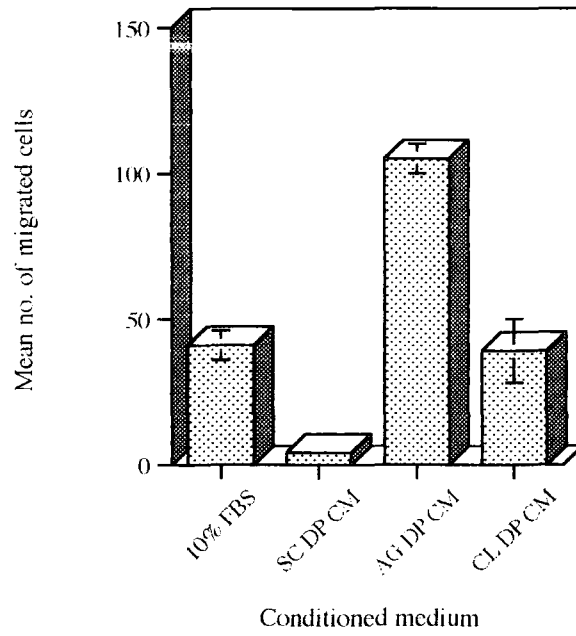


Fig. 35c

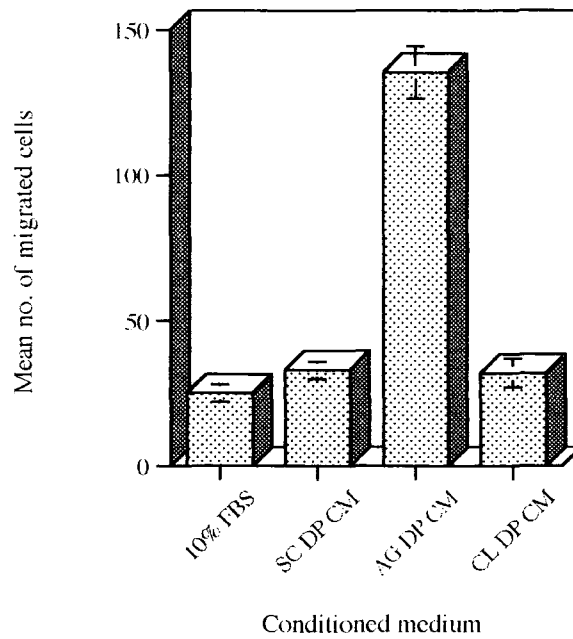


Fig. 36a

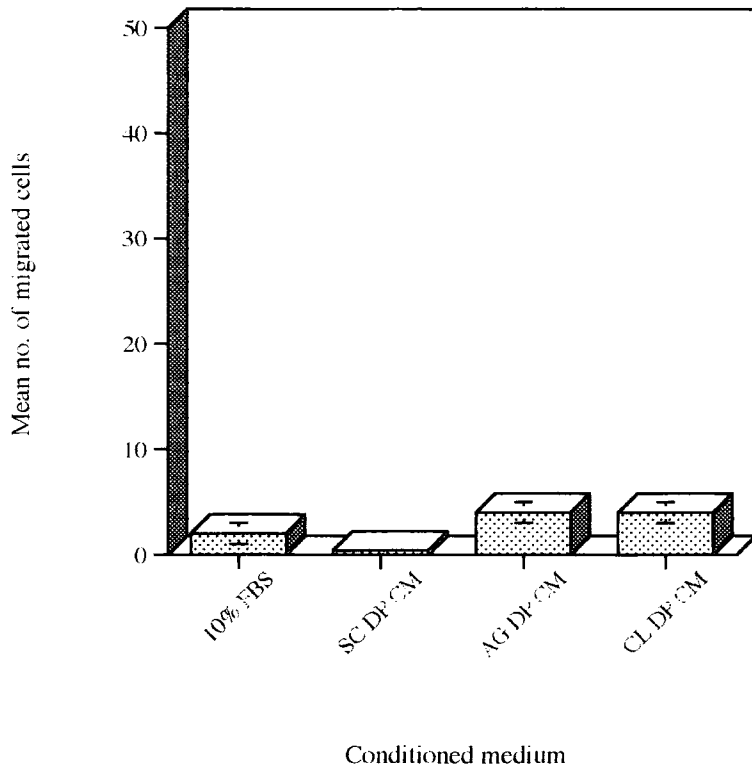


Fig. 36b

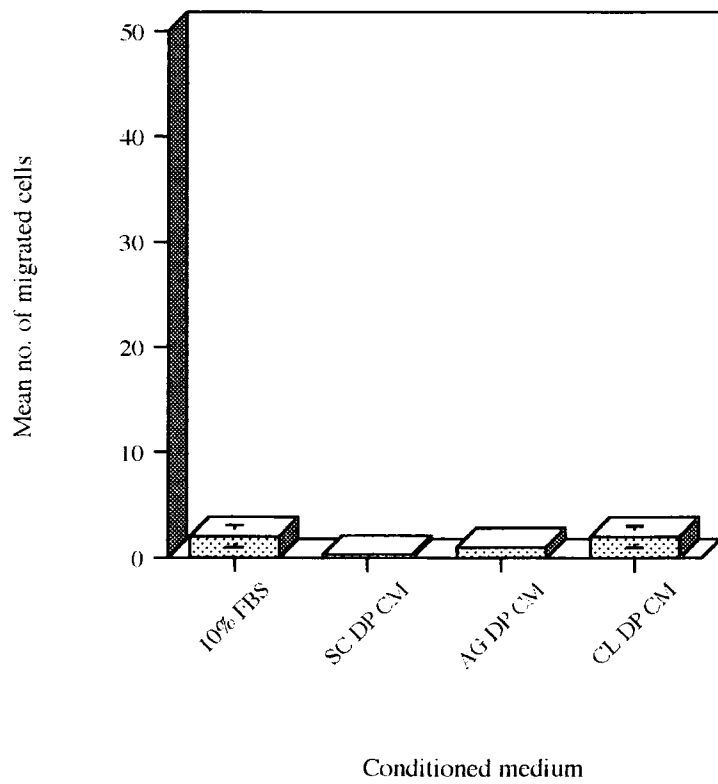


Fig. 37a

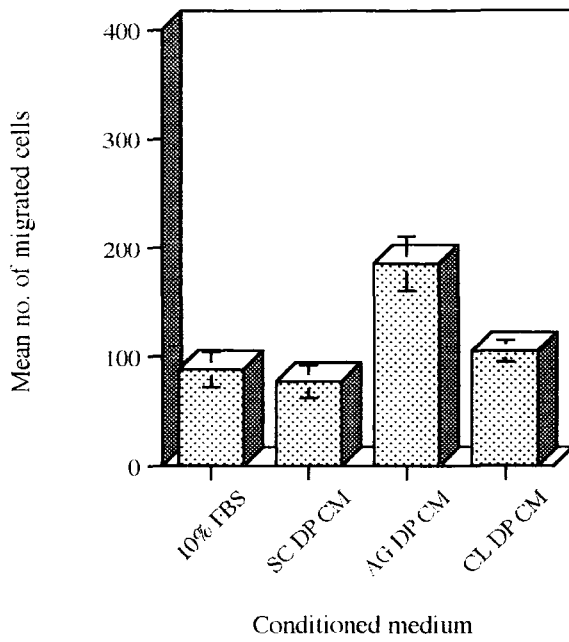


Fig. 37b

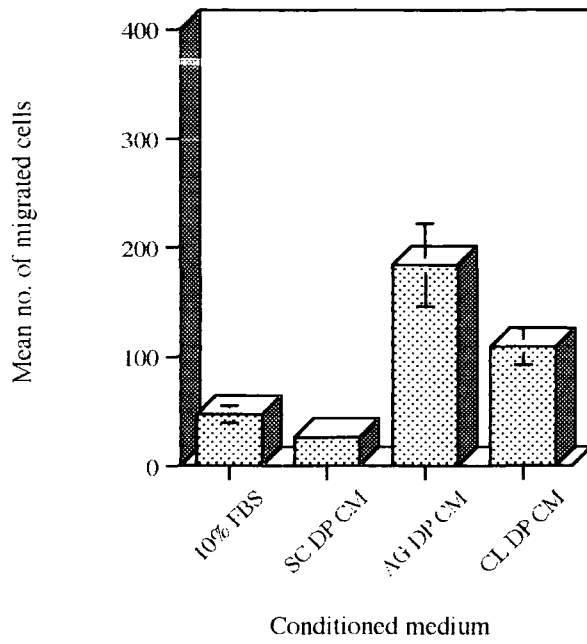


Fig. 37c

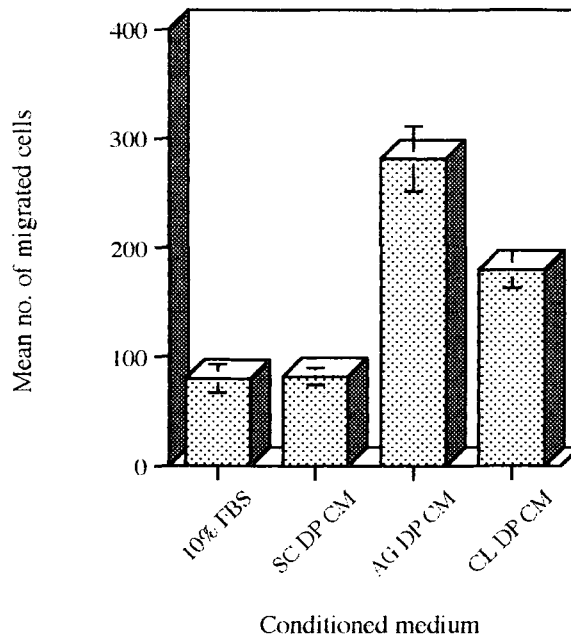


Fig. 38a

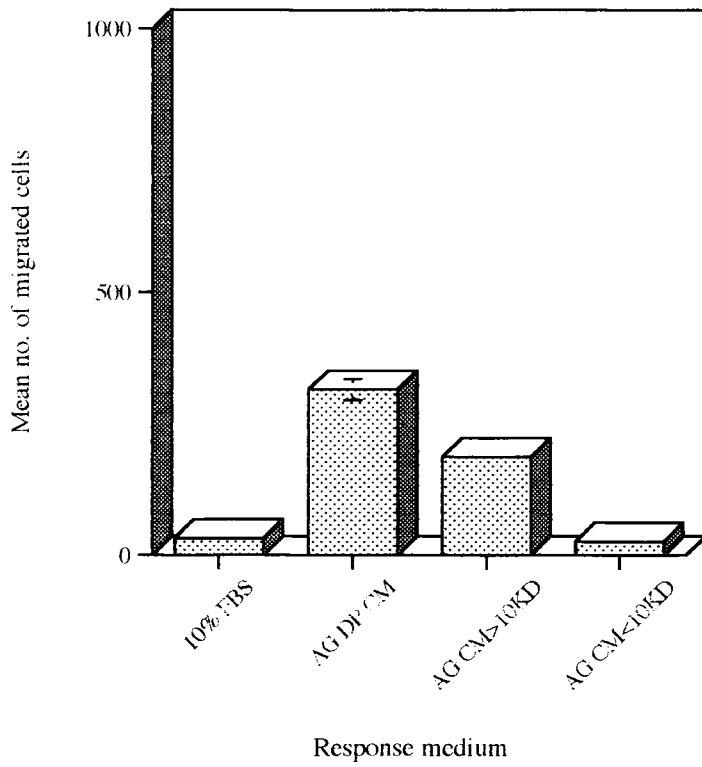


Fig. 38b

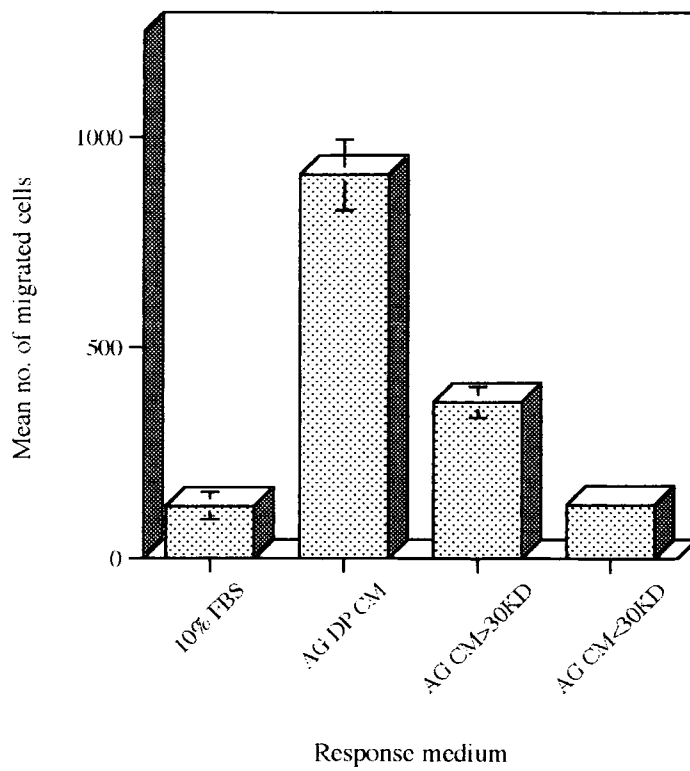


Fig. 38c

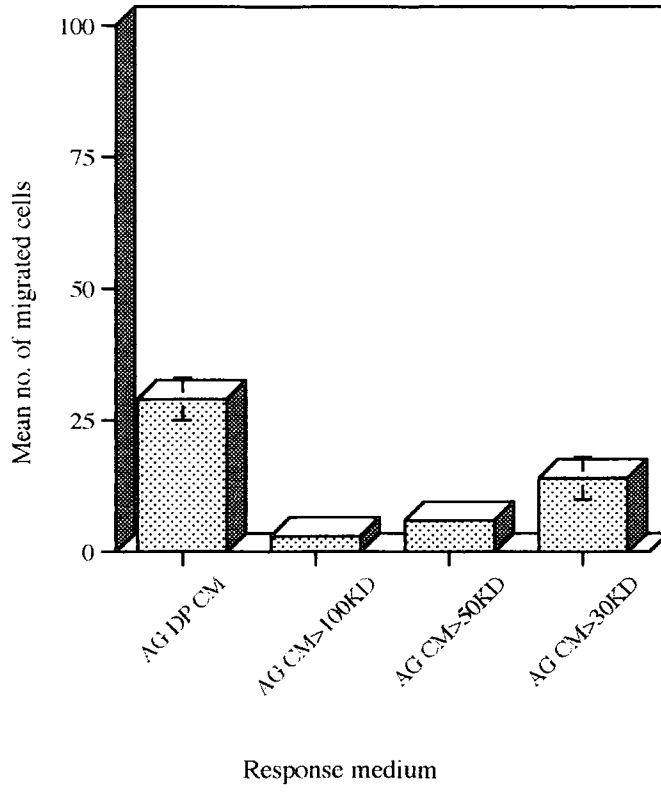


Fig. 38d

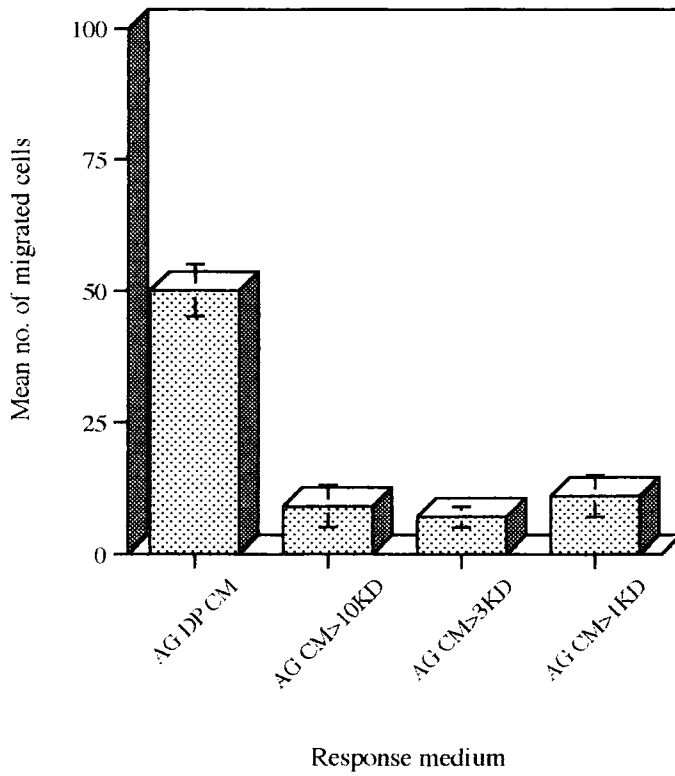
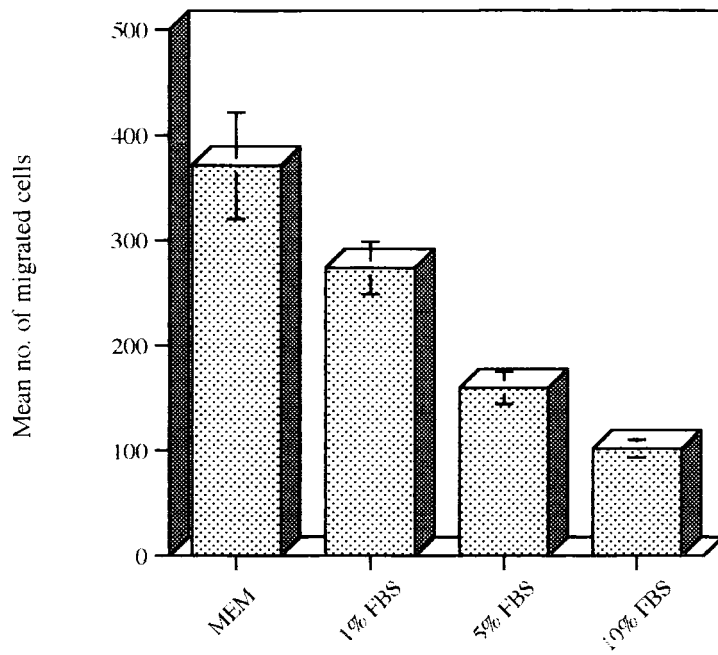
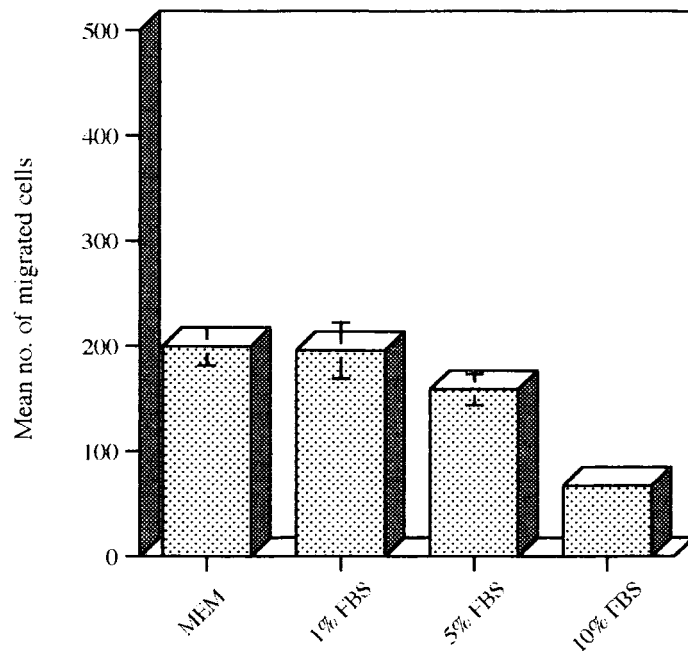


Fig. 39a



Response of DP cells in diluted serum or serum free medium to AG DP CM

Fig. 39b



Response of DP cells in diluted serum or serum free medium to AG DP CM

Fig. 39c

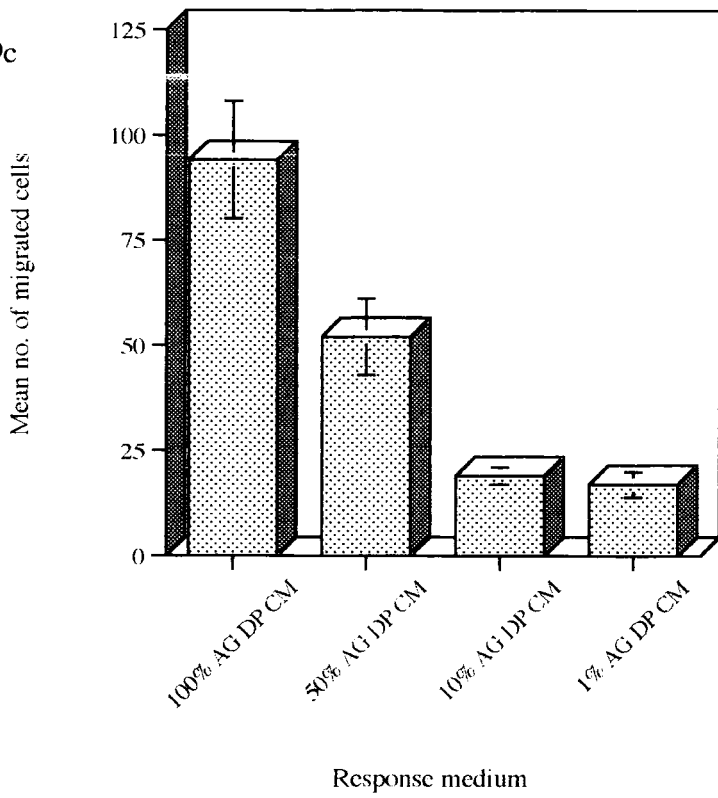


Fig. 40a

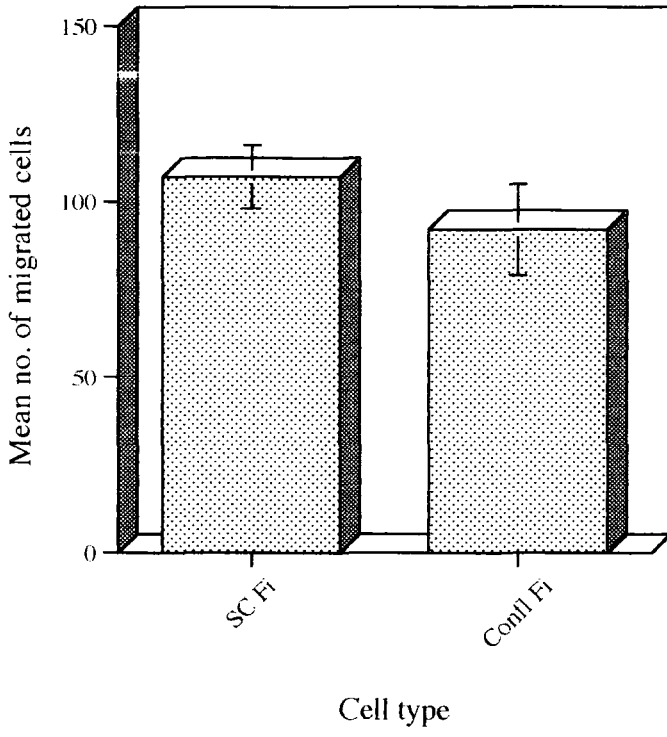


Fig. 40b

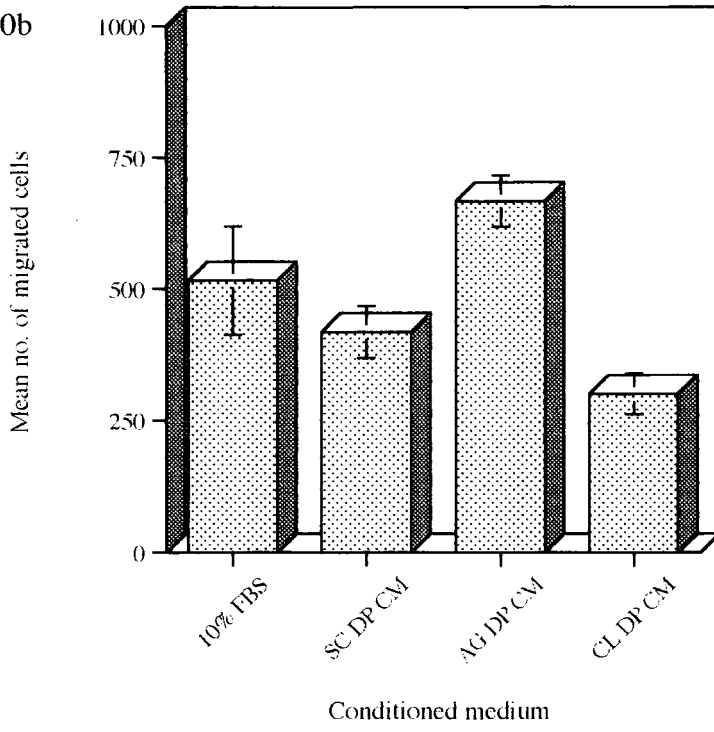


Fig. 40c

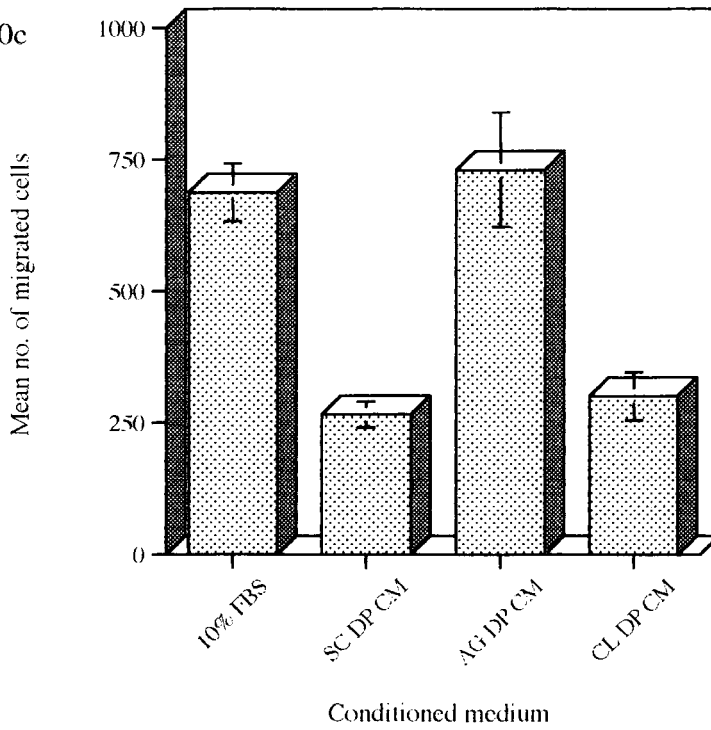


Fig. 41a

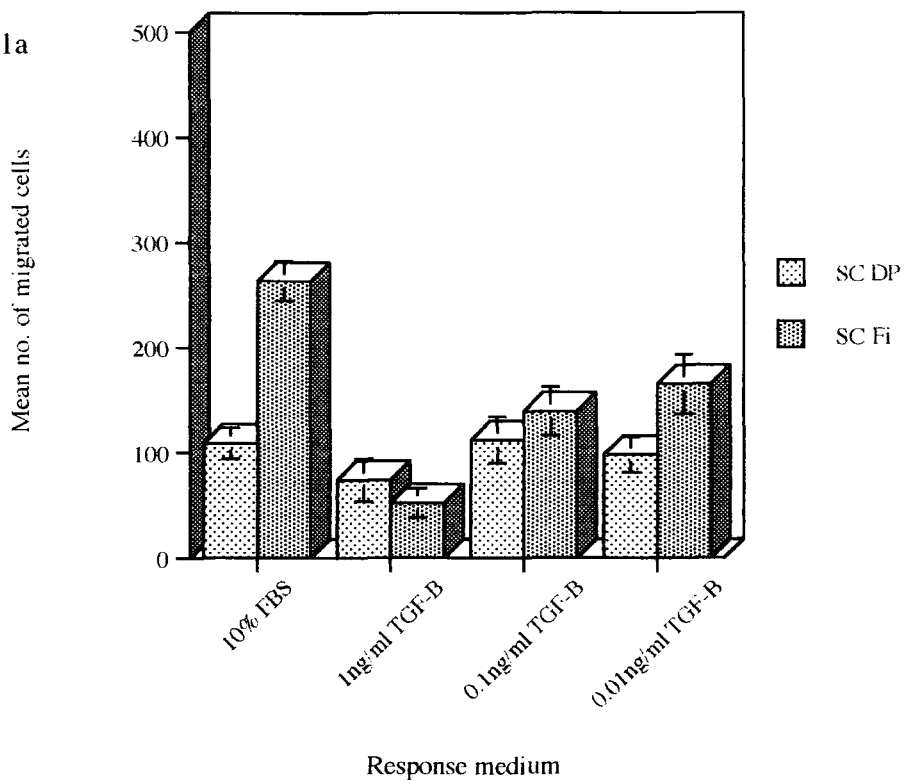


Fig. 41b

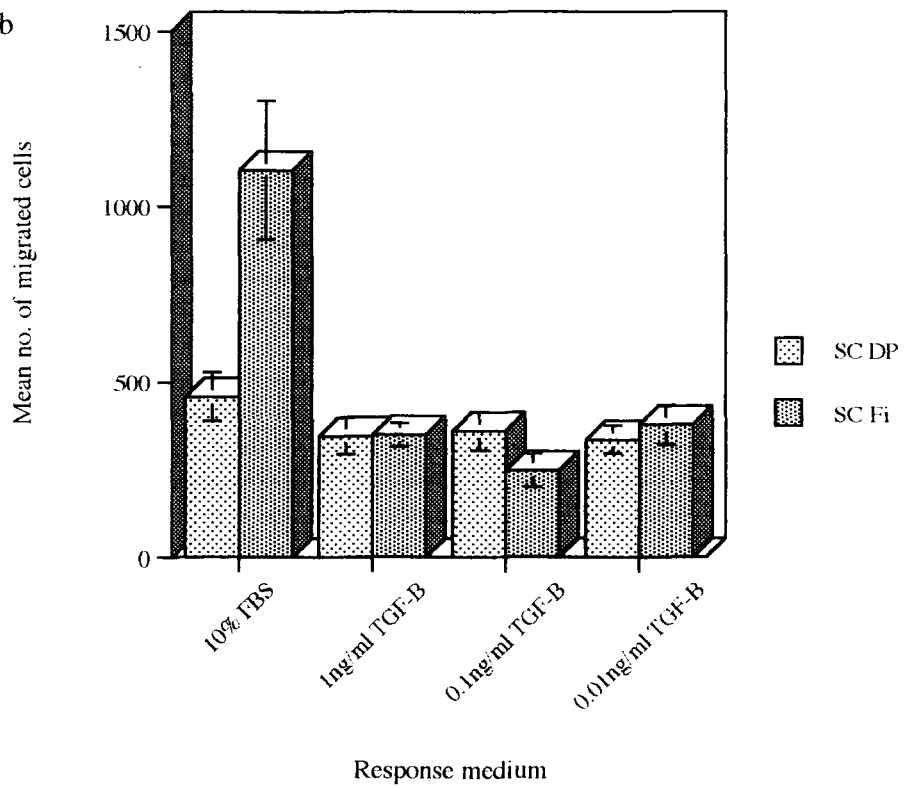


Fig. 42a

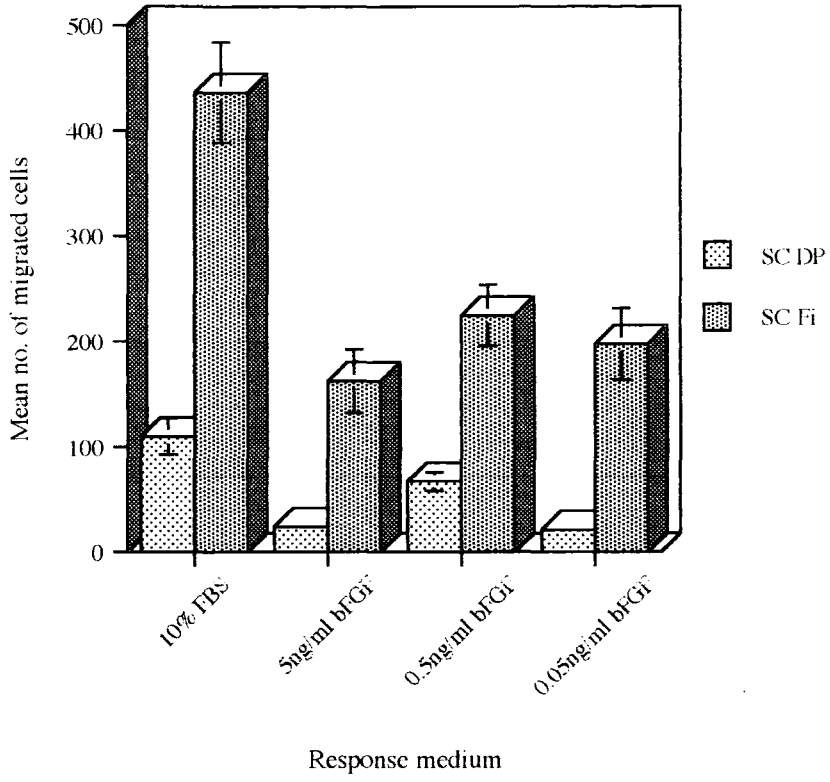


Fig. 42b

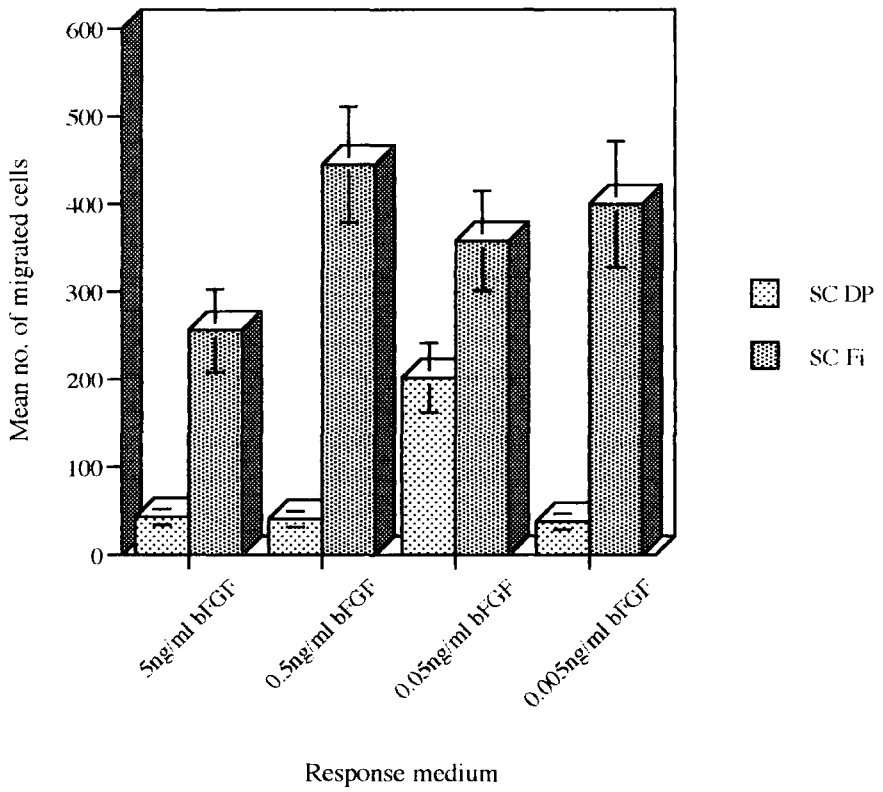


Fig. 43a

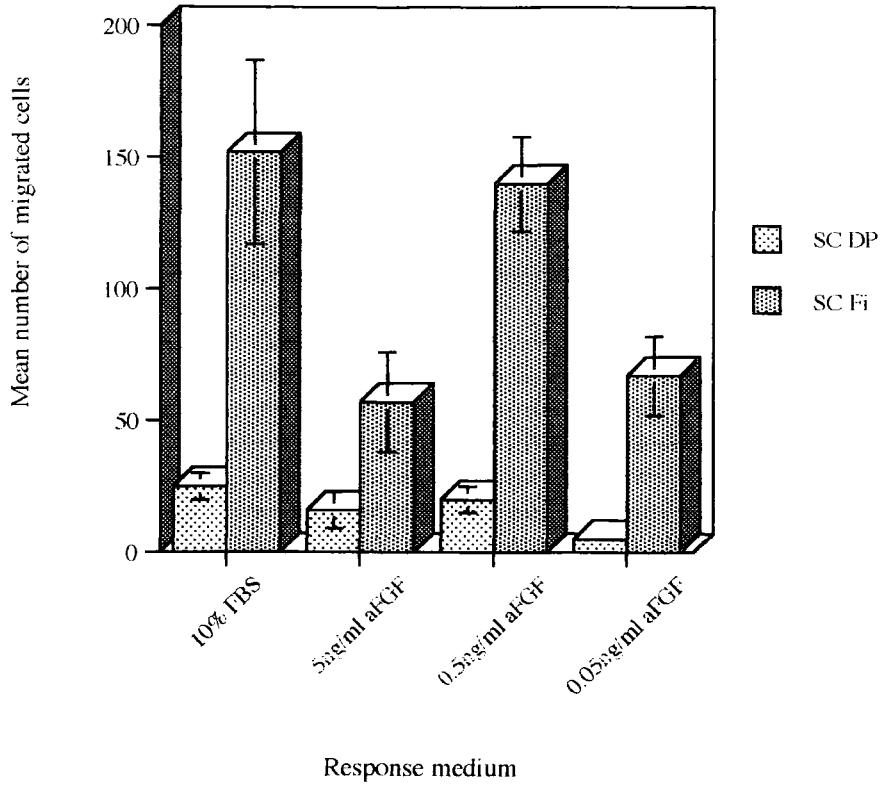


Fig. 43b

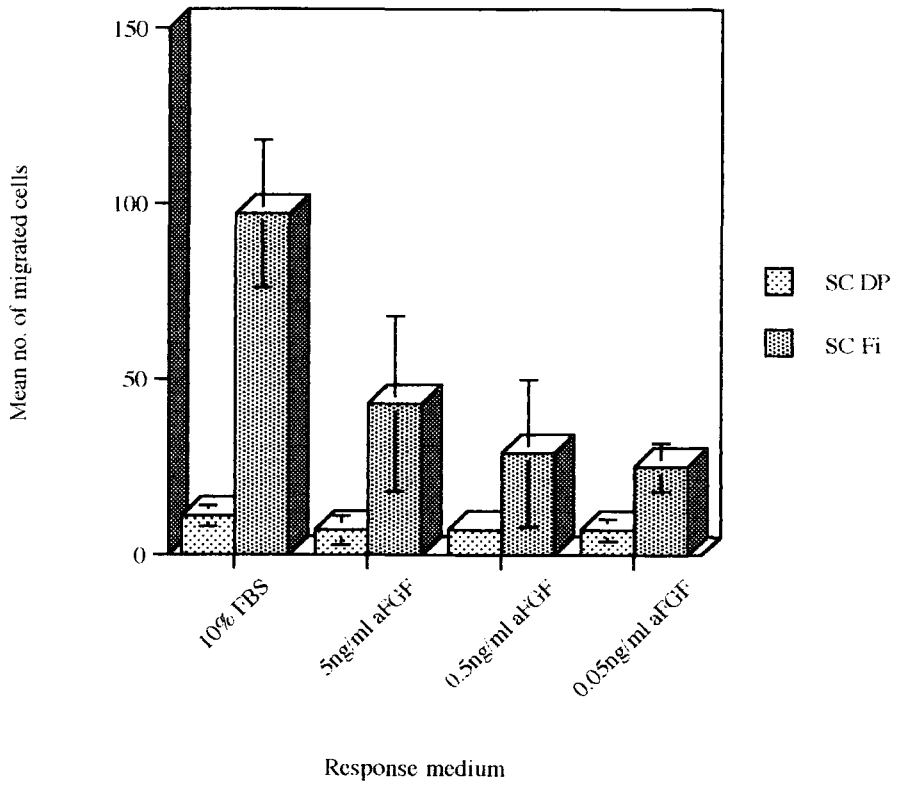


Fig. 44

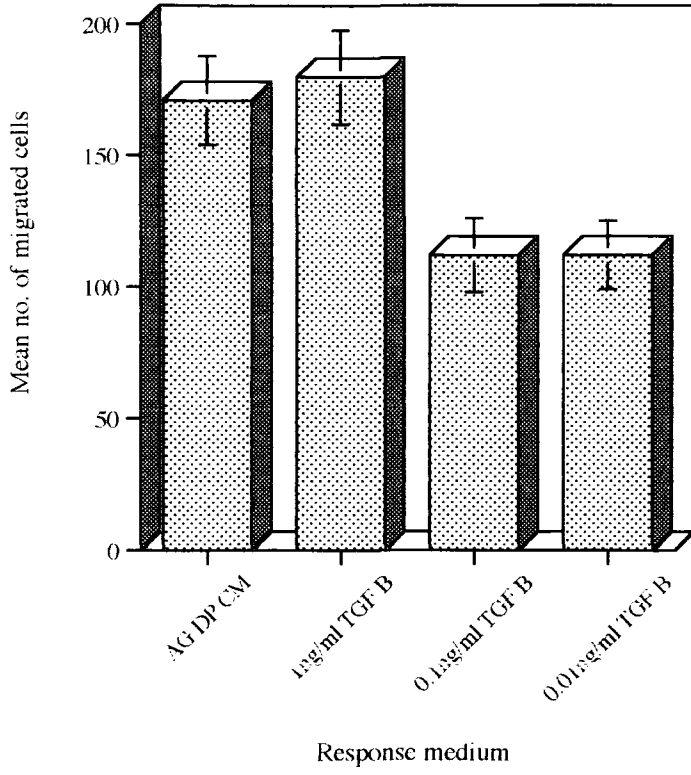


Fig. 45

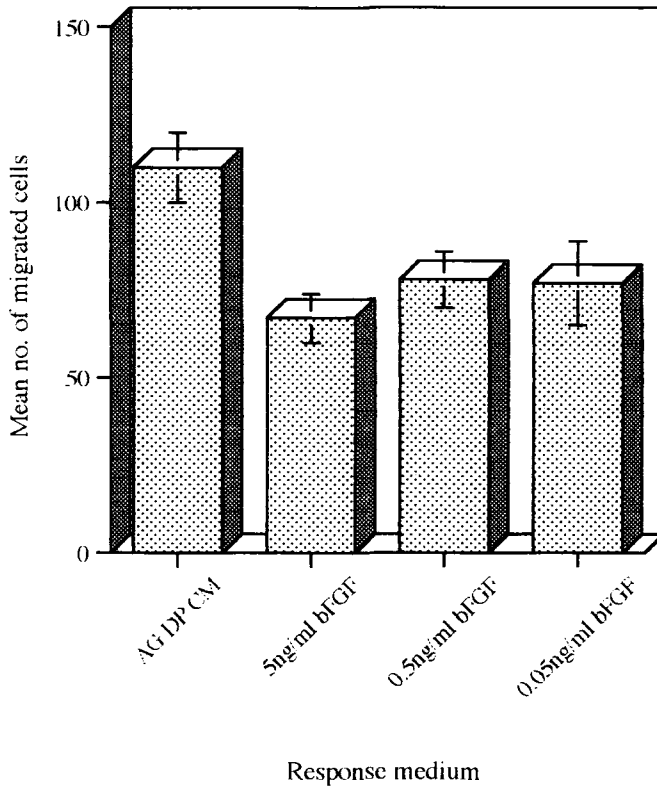


Fig. 46a

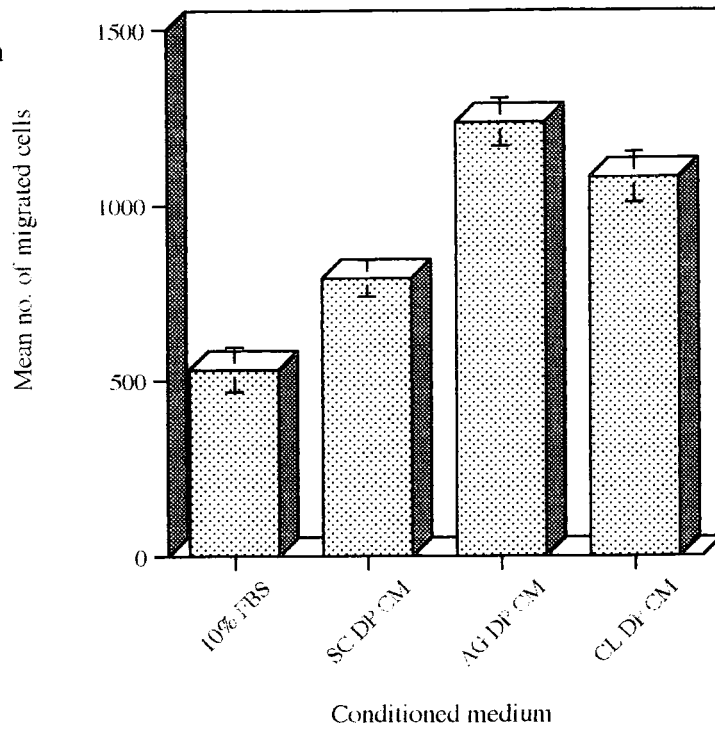


Fig. 46b

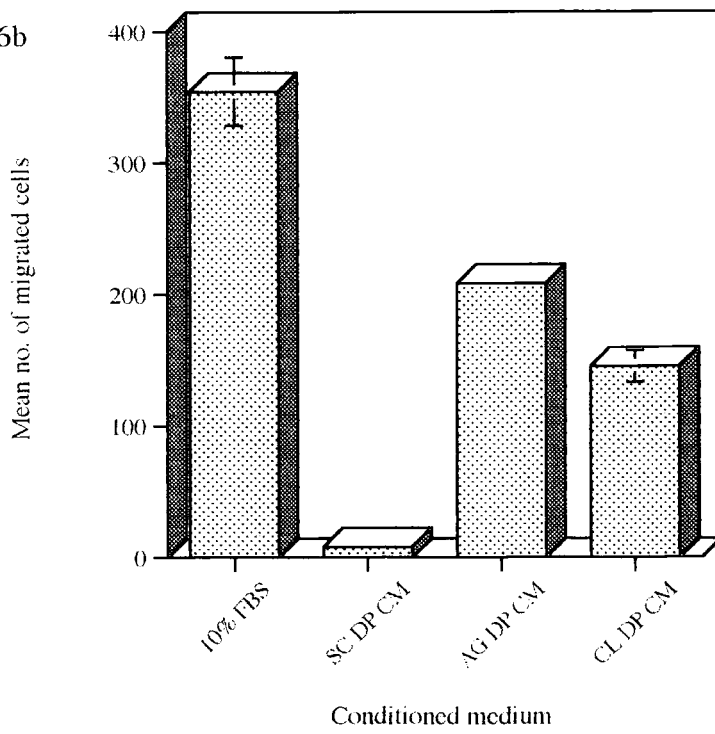


Fig. 46c

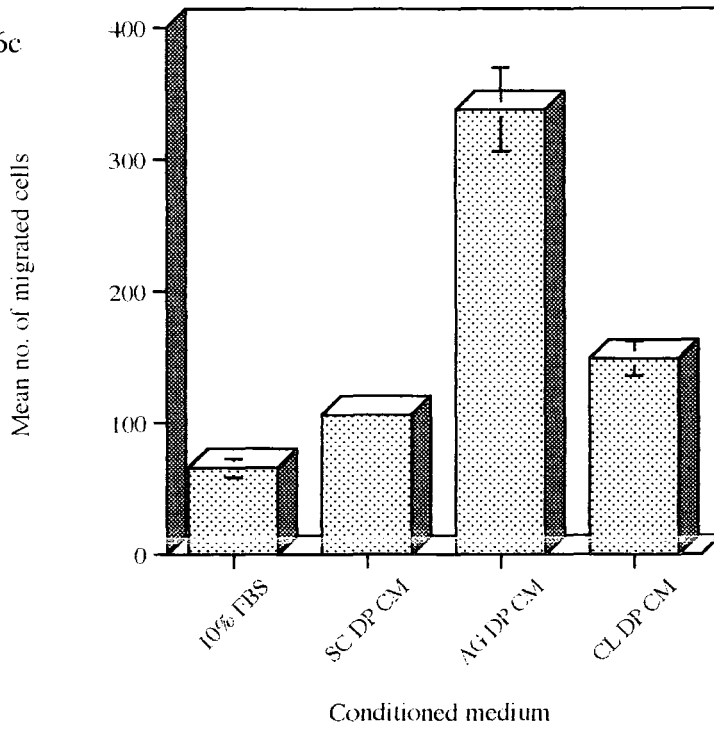
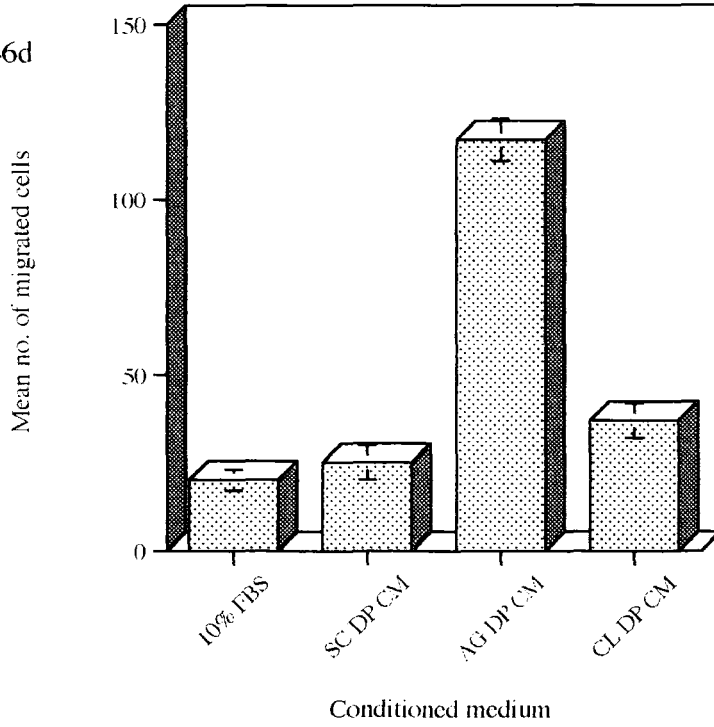


Fig. 46d



## Discussion

### 4.4.1.1 Chemotaxis

During the course of this chapter the words "migrated to" have been used to avoid repetition of other words. However, these words imply that the cells are moving towards a focal stimulus, and are therefore chemotactic. The main limitation of the modified boydens chamber is that it can only give details on the number of cells that pass through the membrane, and although the stimulus maybe a chemotactic, one cannot separate this type of movement from just an overall increase in cell motility. Furthermore, the exact nature of the concentration gradient is unknown, with cells possibly blocking pores and altering the flow conditions between the two wells. Therefore, when interpreting these results this should be borne in mind.

### 4.4.1.2 DP cell *in vitro* motility behaviour from the three *in vitro* DP cell phenotypes

Dermal condensation is a fundamental property of all skin appendage morphogenesis, however little is known about how condensations are formed, and once formed, how they are maintained. Using DP cells isolated from the vibrissa hair follicle a good model of these early embryonic events has been established. When in culture the DP cells form distinct spatially arranged aggregates (Jahoda and Oliver, 1984) which superficially resemble dermal condensates. Indeed, dermal papilla cells maintain an array of interesting properties in culture that support the use of them as a model for dermal condensation. Oliver (1970, 1973) discovered that implanted dermal papillae from whole follicles remained as a uniform structure when isolated from the epidermis. Likewise, Jahoda et al., (1993) found a similar property of the cells after they had been in culture and put back in skin. Therefore, it appears that DP cells maintain some specialized characteristics after they have been isolated from the hair follicle.

Furthermore, they appear to have an inbuilt ability to form aggregations as if trying to form an intact papilla. Jahoda et al., (1993) also noted that these implantations showed a limited capability to cycle, with fluctuations in the amount of glycosaminoglycan production, as if they were associated with the follicle. Further evidence of the cells maintaining much of their *in vivo* functions came from a series of experiments in which Reynolds and Jahoda (1992) discovered that these cells could stimulate the production of follicles in glabrous rat skin, with fibre specificity dependant on whether the dermal papilla tissue was derived from pelage or vibrissa follicles. If vibrissa DP cells were implanted, then a vibrissa follicle was produced, however if pelage DP cells were introduced, then there would be pelage fibres produced.

As these DP cells are in direct lineage from an early dermal condensation event and because they illustrate an interesting array of characteristics, it was felt that they may be a useful tool in answering questions about condensation events. Pilot experiments were initially aimed at investigating whether papilla cells produced conditioned medium with migration stimulating properties. However, it rapidly became apparent that the results varied according to the density, or more specifically the state of aggregation, that the DP cells were in, just prior to being used. In other words, the aggregation status influenced both the properties of the conditioned medium, and the way the cells themselves would act in the test chambers. From the preliminary data, the idea evolved that experiments into the aggregation problem might involve examination of two interlinked phenomenon; 1) a motility factor appeared to be secreted by DP cells during aggregate formation, as a possible part of the aggregation process and 2) that there were intrinsic fluctuations in motility dependent on the aggregation status of the DP cells. Therefore the main series of experiments was designed to test these two ideas.

From the first motility experiments it can be concluded that there are distinct differences in cell movement, between the phenotypic stages of the cells in culture. When papilla cells at different behavioural states were presented with a common influence (10% FBS), migration was highest in cells that were in the process of

forming aggregates. As both subconfluent (SC) and clumped (CL) revealed less movement towards 10% FBS, one interpretation is that at some point between non-aggregation and aggregation the cells are stimulated into motility. The consistently poor migratory abilities of clumped dermal papilla cells, suggests that once clumped structures are formed, the transient motility phase is completely switched off.

Papilla cell migration, in general was found to be lower than that of fibroblasts, and only with aggregating cells were levels of migration comparable. The most likely explanation for the increased motility of aggregating cells was that a molecular stimulus was being produced by the papilla cells themselves. This question was addressed next.

A fluctuating, increase and decrease in motility has not been reported for cultured fibroblasts, where the reported trend is for cells to become less motile from subconfluency to confluency (Abercrombie and Heaysman, 1953, Carter, 1965, Elsdale and Foley, 1969, Elsdale and Bard 1972, ). However, this reduction in motility has been noted using cinephotography, rather than by quantitative methods. Therefore, it was felt important to test this with respect to the chemotactic protocols described in section (4.2.1.2)

When subconfluent fibroblast motility was compared with confluent fibroblast motility, as a comparison for the *in vitro* motility behaviour of the DP cells, the confluent fibroblast cells were maintained in a state of confluency for several weeks, to try to create an equivalent phenotype to CL DP cells. However, although there was a reduction in motility of the confluent fibroblasts the difference between SC and Confl cells was not significant, with high motility occurring in both cases. From the data it is clear that fibroblast cell motility within this type of assay does not show the fluctuations illustrated by the *in vitro* DP cell phenotypes.

#### 4.4.1.3 DP cell *in vitro* response to conditioned medium derived from the three DP cell *in vitro* phenotypes

When subconfluent cells were presented with conditioned medium from the three DP behavioural phenotypes, migration was highest to aggregating conditioned medium, with SC and CL conditioned medium eliciting a poor migratory response. As an alternative to the idea that the aggregating DP cells could be releasing something into the medium that specifically increases motility in the subconfluent cells, it could be argued that the increased density of aggregating cells gives rise to a greater concentration of generally stimulatory molecules in the medium, and as a result of this, the subconfluent cells are more responsive to this increased concentration? If the latter were the case then one would expect that migration to the clumped DP cell conditioned medium would be of a similar magnitude to that of the aggregating DP conditioned medium. The fact that only conditioned medium from aggregating papilla cells produced increased migration was a further indication that the cells from the aggregating phenotype were releasing something into the medium at this time.

Aggregating DP cells showed preferential increased migration to aggregating conditioned medium, and in support of the first experiments, the overall level of migration was greater to all test media. The finding that aggregating cells were further stimulated is not surprising, since these are the cells that would be actively forming clumps *in vitro*. Likewise, it is logical that pre-aggregating cells should be capable of responding to this stimulus.

The CL DP cells were non responsive to any of the conditioned medium presented to them, and consistent with the comparative study migration was generally extremely low. This failure to stimulate clumped cells is particularly interesting, since it illustrates the antagonistic combination of the influence sustaining clump maintenance against the motility stimulus initiation, with clump maintenance dominating. Dermal papilla cells were found to be strongly positive for smooth

muscle  $\alpha$ -actin (Jahoda et al., 1991), an actin form that is generally associated with slow contractile muscle. This actin isoform is also expressed in myofibroblasts (Darby et al., 1990), cells that are highly migratory in wound sites. Recent unpublished data from our group, has noted that smooth muscle  $\alpha$ -actin was not expressed within the clumped dermal papilla cells, hence supporting the termination of migration in the DP cells in the clumped stage.

Thus the previous set of experiments had implied the presence of a molecule or molecules within the conditioned media of aggregating cells that had the ability to induce motility. However, when this media was tested on clumped DP cells, their non-motility could not be reversed.

While not detracting from the main findings, at this stage the most difficult interpretation was of results relating to the dermal papilla cells in the subconfluent state. As seen above, the motility of these cells could vary considerably between experiments. This variation could be due to two possible factors, 1) an inability to accurately detect when the aggregating phase is beginning to occur or 2) phenotypic variation within a single culture vessel, for example in most of the culture vessel the cells might be evenly spaced and subconfluent, whereas in some areas the cells may be closely interacting and initiating the aggregation process. This variation is born out by results from section (4.3.2.5), where pre-aggregating dermal papilla cells displayed far greater motility than the subconfluent cells alone.

Thus, on closer examination of cells between the aforementioned subconfluent and aggregating cells, pre-aggregating DP cells produced a similar response to the DP conditioned medium as that of the aggregating DP cells, but at an even higher level. One must remember that the aggregating phase was adopted on purely visual cues from the cells (section 3.4.1.1), therefore when aggregation was seen to occur was probably not when motility had started. It is therefore, logical that migration should be highest prior to the visual appearance of the full aggregating phenotype, and indeed as the aggregate becomes visible then motility may begin to tail off.

#### 4.4.2.1 AG DP CM molecular weight separation using microconcentrating spin columns and use in the migration assay system

Characterisation of the aggregating dermal papilla conditioned medium was carried further using microconcentrators. From both types of microconcentrator (Amicon and Flowgen) it was discovered that the highest activity was generally between 30KD and 50KD. However, the migration was always greatest towards the unaltered AG DP CM. A possible candidate within this range is that of platelet derived growth factor (PDGF) (Kohler and Lipton, 1974). PDGF was first purified from human platelets and platelet rich plasma (Kohler and Lipton, 1974, Ross et al., 1974) and found to exhibit multiple molecular weights ranging from 28 KD to 35 KD. PDGF was also known to be chemotactic for fibroblasts (Seppa et al., 1982, Senior et al., 1983) and smooth muscle cells (Grotendorst et al., 1981). As DP cells were positive for the smooth muscle  $\alpha$ -actin, it may be a real possibility that the unknown molecule is PDGF. However, PDGF has been implicated in inducing chemotaxis of neutrophils, monocytes and fibroblasts in an excisional wound site (Pierce et al., 1989, Pierce et al., 1991), and yet it inhibits myofibroblast invasion (Pierce et al., 1991), another smooth muscle  $\alpha$ -actin positive cell type.

A further problem still exists within this data, the response within this molecular weight range was never as much as that for the unaltered AG DP CM. It is possible that this increased migration to AG DP CM is a function of multiple molecules, rather than a single specific molecule. Recent research on bFGF and TGF  $\beta$  (Frenz et al., 1994) shows that their chondrogenic inductive activity is increased when these two molecules act in synergy. Therefore, it is possible that the greatest activity occurs in the above size range, however this motility could be further increased in conjunction with other molecules of different sizes. A large number of molecules within the *in vivo* system exist as hetero or homodimers, for example, members of the TGF $\beta$  superfamily (Sampath et al., 1990), therefore creating further complexity within the conditioned

medium. More simply, the conditioned medium activity may be reduced by the physical stresses of the centrifugation processes.

#### **4.4.3.1 Effect of FBS on *in vitro* DP cell motile response**

During the course of the previous experiments, the DP test cells were always put into the upper test wells in 10% FBS in MEM. As FBS is a complex cocktail of growth factors and ECM molecules, it was relatively surprising that the cells showed preferential migration to a variety of different conditioned medium. However, the possibility remained that the presence of FBS in with the test cells in some way enhanced their migration towards the conditioned medium. When the relative amounts of FBS in the MEM was decreased, the resulting higher levels of DP cell migration illustrated that the presence of FBS in the upper test wells had a negative influence on DP cell motility. Nevertheless migration assays utilizing FBS, gave a powerful indication of differential responses of the DP cells to their own conditioned medium.

#### **4.4.4.1 Dilution effect of AG DP CM on DP cell *in vitro* motile response**

Dilution of the AG DP CM produced a corresponding reduction in activity of the motile response, implicating that the effect may act in a dose responsive manner, with the greater the concentration, the greater the motility. However, the conditioned medium was never more concentrated than on extraction from the culture vessel. If the conditioned medium was further concentrated to a higher concentration than that produced in the culture vessel, it would have been interesting to see whether the effects were different.

#### **4.4.5.1 SC Fi cell *in vitro* response to conditioned medium derived from the three DP cell *in vitro* phenotypes**

When subconfluent fibroblast cells were presented with the three conditioned medium types, a similar response to that of DP cells was discovered, with greatest migration occurring to the AG DP conditioned medium. Migration was reduced to the other two conditioned medium types, but this was not comparable to the lower levels of motility shown by the DP cells. Overall the motility of the subconfluent fibroblasts was at a much greater level.

#### **4.4.6.1 *In vitro* DP cell behaviour**

From the above it is apparent that DP cells, although superficially similar to fibroblasts, act very differently to other cell types in culture. DP cells *in vitro* appear to have fluctuations in motility, with the greatest motility occurring when the cells are in the process of forming the 3 dimensional aggregates. Motility is negligible prior to and post to this aggregate formation step. Furthermore, it seems that the DP cells modulate their own motility by secreting certain molecules that can alter motility in the non motile subconfluent cells. Therefore, this *in vitro* DP aggregate formation appears to be under subtle control, indicating that this particular behaviour is intrinsic to these cells.

#### **4.4.7.1 DP cell *in vitro* migration to TGF $\beta$ <sub>1</sub>**

In a number of assays the role of known growth factors were investigated. In the first experiments with DP conditioned medium (sections 4.3.1 & 4.3.2), cells were maintained in 10% FBS during the time course of the experiment, therefore to investigate the comparative effect of growth factors with conditioned medium, DP cells were still maintained in the serum environment. However, if the aggregating

conditioned mediums largest component is a growth factor, then a similar response would be stimulated from the assayed growth factor.

TGF  $\beta_1$ , a molecule highly expressed in dermal condensation events, has already been shown to exhibit chemotactic functions within monocytes and fibroblasts (Wahl et al., 1987 and Postlethwaite et al., 1987). From (Fig. 41), it was clear that there were no peaks in DP cell migration between the 10% FBS and TGF  $\beta_1$  concentrations. However, consistent with Postlethwaite et al., (1987), fibroblast migration was often greatest to 0.01ng/ml TGF  $\beta_1$  (fig. 41a,b). Nevertheless, migration was never greater than the response to 10% FBS. As migration to 10% FBS from DP and Fi cells is generally equivalent to or smaller than the response to AG DP CM, it therefore appears that TGF  $\beta_1$  is not the prime migratory influence.

In a direct comparison of TGF  $\beta_1$  concentrations with AG DP CM (section 4.3.6.2), migration was comparable between 1ng/ml TGF  $\beta_1$  and AG DP CM, however cells were not maintained in 10% FBS throughout the experiment. TGF  $\beta$  is multifunctional and can have different effects on the same tissue at different concentrations and times in development. For example, it can stimulate growth of fibroblasts from early human foetuses and yet inhibit fibroblast growth from later gestational ages (Hill et al., 1986). Nearly all cells that bind TGF  $\beta$ , do so in the picomolar range (Frolik et al., 1984, Tucker et al., 1984), therefore it appears that DP cells are responding in a similar concentration range. What is also apparent is that FBS has a negative response on the action of TGF  $\beta_1$ . As AG DP CM created a response in the presence of 10% FBS, it is possible that TGF  $\beta_1$  is not the effector molecule in migration. This is supported by the molecular weight cut off data (sections 4.3.3.1), that showed that the greatest response is between the 30KD and 50KD, whereas TGF  $\beta_1$  is only 25KD.

However, this does not rule out the action of TGF  $\beta_1$  in aggregate formation. As mentioned previously, TGF  $\beta_1$  molecules are highly expressed during dermal condensation of the hair follicle, therefore, it is possible that TGF  $\beta_1$  acts in another role. A prime function of TGF  $\beta$ , is that it can modulate the expression of the ECM.

Many cell lines show increased collagen type I and fibronectin synthesis following TGF  $\beta$  treatment (Ignotz and Massague, 1986, Roberts et al., 1986). Both these ECM molecules have broad specificity, however TGF  $\beta$  can also modulate tenascin (Pearson et al., 1988), an ECM molecule present during dermal condensation (Chiquet-Ehrismann et al., 1986, Kaplan and Holbrook, 1994). Tenascin has also been implicated in neural crest cell migration (Bronner-Fraser, 1988), therefore it may be possible that TGF  $\beta_1$  regulates the expression of molecules such as tenascin, which in turn regulates the motility of DP cells. Therefore, TGF  $\beta_1$  does not appear to be a primary effector in DP motility, however its parallel expression maybe part of a cascade of events leading to aggregate formation.

#### **4.4.8.1 DP cell *in vitro* migration to bFGF and aFGF**

As with TGF  $\beta$ , bFGF is associated with dermal condensation events in the embryo, whereas aFGF is restricted more to the epidermis (Gonzalez et al., 1990). Furthermore, bFGF has chemotactic properties for fibroblasts (Senior et al., 1986). Overall migration of fibroblasts was consistently at a greater level than that of DP cells. However, from the data, it appeared that both cell types were most responsive to bFGF between 0.5ng/ml and 0.05ng/ml. Likewise, aFGF produced greater motility in fibroblasts rather than dermal papilla cells, however there was no obvious trend in cell migration. Due to the inconsistencies from these results it is not clear that FGFs have a chemotactic function in this particular experimental set up. In support of this, when cells were incubated in an absence of 10% FBS, there was no obvious motile response towards bFGF. The molecular weights of both bFGF and aFGF (16-18KD and 15-16KD respectively) are outside the optimal range of migration discovered in the molecular weight cut off experiments (section 4.3.3.1), and therefore it seems increasingly likely that DP cells do not have a motile response to either aFGF or bFGF.

#### 4.4.9.1 DS cell *in vitro* migratory behaviour

DS cells were also used in comparative studies with DP conditioned medium.

Subconfluent dermal sheath cell response was very similar to that produced by DP cells. Migration was greatest to the AG DP CM, consistent with DP cells. However, there was not the great reduction in migration to the CL DP CM as shown by the DP cells. Transformed DS cells had a similar effect to the dermal papilla conditioned medium, although motility was reduced. Nevertheless, it is encouraging that the proliferative tDS cells (section 3.3.9.2) also illustrated similar functions.

From the *in vivo* manipulations of Oliver (1966b, 1967a) and Horne and Jahoda (1992), the DS cells were capable of migration to replenish an ablated DP, and furthermore were proficient at forming a dermal papilla, when surgically implanted into the base of an amputated follicle. Both these cases, implicate the flexibility of the DS and it's close functional links with the DP. Therefore it is unsurprising that the DS cells responded in the same manner as the DP cells, however what is unclear is the similar response found in subconfluent fibroblasts.

With the discovery of any new growth factor it is originally thought to be highly specific to where it was first isolated, however later research often indicates a wider function within a variety of different tissues. Therefore, what is occurring within the fibroblasts may be the result of a wider specificity. As discussed in the previous chapter, there is often great plasticity between fibroblast types. If all the mesenchymal cells in the embryonic dermis are derived from a fibroblast stem cell population, it could be that fibroblasts, because of this proposed lineage, are sensitive to the action of DP conditioned medium. However, it is not known from which embryonic tissue source the dermal papilla cells are derived.

#### 4.5.1.1 Models of dermal condensation *in vitro*

Harris et al., (1981), showed that motile fibroblasts could create large forces on the extracellular matrix. When investigated further, Harris et al., (1984) were able to simulate mesenchymal condensation, by stabilising a collagen substrate in a culture vessel, by preventing contraction of the gel. Therefore, when stabilized all the contractile forces are transferred directly between cells and hence aggregates are formed. They were also able to manipulate the size and number of cells in the condensation purely by increasing the density of the cells.

In comparison, the model that we suggest has two benefits. Firstly, the dermal papilla cells are able to spontaneously form aggregates on a very smooth surface without the aid of any artificial extracellular matrixes, and secondly that the size of the aggregates are not dependent on the density of the cells. For example, if one increases cell density the size of the aggregates remains relatively uniform, although a specific density of cells may be required before aggregation can occur. Both of these properties indicate that the aggregation of *in vitro* DP cells is an intrinsic property of the cells, rather than a combination of interactions induced by tractional forces. When, Oster et al., (1983) created a mathematical model of mesenchymal condensation, they postulated that these condensation events were formed by a traction induced effect from the substrate. They also proposed that the events leading up to formation of a complete aggregate utilised motility in the cells, although their model also worked if the cells were non-motile (Oster et al., 1985). In this later paper, the authors state that although aggregation can occur, it was felt that it would only be enhanced if the cells had a motile property. In these papers the authors see the motility as a function for distorting the extracellular matrix, and that it is the changes in the structure of the ECM that creates the condensation. However, the dermal condensation system described in previous chapters of this thesis occurs over plastic or glass with no interaction from artificial ECMs, and therefore the traction induced effect must be reduced or negligible in this case.

#### **4.5.1.2 Cell adhesion molecules**

Current investigations *in vivo* indicate a positive role for cell adhesion molecules in the dermal condensation and adjacent epidermis. (Choung & Edelman, 1985 a, 1985 b ; Choung et al., 1991; Takeichi, 1988; Hirai et al., 1989). Monoclonal antibodies against some of the epidermal cell adhesion molecules have been able to disrupt the correct morphogenesis of feather dermal papilla (Gallin et al., 1986), and hence the development of the skin appendage. These cell adhesion molecules tend to be expressed after organ formation has begun, and therefore may not be the initial stimulant of the organ formation. Cell adhesion molecules have an important role to play but their function may be more in maintaining condensation structure rather than a driving force in dermal condensation formation.

#### **4.5.1.3 *In vitro* DP cell motility**

In conclusion, it appears from this model that motility of cells may have a more active part to play in the creation of early *in vitro* dermal condensations. Supporting this work, Jiang et al., (1993) postulated that recruitment of cells from the surrounding tissue may be involved in dermal condensation prior to bone formation, after showing that an increase in the condensation was not due to increased proliferation. Although we have illustrated that DP cells have fluctuating motility within their culture vessels, we have as yet been unable to discover if this motility is random or whether in fact it is to distinct foci on the culture vessel; this we plan to do using time lapse photography. One must remember that dermal condensation formation is inextricably linked with the overlying epidermis (Dhouailly, 1973; Sengel, 1976) and that to eventually work out the processes involved in dermal condensation, one must see how its molecular makeup relates to the instructive and permissive messages from the epidermis.

**Chapter 5**  
**Molecular Biology**  
**Introduction**

**5.1.1.1 Bone morphogenetic proteins (BMP)**

Bone morphogenetic proteins (BMPs), members of the TGF $\beta$  superfamily, have been shown to have close functional links with a variety of mesenchymal condensation events (see chapter 1). During the course of epithelial/ mesenchymal of vibrissa follicle development, BMP 4 is restricted to the condensed dermis (Jones et al., 1991). As events in the hair cycle are thought to be reminiscent of hair follicle development, BMP 4 expression was investigated to establish whether it was restricted to the dermal papilla when a new hair cycle was beginning, as this mimics early follicle embryogenesis.

Bone morphogenetic proteins were first isolated from demineralized bone, that was found to induce bone formation in ectopic sites (Urist, 1965, Urist et al., 1973, Urist et al., 1979). There are currently seven bone morphogenetic proteins (BMPs), BMP1-BMP7 (Rosen and Thies 1992). Of the seven BMPs, BMP 2 to BMP 7 form part of the TGF $\beta$  superfamily, the members of which are characterised by having three regions of conservation in the amino acid sequence. These are, residues 1 to 36, the residues immediately surrounding the cysteine pair at residues 61, 62 and the final ten residues of the carboxy terminus (Wozney et al; 1988). BMP 2 and BMP 4 are the most conserved, with 92% identity at the carboxy terminus, and they also have strong homology with the *Drosophila* Decapentaplegic gene (dpp) (Padgett et al., 1987) and the *Xenopus laevis* Vg-1 gene (Weeks and Melton, 1988). Both BMP 2 and 4 are widely expressed throughout embryogenesis (Lyons et al 1990, Jones et al., 1991). However, BMP 4 was shown to be more involved in mesodermal patterning (Dale et al., 1992, Jones et al., 1992) having a suggested role as a posterior ventralizing factor in mesoderm induction. This correlated with dpp function in establishing the correct

dorsal ventral patterning in *Drosophila* (St Johnston and Gelbart, 1987). Furthermore, BMP 4 has a greater association with the mesoderm at sites of epithelial / mesodermal interactions (Jones et al., 1991, Vainio et al., 1992), whereas BMP 2 adopts a more epithelial expression (Lyons et al, 1990).

In mouse facial development, BMP 4 expression first occurs at 11.5pc in the mesenchyme underlying the epithelium (Jones et al., 1991). By 13.5pc its expression is highest in regions of condensing mesenchyme adjacent to the epithelial placode, the future sites of vibrissa follicles. Concurrent with BMP 4 expression, BMP 2 is localized in the epidermal placode, however at later stages of follicle development, expression is confined to the epithelial matrix adjacent to the DP. Further on in follicle development the highest levels of expression are in the precortex. At these later developmental stages, BMP 2 expression is not present in the inner root, outer root or dermal sheath layers (Lyons et al, 1990). However, BMP 2 expression persists throughout adulthood in the cortex and inner root sheath (Lyons et al., 1989).

As described in (Lyons et al., 1990, Jones et al., 1991), BMP 2 and BMP 4 often have similar distributions of expression in tissues. As TGF  $\beta$  superfamily members are capable of forming homo and heterodimers with opposing actions, the complexity of the system is further increased. (Hsueh et al., 1987, Sampeth et al., 1990). The function of the BMPs within these epithelial / mesenchymal interactions is unclear, particularly since BMPs have a variety of functions depending on cell type. Vukicevic et al. (1989) provided evidence that BMPs stimulate collagen synthesis in osteoblasts and proteoglycan synthesis in chondroblasts. Bone morphogenetic proteins have also been shown to be chemotactic for monocytes (Cunningham et al., 1992). Therefore, extracellular matrix production and cell motility are just two of the possible ways in which BMPs may regulate differentiation in epithelial / mesenchymal interactions.

Receptors for the TGF  $\beta$  superfamily, in particular the serine/ threonine complexes and betaglycans, are only now beginning to be characterized (Massague, 1992). Moreover, receptors to BMP4 have recently been isolated (ten Dijke et al.,

1994a). These receptors are members of the type 1 serine/threonine complex termed activin receptor like kinase (ALK), of which there are 6 members (Franzen et al, 1993, ten Dijke et al, 1994b, Ebner et al, 1993, Attisano et al., 1993, Matsuzaki et al., 1993). In one study, ten Dijke et al. (1994a) found that BMP 4 was specific to ALK-3 and ALK-6 receptor; however, osteogenic protein 1 (OP-1) also binds to both these receptors, therefore adding further complexity to the system. Nevertheless further research into these receptors will give better insights into the role of BMPs in development.

From the above it is apparent that the function and control of the BMP's are only now being clarified. The following chapter describes the isolation of rat BMP2 and 4 homologues from an adult hair follicle cDNA library. In situ hybridization studies using rat BMP 4 non radioactive probes were used to localise the expression of this molecule in the rat embryo. This was then compared with BMP 4 distribution already characterized in the mouse. BMP 4 distribution within the adult rat sinus follicle was then examined.

#### **5.1.2.1 Hair cycle and hair development: a possible link?**

Hair growth is characterized by cyclical periods of activity followed by periods of quiescence. During the intermediate catagen stage of the human hair cycle, the whole follicle shrinks, with an upward migration of both the epidermal and connective tissues. When in telogen, the follicle is  $\frac{1}{3}$  to  $\frac{1}{2}$  of its original length in anagen. (Parrakal, 1990). When the growing anagen phase resumes there is a concomitant down growth of the follicle into the dermis. It is this deep downgrowth into the dermis that is thought to be analogous to mid to late stages in the original morphogenesis of the hair follicle in the embryo. Furthermore, the reactivation of GAG and specialized ECM production in the dermal papilla (Couchman et al., 1991, Messenger et al., 1991, Jahoda et al., 1992, Kaplan and Holbrook, 1994), and redifferentiation of the specialized epidermal constituents (Heid et al., 1988, Coulombe et al., 1989, Baden, 1990, Forslind, 1990,

Lane et al., 1991) of the follicle with the associated manufacture of a new hair fibre, all support this concept.

Therefore, by examining events during the hair cycle, it becomes possible to gain a partial insight into the early embryonic events of hair development. In the previous chapter, aggregate formation of *in vitro* dermal papilla cells was investigated with respect to motility. It was postulated that these aggregates might mimic early mesenchymal condensations during follicle development (Jahoda and Oliver, 1984). Through a variety of experiments (Jahoda et al., 1984, Jahoda, 1992, Reynolds and Jahoda, 1992) it has been shown that *in vitro* , dermal papilla cells maintain a number of their *in vivo* properties. Therefore, a differential screen approach was adopted to screen the hair follicle end bulb cDNA library, with probes derived from the *in vitro* dermal papilla cell populations in order to isolate specific genes involved in the *in vitro* maintenance of the clumped phenotype. This strategy of screening an *in vivo* library with *in vitro* generated probes identifies genetic similarities between the *in vivo* and *in vitro* systems, and should eliminate or reduce the recording of genes which are only expressed culture. Therefore, the differential screen will isolate messages involved in aggregation that are expressed *in vivo*.

#### **5.1.3.1 Differential screens**

The basic principal behind a differential screen is that by constructing two different first strand complementary DNA (cDNA) populations, it is possible to compare the expressed messages from both populations. Moreover, one can then isolate the genes which are specific to, or upregulated in, the population of interest.

#### **5.1.4.1 Maintenance of clumped *in vitro* DP cells**

In the previous chapter the dermal condensation process could be thought of as two distinct stages, firstly, condensation formation and secondly, condensation

maintenance. Condensation formation was partially addressed in the chapter 4. In this chapter the second question is addressed, how is the clump or aggregate maintained?

Cell adhesion molecules and cadherins are obvious candidates in condensation maintenance, with molecules from both groups present at the placode stage of hair development. Consistent with the discovery of many growth factors having tissue type specificity (Heine et al., 1987, Pelton et al., 1989, Gonzalez et al., 1990, Lyons et al, 1990, Jones et al., 1991), cell adhesion molecules often exhibit similar tissue specific behaviour, with the expression of certain cell adhesion genes being distinct to the epidermis or the dermis.(Hirai et al., 1989, Choung et al., 1991, Kaplan and Holbrook, 1994). In hair and feather dermal condensation, a prominent ligand is the neural cell adhesion molecule or N-CAM (Choung and Edelman, 1985a, Choung et al, 1991; Kaplan & Holbrook, 1994). In the developing hair follicle, N-CAM expression was identified in the dermal papilla and dermal sheath tissues (Choung et al, 1991; Kaplan & Holbrook, 1994) and in the dermal papilla of the adult follicle. N-CAM was also expressed in cultured feather dermal papilla cells (Choung et al., 1991), although expression was restricted to the aggregated clumps. Expression of N-CAM has also been identified in the mesenchymal condensations of bone. (Choung, 1990). Bard (1990) proposes that cell adhesion molecules are a major influence in mesodermal condensation formation and maintenance.

Recent research has also illustrated a growing function for the extracellular matrix in condensation formation. Tenascin, a molecule commonly found in condensation events, was first isolated by Chiquet & Famborough (1984). Expression of tenascin occurs in the condensing mesoderm in a variety of developing tissues, such as tooth, hair and mammary gland (see chpt 1).(Chiquet-Ehrismann et al, 1986, Thesleff et al, 1987, Vainio & Thesleff, 1992 and Kaplan & Holbrook, 1994). Tenascin, first appears in the human hair follicle at the epidermal-dermal interface prior to hair follicle initiation; at later stages in development, expression is apparent in the dermal sheath and basal lamina (Kaplan & Holbrook, 1994). Other studies have also implicated proteoglycans as having roles in regulating condensation events (Goetinck

and Carlone, 1988). Primary interest has involved the cell surface proteoglycan syndecan (Bernfield and Sanderson, 1990), which binds extracellular matrix molecules and growth factors. With this complex array of cell to cell and cell to matrix interactions forming a dense network of scaffolding, dermal condensations could be easily maintained.

Although a wide variety of molecules have been identified within the condensing dermis, their exact function within the condensation is far from ascertained. Using a differential screening approach on our *in vitro* DP dermal condensation model we hope to discover new molecules, that are directly involved in the maintenance of the clumped phenotype, in an unbiased fashion.

## Materials and Methods

### 5.2.1.1 *Escherichia coli* (*E. coli*) strains

The *E. coli* strains used in this research were: XL1-Blue [ *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, {F', *proAB*, *lacI*<sup>q</sup> *ZDM15*, Tn 10 (tet<sup>r</sup>)]; SOLR<sup>TM</sup> strain: e14<sup>-</sup> (*mcrA*), D (*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn5(kan<sup>r</sup>), *uvrC*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, λ<sup>R</sup>, {F' *proAB*, *lacI*<sup>q</sup> *ZDM15*}. Su<sup>-</sup> (non-suppressing); SURE<sup>TM</sup> strain: e14<sup>-</sup> (*mcrA*), D (*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn5(kan<sup>r</sup>), *uvrC*, *supE44*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, {F' *proAB*, *lacI*<sup>q</sup> *ZDM15*}, Tn 10(tet<sup>r</sup>). All strains were obtained from Stratagene.

### 5.2.1.2 *E. coli* growth medium

Luria-Beltrami (LB) medium, NZY broth, super broth and 2 x YT medium were prepared as described in ZAP-cDNA synthesis kit protocol (Stratagene) and Sambrook et al., (1989).

### 5.2.1.3 Plasmids

The following commercially available plasmid was used: pBLuescript SK (+/-) (Stratagene).

Plasmids (pGEM3 and pSP72) containing the mouse cDNA clones for BMP-2 and BMP-4 were a gift from Dr B. Hogan, Vanderbilt University, Nashville, Tennessee, USA. Plasmids were isolated in one of two ways, as described in section (5.2.2.2 & 5.2.2.3).

#### **5.2.1.4 Buffers used in DNA and RNA manipulations**

Restriction enzymes and DNA modification enzyme reaction buffers were obtained from; Promega; Gibco; NBL and BRL.

#### **5.2.2.1 Plasmid Isolation**

Plasmid isolation was as described in the following two sections.

#### **5.2.2.2 Plasmid mini-preps from *E. coli* by alkaline lysis**

This method was modified from Birboim and Doly (1979). *E. coli* (5ml) were grown in LB broth (section 5.2.1.2) for 16 hrs at 37°C with 100µg/ml ampicillin. An aliquot (1.5ml) was removed to an Eppendorf microfuge tube and the cells harvested by microcentrifugation (13 000g for 1-2min), (Heraeus bio-fuge 13). The supernatant was then removed, and the cells were resuspended in 100µl of 50mM Glucose, 10mM EDTA (pH 8) and 25mM Tris-HCl (pH 8) and incubated for 5 minutes at room temperature. 200µl of 0.2N NaOH, 1% SDS was added and samples placed on ice for 10 minutes. 150µl of ice cold 5M potassium acetate/glacial acetic acid was then added and the sample spun down in a microcentrifuge for 5 minutes. The supernatant was then removed to a new Eppendorf and incubated for 30 minutes at 37°C with 20µg/ml RNase A (Sigma). This solution was then extracted with 500µl of phenol/chloroform. Samples were spun down and the aqueous phase removed. The plasmid DNA was then precipitated with 100% ethanol at -80°C for 10 minutes. The plasmid was then rescued by high speed microcentrifugation for 5 minutes, and the pellet washed with 1ml of 70% ethanol and dried under a vacuum. The DNA was resuspended in 16µl of ddH<sub>2</sub>O and subjected to a further precipitation with 4µl 4M NaCl and 20µl of 13% polyethylene glycol (PEG). The plasmid was then recovered by microcentrifugation,

washed with 1ml of 70% ethanol and dried under vacuum The DNA was then resuspended in 20µl ddH<sub>2</sub>O and stored at -20°C.

### **5.2.2.3 Plasmid Minipreps with the Magic™ Minipreps DNA purification System**

Plasmids were recovered using a modification of Magic™ Minipreps DNA purification system described by Promega (Technical bulletin No 117). *E. coli* (10ml) were grown in LB broth (section 5.2.1.2) for 16 hrs at 37°C with 100µg/ml ampicillin. Cells were spun down and resuspended in 300µl resuspension buffer (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100ug/ml RNase A). An equal volume of cell lysis buffer (0.2M NaOH; 1% SDS) was added, and the solution inverted until suspension cleared. 300µl neutralization buffer (2.55M Potassium acetate, pH 4.8) was then added. Samples were then centrifuged for 3 minutes in a microcentrifuge at 12000rpm. The supernatant was removed and re-spun. Cleared supernatant was then transferred into two microcentrifuge tubes ( 400µl/tube). "Magic Miniprep" DNA Purification resin (500µl) was added to each tube, and incubated at room temperature for 5 mins. Resin/DNA mix was pipetted into a 5ml syringe barrel. A minicolumn was attached to the barrel and the resin/DNA mix pushed into the column. The mini column and resin/DNA mix was then washed with 3-4ml of column wash (200mM NaCl; 20mM Tris-HCl, pH 7.5; 5mM EDTA; diluted 1:1 with 95% ethanol). The syringe barrel was removed and the minicolumn transferred to a 1.5ml microcentrifuge tube and spun down at top speed for 1 minute to remove all the column wash solution. The minicolumn was transferred to a new microcentrifuge tube and 100µl of sterile ddH<sub>2</sub>O heated to 70°C was added. After a 1 min incubation, the DNA was collected by centrifuging the minicolumn at top speed in a microcentrifuge for 1 min. The elutant was then re-applied to the top of the minicolumn and spun through again. DNA was then analysed for purity and quantity via agarose gel electrophoresis against standards.

#### **5.2.2.4 NaI solution**

90.8g NaI and 1.5g Na<sub>2</sub>SO<sub>3</sub> were added to 100ml distilled water (final volume was greater than 100ml). The solution was filter sterilized and a further 0.5g Na<sub>2</sub>SO<sub>3</sub> added (the final solution should be saturated). The resulting solution was stored in the dark at 4°C.

#### **5.2.3.1 Agarose Gel Electrophoresis**

TAE agarose gel electrophoresis was carried out as described in Sambrook et al., (1989). cDNA was released from plasmid by digestion with restriction enzymes (Sambrook et al., 1989), and the digested products run on a 0.8% agarose/1xTAE gel (0.04M Tris-acetate, 0.001M EDTA) with DNA markers.

#### **5.2.3.2 Isolation of DNA fragments from agarose gels**

Isolated fragments were cut from the TAE gel and dissolved by incubation with NaI solution (Section 5.2.2.4) at 60°C for 10 minutes and a further 5 minutes at 5°C. Once the gel was completely dissolved, 10µl of silica fines (donated by Prof. N. Robinson, University of Newcastle-Upon-Tyne) was added and the solution incubated at room temperature for 10 minutes with frequent mixing. The fines were collected by centrifugation and washed in 1ml of 70% ethanol, after which, the fines were resuspended in 50µl of ddH<sub>2</sub>O, and incubated at 37°C for 15 minutes with occasional vortexing. The fines were pelleted and the isolated DNA aspirated off.

#### **5.2.3.3 Labelling of DNA fragments**

Probes were constructed by random priming (Feinberg & Vogelstein, 1983). DNA was first denatured at 100°C for 5 minutes, and snap cooled on ice to prevent renaturation.

Probes were constructed on addition of labelling buffer (250mM Tris-HCl, pH 8.0; 25mM MgCl<sub>2</sub>, 10mM DTT, 1mM HEPES, pH 6.0; 26 A<sub>260</sub> units/ml random hexadeoxyribonucleotides), unlabelled dNTP's (ATP, GTP & TTP), 10mg/ml acetylated BSA, DNA template (500ng/ml), 50mCi [ $\alpha$ -<sup>32</sup>P] dCTP and 5 units of Klenow enzyme (100u/ml). Reactions were incubated at room temperature overnight. Unincorporated radioisotope was removed using a sephadex G-50 gel permeation chromatography column.

#### **5.2.4.0 Screening of a cDNA library with radiolabelled probes**

Screening of a cDNA library with radiolabelled probes was as described in Sambrook et al., (1989). The following sections (5.2.4.1-5.2.4.3), describe this process in detail.

#### **5.2.4.1 cDNA library plating**

LB broth (50 mls), supplemented with 0.2% maltose and 10mM MgSO<sub>4</sub>, was inoculated with a single colony of SURE cells (Stratagene), and grown overnight at 30°C with vigorous shaking. Cells were then collected by centrifugation for 10 minutes at 2000 rpm. The medium was then decanted and the pellet resuspended in 15mls of 10mM MgSO<sub>4</sub>. Cells were then diluted to an OD<sub>600</sub> = 0.5 in 10mM MgSO<sub>4</sub>.

Aliquots of cDNA library, to yield approximately 100 000 plaque forming units (pfu) per 500cm<sup>2</sup> plate, were mixed with 600µl of host (OD<sub>600</sub> = 0.5) SURE cells.

Bacteriophage and host cells were then incubated for 15 minutes at 37°C. Each aliquot of bacteriophage and host cells was then mixed with 40 mls of molten top agar (NZY broth {section 5.2.1.2} with 0.7% agarose), at approx. 48°C, and poured onto bottom agar plates (NZY broth {section 5.2.1.2} with 1.5% agar). Plates were then incubated at 39°C for 6-8 hours.

#### **5.2.4.2 Plaque lifts**

Four plates each containing 100 000 plaques were used for the primary screen. Duplicate lifts were carried out using nitrocellulose membranes (Schleicher & Schuell). Filters were placed on plated libraries for 1 min (first lift) and 3 minutes (second lift) respectively. Filters were then denatured for 1.5 minutes in 1.5M NaCl, 0.5M NaOH and neutralized for 5 minutes in 1.5M NaCl, 0.5M Tris-Cl pH 8 after which they were soaked in a solution of 3x SSC. DNA was then fixed to the membrane by baking in a vacuum oven at 80°C for 1 hr.

#### **5.2.4.3 Hybridization of radiolabelled probes on to nitrocellulose membranes**

Filters were prehybridized with a solution of 6x SSC, 5X Denhardt's reagent (Sambrook et al., 1989), 0.5% SDS, 100µg/ml of denatured herring or salmon sperm DNA and 0.1% sodium pyrophosphate at 62°C for 1.5-2 hours. Probes were hybridized to the filters overnight in a solution of 6x SSC, 5X Denhardt's reagent, 0.5% SDS, 1mM EDTA, 0.1% sodium pyrophosphate at 62°C. Filters were then washed at 62°C, with increasing stringency washes of 2x SSC, 0.1% SDS and 1x SSC, 0.1% SDS. Filters were then exposed to X-ray film. Plaques were identified by aligning the developed film with the plates. Positive plaques were cored and stored at 4°C in SM buffer (0.1M NaCl, 0.02M MgSO<sub>4</sub>, 0.05M Tris-HCl pH 7.5, 0.01% gelatin) and chloroform. To isolate single plaques the primary positive plaques were subjected to secondary and tertiary screens.

#### **5.2.5.1 *In vivo* excision of pBluescript phagemid clones from Uni-ZAP XR vector**

*In vivo* excision of clones was as described in ZAP-cDNA synthesis kit protocol (Stratagene). Phage, in SM buffer and chloroform, were released from cores by

vortexing. 100µl of the phage stock was then incubated with with 200µl of XL-1 Blue cells(OD<sub>600</sub>= 1.0) and 1µl ExAssist Helper phage (Stratagene) at 37°C for 15 minutes. Cells were then grown in 2x YT media for 2-2.5 hrs at 37°C, heated to 70°C for 20 mins and centrifuged for 15 mins at 4000g. The supernatant contained the rescued phagemid and could be stored at 4°C for 1-2 months. To plate out rescued phagemid 200µl of SOLR cells (OD<sub>600</sub>= 1.0) was added to two 1.5 ml tubes. 1µl and 50µl, respectively, of phage stock were added to two 1.5ml tube and incubated at 37°C for 15 minutes. SOLR cells were then plated on LB/ampicillin plates and incubated overnight at 37°C. Plasmid recovery from infected SOLR was as described in sections 5.2.2.3. Recovered cDNA was sequenced as described in section (5.2.5.2)

#### **5.2.5.2 DNA sequencing**

Direct sequencing of plasmid clones was performed by the dideoxy-sequencing method of Sanger et al., (1977) using fluorescent dye-linked universal M13 primers.

Sequences were analysed using an Applied Biosystems 370A DNA sequencer.

Reactions were prepared according to protocols provided by the manufacturer (Model 370A DNA Sequencing System, User's Manual Version 1.3A, October 1988).

Sequences were analysed using a combination of the GenEMBL data bases and DNA Strider

### **5.2.6.1 Wholemout In Situ Hybridization of Embryo's**

In situ hybridization was performed essentially as described by Wilkinson (1992).

### **5.2.6.2 Riboprobe construction**

Anti-sense riboprobes were transcribed *in vitro*. Plasmids were isolated from XL1 blue cells as previously described in section (5.2.2.3). Plasmid was then linearized by a restriction digest using EcoRI. T7 RNA polymerase (Promega), transcription buffer (200mM Tris-HCl pH 7.5, 30mM MgCl<sub>2</sub>, 10mM spermidine and 50mM NaCl), and an NTP labelling mixture containing 10 mmol/ ATP, 10 mmol/l CTP, 10 mmol/l GTP, 6.5 mmol/l UTP and 3.5 mmol/l digoxigenin labelled UTP (Boehringer) was added to transcribe the antisense riboprobe. To inhibit RNase activity, 1µl of RNase guard (Pharmacia) was then added. The reaction was incubated for 2 hrs at 37°C. DNA template was then removed by digestion with DNase I (Promega) at 37°C for 15 minutes. The reaction was then terminated by the addition of 2µl of 0.2 M EDTA. Transcribed antisense mRNA was then precipitated in 1ml of ethanol at -20°C overnight with 4µl of 4M LiCl; washed in 1ml of 70% ethanol; and resuspended in 100µl of DEPC treated ddH<sub>2</sub>O. A sample of RNA was then run out on a 1.2% agarose/ TAE gel to assess its concentration. Probe was used at a concentration of 20µg/ml of hybridization solution.

### **5.2.6.3 Embryo Fixation and hybridization**

Embryo fixation was as described by Wilkinson (1992). Rat embryos, of 14.5 day gestation, were rapidly dissected into sterile phosphate buffered saline (PBS)(PBS; 0.14 M NaCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub> and 3.2mM Na<sub>2</sub>HPO<sub>4</sub>), and kept on ice. Embryos were fixed at 4°C for 4hrs in 4% paraformaldehyde in PBS; washed in PBT (PBS + 0.1% Tween-20) at 4°C; and then dehydrated and rehydrated through a series of

concentrations of methanols in PBT from 25% - 100% and 100 - 25%. The samples were then washed again in PBT. To remove pigment, embryos were bleached in 6% hydrogen peroxide in PBT for 1 hr. Hydrogen peroxide was then removed by washing in PBT. Cellular membranes were then broken down by treatment with 10 $\mu$ g/ml of proteinase K (Sigma) in PBT for 15 mins. Proteinase K treatment was stopped with a 2mg/ml glycine in PBT solution for 5 minutes, and the specimens washed twice in PBT. They were then refixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBT for 20 mins. After fixation the embryos were washed in PBT and then prehybridized for 1 hr at 70°C in 50% formamide, 5 x SSC (pH 5), 50mg/ml yeast tRNA, 1% SDS and 50mg/ml heparin. Samples were then hybridized with 1mg/ml digoxigenin labelled antisense RNA probe in fresh prehybridization solution overnight at 70°C with gentle rocking. Control embryos were treated in the same manner but with no probe in the hybridization step.

#### **5.2.6.4 Post Hybridization**

Embryos were washed twice in 50% formamide, 5x SSC (pH 5) and 1% SDS (solution a) for 30 mins at 70°C. This was followed by a 1:1 wash with the above washing solution<sup>a</sup> and 0.5M NaCl, 10mM Tris-HCl pH 7.5, 0.1% Tween-20 (solution b) for 10 mins at 70°C. Following three successive washes in solution<sup>b</sup>, specimens were incubated twice with 100mg/ml ribonuclease A (Sigma) in solution<sup>b</sup> for 30 mins at 37°C. After washing again with solution<sup>b</sup>, the embryos were then washed in 50% formamide, 2x SSC pH 5 (solution c). They were then washed twice for 30 mins in solution<sup>c</sup> at 65°C after which they were washed in TBST (0.14M NaCl, 2.7mM KCl, 0.025M Tris-HCl pH 7.5, 0.1% Tween-20). The samples were then blocked with heat inactivated 10% sheep serum in TBST for 1.5hrs, and incubated overnight at 4°C with the preabsorbed antidioxigenin antibody (1:2000 dilution), 1.5mg/ml rat embryo powder, 1% sheep serum in TBST.

### **5.2.6.5 Histochemistry**

Embryos were washed with TBST and then three times in NTMT (100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl<sub>2</sub>, 0.1% Tween-20). To reveal the antibody the embryos were then incubated (with rocking) in NTMT with 4.5µl NBT<sup>a</sup> and 3.5µl BCIP<sup>d</sup>. When the colour had developed the reaction was stopped by washing in NTMT and storing in PBT at 4°C. Embryo's were photographed under a dissecting microscope (Nikon SMZ-10).

<sup>a</sup> NBT (nitroblue tetrazolium salt) is 75mg/ml in 70% dimethylformamide.

<sup>d</sup>BCIP (5-bromo-4-chloro-3-indolyl phosphate) is 50mg/ml in dimethylformamide.

### **5.2.6.6 Sectioning of wholemount in situ hybridization embryos**

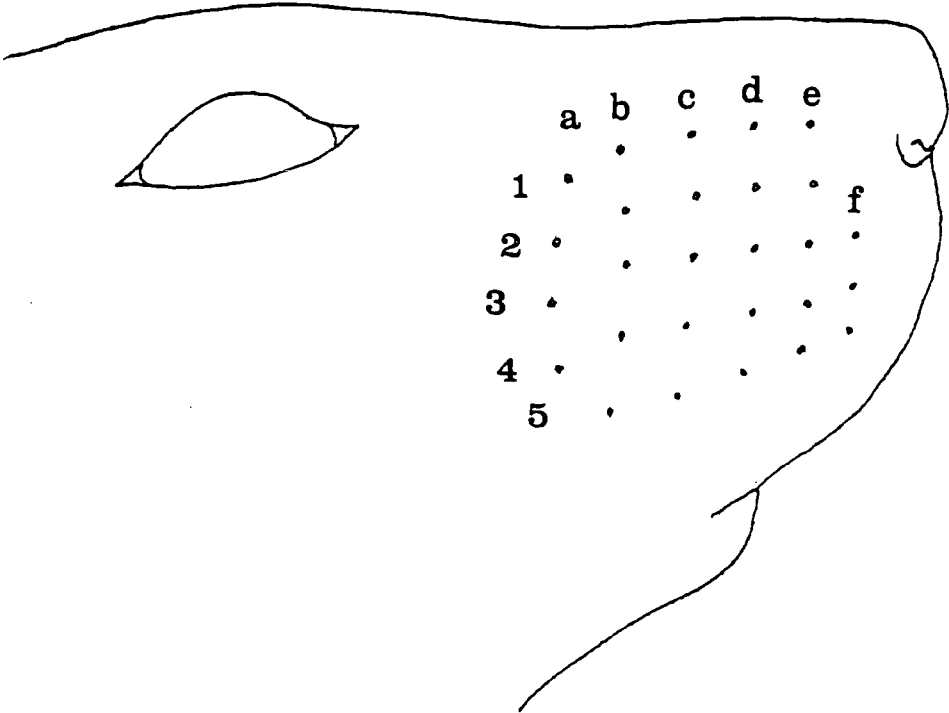
Embryo heads were sectioned using a vibratome (Campden). The heads were dissected from the body and mounted on a 1% agarose gel with "super glue" (Loctite). Heads were quickly orientated prior to the glue setting. Crude 200µm sections were obtained through the the head, and floated in PBS. Sections were carefully removed and examined under dissection microscope (Zeiss) whilst immersed in saline. Sections were photographed on a Zeiss dissecting microscope.

### **5.2.7.1 Follicle Identification and Nomenclature**

Identification of follicle position in the developing mystacial pad was as described by Oliver (1965) [figure 47]. Whisker follicles develop in a pattern of well defined rows. The five horizontal rows are numbered 1 to 5 in a dorso-ventral direction, and the six vertical rows lettered a to f in the posterior anterior direction.

**Figure 47**

A schematic representation of follicle position on the rat mystacial pad as described by Oliver, (1965). Follicles are labelled as coordinates. Horizontal rows are 1 to 5, and vertical rows lettered a to f.



### **5.2.8.1 Differential screen of clumped DP cells against subconfluent DP cells**

### **5.2.8.2 Cell culture**

Rat (PVG) dermal papilla cell cultures were routinely grown in Eagle's MEM supplemented with 10% foetal calf serum and antibiotics (Jahoda and Oliver, 1981). Cells were grown until the cells had reached the required subconfluent or aggregated state as previously described in section (3.4.1.1).

### **5.2.8.3 Poly (A)<sup>+</sup> RNA isolation**

When isolating, or working with, poly (A)<sup>+</sup> RNA, all glassware, plasticware and solutions should be free from ribonucleases (RNAases). Glassware should be baked at 180°C or more for 8 hours and plasticware treated with a 0.1% solution of diethyl pyrocarbonate (DEPC), overnight at 37°C. All solutions, except buffers containing Tris, can be DEPC treated in this manner. After incubation, all plastics and solutions are autoclaved (Sambrook et al., 1989).

Poly (A)<sup>+</sup> RNA was isolated using a Pharmacia Quickprep<sup>TM</sup> Micro mRNA Purification Kit. Cells were washed and scraped into PBS and spun down. Nucleic acids were extracted using Pharmacia extraction buffer ( a buffered aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine) and elution buffer (10mM Tris-HCl (pH 7.4), 1mM EDTA). The homogenate was then cleared by centrifugation and the supernatant mixed with Oligo(dt) cellulose resin (Pharmacia). The Oligo(dt) cellulose was then pelleted, by centrifugation, with the bound mRNA and the supernatant discarded. The pellet was then sequentially washed, by resuspension and centrifugation, through a series of high and low salt buffers provided in the Pharmacia kit. After the final wash, the pelleted material was transferred to a MicroSpin Column (Pharmacia) in a small volume of low salt buffered solution, and washed a further 2

times, with a brief centrifugation between washes. The poly (A)<sup>+</sup> RNA was finally eluted with pre-warmed elution buffer by centrifugation. Once eluted the mRNA was frozen or used immediately. The whole extraction and recovery was performed on ice in a 4°C room.

#### **5.2.8.4 Probe construction**

Poly (A)<sup>+</sup> mRNA was extracted from the subconfluent and clumped *in vitro* dermal papilla cells (section 3.4.1.1). Poly (A)<sup>+</sup> mRNA from both populations was heated to 70°C for 3 minutes and placed on ice. First strand cDNA was constructed using, random primers (pdN6), MMLV 5x buffer (250mM Tris-HCl pH 8.3, 375mM KCl, 15mM MgCl<sub>2</sub>, 50 mM DTT), dNTP's(CTP,GTP,TTP), MMLV reverse transcriptase (Promega) and radiolabelled ATP. The positive clumped cDNA probe was constructed with [ $\alpha$ -<sup>35</sup>S] dATP (low energy  $\beta$  emitter)( $> 400$ Ci/mmol). The negative or SC cDNA probe was labelled with [ $\alpha$ -<sup>32</sup>P] dATP (high energy  $\beta$  emitter),( $> 400$ Ci/mmol). Each probe was constructed by incubation for 1.5hrs at 37°C. Unincorporated nucleotide was removed using chromatography biospin columns (Biorad). Probes were denatured by heating for 10 minutes at 70°C in 400mM NaOH\40mM Na<sub>2</sub>EDTA, after which they were treated with 1M Tris-Cl pH=7.2 and 20mM Tris-Cl pH=8\2% SDS\50mM Na<sub>2</sub>EDTA pH=8\200mM NaCl. Positive and negative probes were then counted using a scinitillation counter.

#### **5.2.8.5 Differential screen**

A hair vibrissa end bulb cDNA library, constructed by Dr J. Huckle and Miss C.J. Whitehouse, was differentially screened by adapting a method described by Olszewski et al(1989). Phage were plated at a low density (20 000 pfu) in 500cm<sup>2</sup> dishes (Nucleopore). Duplicate lifts of nitrocellulose were taken from the dish as previously described in section (5.2.4.2). Filters were prehybridized with 6x SSC, 5X Denhardt's

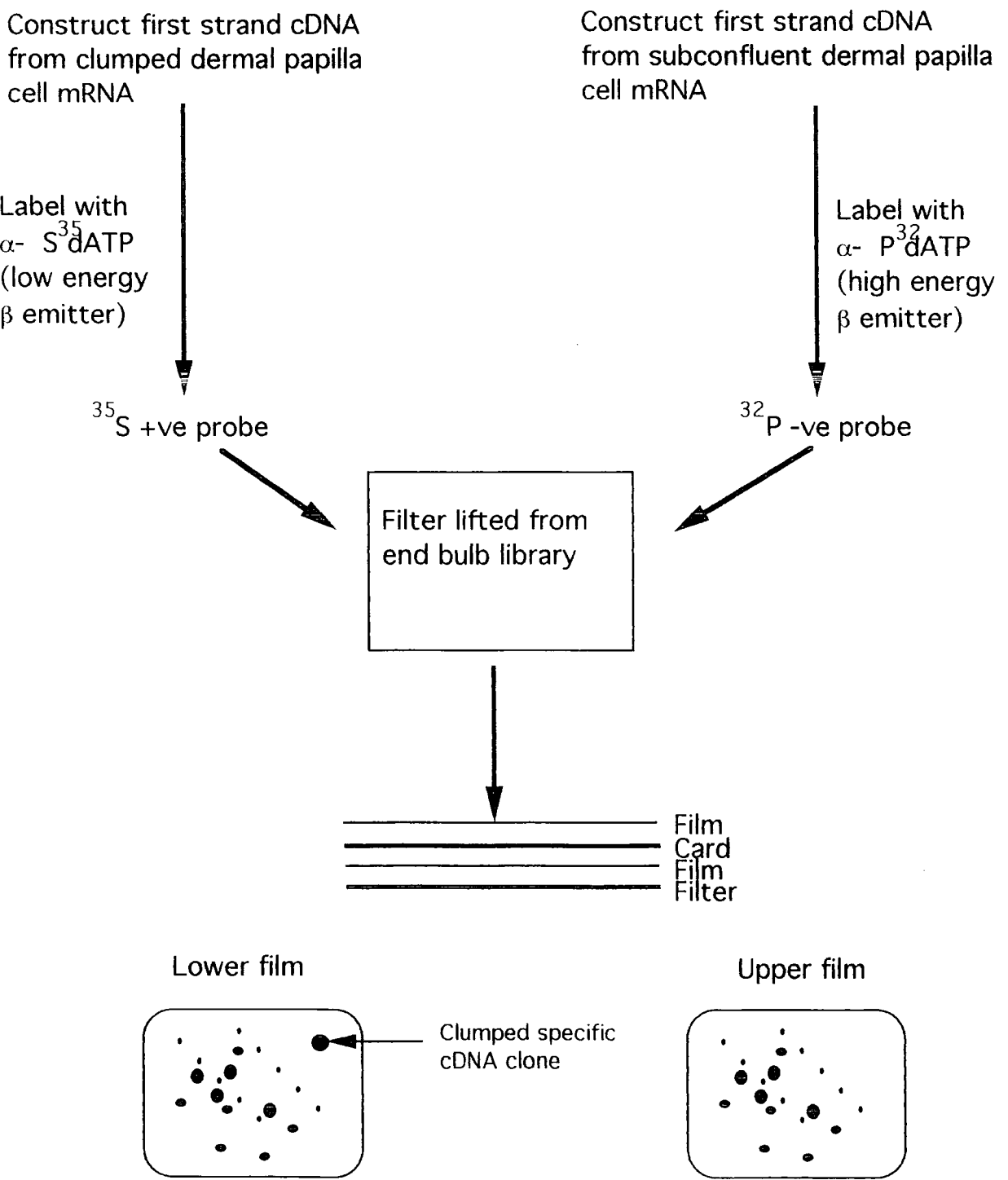
reagent, 0.5% SDS, 100µg/ml herring sperm DNA and 0.1% sodium pyrophosphate at 62°C for 1.5-2 hours. Both filters were hybridized with [ $\alpha$ -<sup>35</sup>S] and [ $\alpha$ -<sup>32</sup>P] cDNA probes simultaneously in 6xSSC, 5X Denhardt's reagent, 0.5% SDS, 1mM EDTA, 0.1% sodium pyrophosphate at 62°C overnight. Filters were then washed in 2x SSC, 0.1% SDS followed by 1X SSC, 0.1% SDS; mounted on Whatman 3mm paper; and exposed to 2 X-ray films. The first film was placed next to the filter, with the second film separated from the first film by an attenuator. The attenuator was able to block the emissions of the positively [ $\alpha$ -<sup>35</sup>S] (low energy  $\beta$  emitter) labelled CL DP cDNA clones from the top film. Plaques were identified by aligning both films with the plate and coring the plaques that were a) not present on both filters or b) that had differential expression. A diagrammatic representation of this procedure can be seen in figure (48). Plaques were cored as described in section (5.2.4.3). Secondary screens were performed to isolate single plaques.

Single putative differential clones were isolated using the plasmid preparation described in section (5.2.2.3). Isolated plasmids were then digested with EcoRI or Pst I and Xho I. Putative clones were run on a 1.2% agarose/ 1x TAE gel to estimate the size of the inserts. Complementary DNA clones were sequenced as described in section (5.2.5.2).

#### **5.2.8.6 Wholemout in situ hybridization of DP cells on coverslips.**

Cells were prepared for whole mount in situ hybridization as described by Dirk et al. (1993). DP cells were grown on coverslips as described in section (3.3.4.3), and fixed while displaying the different degrees of the aggregation process (section 3.4.1.1), subconfluent, aggregating and clumped.

Fig. 48 A diagrammatic representation of a dual labelling method for the differential screen of an end bulb cDNA library, as described by Olszewski et al., 1989



#### **5.2.8.7 Fixation of *in vitro* DP cells on coverslips**

Cells were briefly rinsed in PBS (3 x 3min washes) and fixed in 4% formaldehyde, 5% acetic acid in 0.9% NaCl for 20 min at room temperature. After fixation cells were rinsed in PBS for 5 min, dehydrated in ethanol (50, 70, 90, 95 and 100% ethanol for 5 mins each) and washed for 10 min in Histoclear. Cells were then washed for 5min in 100% ethanol, before rehydration back to PBS. Cells were then treated with 0.1% pepsin (Sigma) in 0.01M HCl for 10 min at 37°C. Cells were then washed and post fixed in 1% formaldehyde in PBS for 5 min to inactivate the pepsin. Cells were then rinsed and dehydrated through to ethanol.

#### **5.2.8.8 In situ hybridization of DP cells *in vitro***

Anti-sense RNA probes were constructed as described in section (5.2.6.2). Probes were incubated at 80°C for 10 minutes to remove secondary RNA structure, and used at a final concentration of 5ng/μl. Hybridization was performed overnight at 50°C in 60% deionized formamide, 2 x SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0), 50mM sodium phosphate and 250 ng/ml of salmon sperm DNA. After addition of hybridization solution the coverslips were covered with Parafilm (Whatman) to maintain an even distribution. Once hybridization was completed the cells were washed in 50% formamide at 60°C (3 x 10 min). Cells were then rinsed in 0.1M Tris-HCl, pH 7.4, 0.15M NaCl and 0.05% Tween-20 for 5 mins.

#### **5.2.8.9 Immunocytochemical detection of digoxigenin labelled RNA probes in *in vitro* DP cells**

Specimens were incubated for 30 mins at room temperature with the preabsorbed antidioxigenin antibody(1:2000 dilution)(Boehringer) and 1% sheep serum in TBST (section 5.2.6.4). The coverslips were maintained in a humidity chamber to prevent evaporation of the antibody solution from the cells. After incubation cells were washed (3 x 10 min) in 0.1M Tris-HCl, pH 7.4, 0.15M NaCl, 0.05% Tween-20. The digoxigenin antibody was revealed as described in section (5.2.6.5). When the colour had developed the reaction was stopped by washing in NTMT and storing in PBT at 4°C. Coverslips were then mounted on slides with glycergel (Sigma) and photographed.

## Results

### 5.3.1.1 Isolation of rat BMP 2 and BMP 4 cDNA clones

Screening of a rat vibrissa follicle end bulb cDNA library with mouse BMP 2 and BMP 4 probes, as described in section (5.2.4.0 - 5.2.5.2), resulted in the isolation of 6 putative clones and 5 putative clones respectively (Fig. 49e). Two putative clones that hybridised to each mouse probe were further characterized via sequencing of their 5' ends. Resulting sequences were then used to search the GenEMBL data base, Daresbury. The mouse BMP 2 cDNA probe hybridised to a sequence that was 93.3% homologous over 683bp to rat BMP 2 (fig 49a), human-81.9% over 689bp, *Xenopus laevis*-62.8% over 691bp and chick 75.9% over 403bp (fig 49b).

The mouse BMP 4 cDNA probe hybridised to a clone that was 80.4% homologous to human BMP2 over 372bp (Fig 49c) and a clone that was 94.5% homologous in 344bp to mouse BMP 4 (fig 49d), 91.3% homologous in 343bp to human BMP 4 and 79.4% in 209bp to *X. laevis* BMP 4 at the 5' end.

The largest rat BMP 4 and BMP2 cDNA clones were both approximately 1.7kb (fig 49f)

### 5.3.1.2 Distribution of BMP 4 transcripts using whole mount in situ hybridization of the developing mystacial pad of the 14.5 D embryonic rat

Rat BMP4 expression was visible on the integument of the animal. Consistent with Jones et al., (1991), expression on the face was restricted to the developing nasal passages and the mystacial pad (fig 50b). Mystacial pad staining was restricted to follicle placodes. Follicles develop in a wave along the posterior anterior and dorso-ventral axis, and BMP 4 staining was generally present in a band from the posterior dorsal region through to the anterior ventral region (fig 50b).

Using Oliver's (1965) nomenclature (section 5.2.71), in most embryos the strongest labelling was for follicles 1b+c, 2 b+c, 3 c+d, 4 d+e+f and 5 d+e+f, although slight variation was apparent. Placodes adjacent to this diagonal banding had more diffuse labelling, and it was particularly noticeable that the "a" row follicles were virtually free of label. Staining was symmetrical on each whisker pad (fig 50c). The developing whisker below the eye was also positive for BMP 4 (fig 50b). Subtle BMP 4 expression was visible in the nose as the nostrils invaginate. The developing ear also stained positive for BMP 4 (fig 50c), with the strongest stain in the pinna of the ear. Control embryo's, that were hybridized in the absence of the BMP4 antisense probe, illustrated no specific binding (fig 50a).

Thick sections of the snout and mystacial pad showed a marked mesenchymal distribution of stain, in the region of the developing follicles (fig 50d,e). Staining was restricted to the basal lamina and condensed mesenchyme immediately below the epidermal placode. Staining was strongest in the condensed mesenchyme of rows 'b', 'c' and 'd' (Fig. 50e), whereas follicles in the earliest stages of condensation, row "f", had reduced expression in the basal lamina region (Fig. 50d). By contrast, labelling in the invaginating nostrils was strictly epidermal with no expression in the dermis. Both these distributions were consistent with BMP 4 expression studies in the mouse embryo (Jones et al., 1991).

#### **5.3.1.3 Distribution of BMP4 transcripts in the sectioned adult rat vibrissa follicle.**

Dr J. Huckle (Durham University, UK) probed telogen sections of rat sinus follicles with the rat homologue to BMP 4, as described in Wilkinson et al., (1992). Staining was positive throughout the outer root sheath cells of the epidermis (fig 51a,b). A line of ORS cells were positive adjacent to the GM (fig 51c). The dermal components of the follicle including the dermal papilla were not labelled and, there was no positive staining

in the club fibre. BMP 4 expression was also visible in the sebaceous gland in the isthmus of the follicle (fig 51d).

### **5.3.2.1 Identification of differentially expressed cDNA clones using *in vitro* generated probes from *in vitro* CL and SC DP cell probes**

40 putative differential plaques were isolated from the primary screen of the vibrissa end bulb cDNA library and designated mas CL-1 to mas CL-40. Only 11 of the 40 have been further characterised. Of these 11, only 5 were shown to be differentially expressed in the secondary screens (Fig. 52).

### **5.3.2.2 Sequence analysis of cDNA inserts isolated from the differential screen of *in vitro* clumped and subconfluent DP cells**

cDNA clone, mas-CL 32, when identified on an agarose gel (section 5.2.3.2) was found to be approximately 430bp in length (fig 52a). After sequencing mas-CL 32 had 91.9% homology with *Mus musculus* glycosylation inhibiting factor over 420bp, 93% homology with *Mus musculus* growth factor-induced delayed early response protein over 385bp and 93% homology with *Mus musculus* macrophage migration inhibitory factor (MIF) over 385bp (Fig 52b). However, the sequence was not full length and was missing approximately 60 bases of the 5' end. The deduced amino acid sequence was then used to search the Swissprot data base and found to have 98.8% homology over 83 amino acids with mouse macrophage inhibitory factor only (fig 52c). Moreover, the protein sequences from the remaining closely matched nucleotide sequences were not present in this data base. *Mus musculus* glycosylation inhibiting factor protein sequence has been reported by Mikayama et al., (1993), however subsequent papers on its homology with macrophage migration inhibitory factor,

indicate that it may be the same protein. This will be covered in greater detail in the subsequent discussion.

cDNA clone, mas- CL 12, was approximately 450bp in length (Fig 53a). When 155bp of this clone was sequenced it was found to have 78.7% sequence homology with the mouse gene, T-cell specific protein 30 (Tcl-30) (fig. 53b) (Harrigan et al., 1989, Baughman et al., 1992 ). As with mas-CL 32, mas- CL 12 sequence was not full length. Clone mas-CL 12 matched to the mouse sequence in the 3' untranslated region from nucleotides 2179-2331.

Two cDNA clones isolated from the library were found to have no significant homology with any genes in the GenEMBL data base. Clones mas CL- 10.5 (approx. 450bp in length), (Fig. 53a,c) and mas CL- 23 (approx 600bp in length), (Fig. 54a,b) had no homology with any previously described gene in the data base. Therefore, these may be novel genes.

#### **5.3.3.1 In situ hybridization of clone mas-CL 32 (MIF) on cultured DP cells**

#### **5.3.3.2 Subconfluent DP cells**

In subconfluent cells, mas-CL 32 expression was variable, however it was generally at a high level (fig. 55a). Expression was particularly intense in the perinuclear regions (fig 55b), with a gradual decrease in expression at the edges of the lamellipodia. Some subconfluent DP cells had markedly less labelling (fig. 55c), with a more diffuse expression throughout the cytoplasm. SC DP cells labelled with a 16s probe, a ribosomal gene expressed within mitochondria, were also positive, but expression was less than that for mas-CL 32 (fig. 55d). Control cells with no digoxigenin probe showed no labelling within the cells (fig 55e).

### **5.3.3.3 Aggregating DP cells**

Expression of mas-CL 32 did occur within the AG DP cells, however the labelling was far less intense than that illustrated by the SC cells (fig. 56a). Colouring was diffuse throughout the cells with no obvious increase in the perinuclear area (fig. 56b). In more pronounced aggregating regions, there was an increase in intensity of label, however this is only slight and probably due to the layering effect of the cells within these regions (fig 56c), and again the distribution of label within the cells was even. Control cultures with the probe omitted, had no background stain (fig. 56d).

### **5.3.3.4 Clumped DP cells**

Mas-CL 32 expression within the CL DP cultures was different to that described for SC and AG DP cultures. Labelling was most intense in cells within fully established clumps of the culture, with a relatively feeble expression visible in the cells between the clumped structures (fig. 57a). A number of cells within these clumps were expressing greater quantities of mas-CL 32 than the surrounding cells within the clump (fig 57b). This increased intensity within clumps was not due to the piling effect of cells, as single cells on the periphery of clumps were also strongly positive for mas-CL 32 (fig. 57c). In a few clumps, expression had dropped considerably, however a number of individual cells were still clearly expressing mas-CL 32 (fig. 57d). Due to the high density of cells within the clump it is not apparent whether labelling is throughout the whole cytoplasm of the cell or whether it has a perinuclear distribution. Control cultures had no background labelling.

Fig 49a

```

                                10      20      30
dubmp2.      GGCACGAGCCGGGACCCGCTGTCTTCTAGTGTGCTG
              |||
rtbmp2  ACTGCGGTCTCCTAAAGGTCGACCATGGTGGCCGGGACCCGCTGTCTTCTAGTGTGCTG
              50      60      70      80      90      100

              40      50      60      70      80      90
dubmp2.  CTTCCCCAGGTCCTCCTGGGCGGGCGGGCCGGCCTCATTCCGGAGCTGGGCCGCAAGAAG
              |||
rtbmp2  CTTCCCCAGGTCCTCCTGGGCGGGCGGGCCGGCCTCATTCCGGAGCTGGGCCGCAAGAAG
              110     120     130     140     150     160

              100     110     120     130     140     150
dubmp2.  TTCGCCGGGGCATCCGGCCGCCCTTGTCCCGGCCTTCGGACGACGT-CTCAGCGAGTTT
              |||
rtbmp2  TTCGCCGGGGCATC---GCGCCCTTATCCCGGCCTTCGGAGGACGTCTCAGCGAGTTT
              170     180     190     200     210

              160     170     180     190     200     210
dubmp2.  AAGTTGAGGCTGCTCAGCATGTTTGGCCTGAAGCAGAGACCCACCCCAAGGACGTC
              |||
rtbmp2  GAGTTGAGGCTGCTCAGCATGTTTGGCCTGAAGCAGAGACCCACCCCAAGGACGTC
              220     230     240     250     260     270

              220     230     240     250     260     270
dubmp2.  GTGGTGCCCCCTATATGCTCGACCTTGTACCGCCGGCACTTGGGCCAGTCAGGGAGCGT
              |||
rtbmp2  GTGGTGCCCCCTATATGCTCGACC-TGTACCGCCGGCACTCGGGCCAGCCA-GGAGCGC
              280     290     300     310     320     330

              280     290     300     310     320     330
dubmp2.  CCGTCCCAAACCACCGGTTTTGAGAGGGGCAGCCAACCGGGCCAAATAACGTGCGCAGTT
              |||
rtbmp2  TCGCCCCAGACCACC-GGCTGGAGA-GGGCAGCCAGCCGCGCC-AACACCGTGCTCAGCT
              340     350     360     370     380     390

              340     350     360     370     380     390
dubmp2.  TCCATCACGAAGAAGCCATCGAGGAAC TTCAGAAATGAGTGGGAAAACGTCCCGACGCT
              |||
rtbmp2  TCCATCACGAAGAAGCCATCGAGGAAC TTCAGAAATGAGTGGGAAAAC TTCCCGACGCT
              400     410     420     430     440     450

              400     410     420     430     440     450
dubmp2.  TCTTCTTCAATTTAAGTTCTGTCCCTACTGATGAGTTTCTCACATCTGCGGAGCTCCAGA
              |||
rtbmp2  TCTTCTTCAATTTAAGTTCTGTCCCTACTGATGAGTTTCTCACATCTGCAGAGCTCCAGA
              460     470     480     490     500     510

              460     470     480     490     500     510
dubmp2.  TTTTTCGGGAACAAATGCAGGAAGCTTTGGGAAATAGTAGTTTCCAGCACCGAATTAATA
              |||
rtbmp2  TTTTTCGGGAACAAATGCAGGAAGCTTTGGGAAATAGTAGTTTCCAGCACCGAATTAATA
              520     530     540     550     560     570

              520     530     540     550     560     570
dubmp2.  TTTATGAAATTATAAAGCCTGCCACAGCCAGCTCAAAATTTTCTGTGACCAGACTATTG
              |||
rtbmp2  TTTATGAAATTATAAAGCCTGCCACAG-CCAGCTCAAAATTTTCTGTGACCAGACTATTG
              580     590     600     610     620     630
```

```

      580      590      600      610      620      630
dubmp2. GACACCAGGTTAGTGGACTCAGAACACAAGTCAGTGGGAGAGCTTTTGTGTCAACCCGG
          |||
rtbmp2  GACACCAGGTTAGT-GACTCAGAACACAAGTCAGTGGGAGAGC-TTGATGTCACCCCGG
          640      650      660      670      680

      640      650      660      670      680
dubmp2. TTGTTGATGCGATGGGACAGGACAGGGGACAACCAACC-TTGGTTTTTGGTGG
          || |||
rtbmp2  CTG-TGATGCGAT-GGACAGCACAGGGACACACCAACCATGGGTTTGTGGTGGGAAGTGGC
          690      700      710      720      730      740

rtbmp2  CCACTTAGAGGAGAAGCCAGGTGTCTCCAAGAGACATGTGAGGATTAGCAGGTCTTTGCA
          750      760      770      780      790      800

```

Fig 49b

|        |            |             |             |            |            |
|--------|------------|-------------|-------------|------------|------------|
|        | 351        |             |             |            | 400        |
| hbmp2  | GGCCGGGACC | CGCTGTCTTC  | TAGCGTTGCT  | GCTTCCCCAG | GTCCTCCTGG |
| rtbmp2 | GGCCGGGACC | CGCTGTCTTC  | TAGTGTGCT   | GCTTCCCCAG | GTCCTCCTGG |
| dubmp2 | AGCCGGGACC | CGCTGTCTTC  | TAGTGTGCT   | GCTTCCCCAG | GTCCTCCTGG |
| chbmp2 | .....      | .....       | .....       | .....      | .....      |
| xbmp2  | CGCTGGGATC | CACTCTCTGC  | TCCTGCTGCT  | GTTTTACCAG | GTCTTGCTGA |
| CONS   | --C-GGGA-C | C-CT-TCT-C  | T---G-TGCT  | G-TT--CCAG | GTC-T-CTG- |
|        | 401        |             |             |            | 450        |
| hbmp2  | GCGGCGCGGC | TGGCCTCGTT  | CCGGAGCTGG  | GCCGCAGGAA | GTTCGCGGCG |
| rtbmp2 | GCGGCGCGGC | CGGCCTCATT  | CCGGAGCTGG  | GCCGCAAGAA | GTTCGCCGGG |
| dubmp2 | GCGGCGCGGC | CGGCCTCATT  | CCGGAGCTGG  | GCCGCAAGAA | GTTCGCCGGG |
| chbmp2 | .....      | .....       | .....       | .....      | .....      |
| xbmp2  | GTGGCTGCAC | CGGACTGATA  | CCCGAGGAAG  | GCAAGCGCAA | GTATACGGAA |
| CONS   | G-GGC----C | -GG-CT--T-  | CC-GAG---G  | GC-----AA  | GT---C-G-- |
|        | 451        |             |             |            | 500        |
| hbmp2  | GCGTCGTCGG | GCCGCCCTC   | ATCCCAGCCC  | TCTGACGAGG | TCCTGAGCGA |
| rtbmp2 | GCATCG.... | ..CGCCCCCTT | ATCCCGGCT   | TCGGAGGACG | TCCTCAGCGA |
| dubmp2 | GCATC...CG | GCCGCCCTT   | GTCCCGGCT   | TCGGACGACG | T.CTCAGCGA |
| chbmp2 | .....      | .....GGTTC  | GCTGAAGCGC  | CCCAGGACG  | TCCTGGGCGA |
| xbmp2  | TC.....GG  | GTGCTGCTC   | TCCCAGCAG   | TCCAGAGGG  | TCCTCAACCA |
| CONS   | ---TC----G | G-CGC---T-  | -----G---   | -C--A----- | TCCT---C-A |
|        | 501        |             |             |            | 550        |
| hbmp2  | GTTCGAGTTG | CGGCTGCTCA  | GCATGTTTCGG | CCTGAAACAG | AGACCCACCC |
| rtbmp2 | GTTTGAGTTG | AGGCTGCTCA  | GCATGTTTGG  | CCTGAAGCAG | AGACCCACCC |
| dubmp2 | GTTTAAGTTG | AGGCTGCTCA  | GCATGTTTGG  | CCTGAAGCAG | AGACCCACCC |
| chbmp2 | GTTCGAGCTG | CGCCTGCTCC  | ACATGTTTCGG | GCTGAAGCGG | CGGCCGAGCC |
| xbmp2  | GTTCGAGCTC | CGACTGCTCA  | GTATGTTTGG  | CTTGAAGAGG | CGTCCGACGC |
| CONS   | GTT--AG-T- | ---CTGCTC-  | --ATGTT-GG  | --TGAA---G | ---CC-A--C |
|        | 551        |             |             |            | 600        |
| hbmp2  | CCAGCAGGGA | CGCCGTGGTG  | CCCCCTACA   | TGCTAGACC. | TGTATCGCAG |
| rtbmp2 | CCAGCAAGGA | CGTCGTGGTG  | CCCCCTATA   | TGCTCGACC. | TGTACCGCCG |
| dubmp2 | CCAGCAAGGA | CGTCGTGGTG  | CCCCCTATA   | TGCTCGACCT | TGTACCGCCG |
| chbmp2 | CCGGCAAGGA | CGTCGTCATC  | CCCCCTACA   | TGTTGGACC. | TCTATCGCCT |
| xbmp2  | CTGGCAAAAA | TGTTGTGATC  | CCACCCTACA  | TGCTGGACT. | TGTACCACCT |
| CONS   | C--GCA---A | -G--GT--T-  | CC-CCCTA-A  | TG-T-GAC-  | T-T--C-C-- |
|        | 601        |             |             |            | 650        |
| hbmp2  | GCACTCAGGT | CAG.....    | .....CCGGG  | CTCACCCGCC | CCAGACCACC |
| rtbmp2 | GCACTCGGGC | CAG.....    | .....CCAGG  | AGCGCTCGCC | CCAGACCACC |
| dubmp2 | GCACTTGGGC | CAG.....    | .....TCAGGG | AGCGTCCGTC | CCAAACCACC |
| chbmp2 | GCACGCCGGC | CAGCAG....  | .....       | .....      | CTGGGCTACC |
| xbmp2  | GCACTTGGCT | CAGTTGGCTG  | CTGATGAAGG  | CACTTCTGCC | ATGGACTTCC |
| CONS   | GCAC-----  | CAG--G----  | ----T---GG  | --C----G-C | -----C--CC |
|        | 651        |             |             |            | 700        |
| hbmp2  | GG..TTGGAG | AGGGCAGCCA  | GCCGAGCC.A  | ACACTGTGCG | CAGCTTCCAC |
| rtbmp2 | GG..CTGGAG | AGGGCAGCCA  | GCCGCGCC.A  | ACACCGTGCT | CAGCTTCCAT |
| dubmp2 | GGGTTTGAGA | GGGGCAGCCA  | ACCGGGCCAA  | ATAACGTGCG | CAGTTTCCAT |
| chbmp2 | CG..CTGGAG | AGGGCCGCT   | GCCGCGCC.A  | ACACCGTGTG | CAGCTTCCAC |
| xbmp2  | AG..ATGGAG | CGGGCAGCCA  | GCCGAGCA.A  | ATACAGTGAG | GAGTTTCCAC |
| CONS   | -G---TG--- | -GGGC-GCC-  | -CCG-GC--A  | A-A--GTG-- | -AG-TTCCA- |

|        |            |            |            |            |            |     |
|--------|------------|------------|------------|------------|------------|-----|
|        | 701        |            |            |            |            | 750 |
| hbmp2  | CATGAAGAAT | CTTTGGAAGA | ACTACCAGAA | ACGAGTGGGA | AAACAACCCG |     |
| rtbmp2 | CACGAAGAAG | CCATCGAGGA | ACTTTCAGAA | ATGAGTGGGA | AAACTTCCCG |     |
| dubmp2 | CACGAAGAAG | CCATCGAGGA | ACTTCCAGAA | ATGAGTGGGA | AAACGTCCCG |     |
| chbmp2 | CACGAAGAAG | TTTTGGAAGA | ACTGCCAGAA | ACAAGTGGGA | AAACAGCACG |     |
| xbmp2  | CATGAAGAAT | CCATGGAAGA | AATTCCTGAG | TCTCGTGAGA | AAACAATCCA |     |
| CONS   | CA-GAAGAA- | ---T-GA-GA | A-T--C---- | ----GTG--A | AAAC----C- |     |

|        |            |            |            |            |            |     |
|--------|------------|------------|------------|------------|------------|-----|
|        | 751        |            |            |            |            | 800 |
| hbmp2  | GAGATTCTTC | TTTAATTTAA | GTTCTATCCC | CACGGAGGAG | TTTATCACCT |     |
| rtbmp2 | ACGCTTCTTC | TTCAATTTAA | GTTCTGTCCC | TACTGATGAG | TTTCTCACAT |     |
| dubmp2 | ACGCTTCTTC | TTCAATTTAA | GTTCTGTCCC | TACTGATGAG | TTTCTCACAT |     |
| chbmp2 | ACGTTTCTTC | TTTAATTTAA | CTTCCATCCC | TAATGAGGAG | TCTGTACACT |     |
| xbmp2  | ACGATTCTTC | TTCAACCTTT | CTTCAATTCC | AAATGAGGAG | CTGGTCACTT |     |
| CONS   | --G-TTCTTC | TT-AA--T-- | -TTC--T-CC | -A--GA-GAG | ----TCAC-T |     |

|        |            |            |            |            |            |     |
|--------|------------|------------|------------|------------|------------|-----|
|        | 801        |            |            |            |            | 850 |
| hbmp2  | CAGCAGAGCT | TCAGGTTTTT | C...GAGAAC | AGATGCAAGA | TGCTTTAGGA |     |
| rtbmp2 | CTGCAGAGCT | CCAGATTTTT | C...GGGAAC | AAATGCAGGA | AGCTTTGGGA |     |
| dubmp2 | CTGCGGAGCT | CCAGATTTTT | C...GGGAAC | AAATGCAGGA | AGCTTTGGGA |     |
| chbmp2 | CAGCTGAACT | CCAGATTTTT | CCGGGGGAGC | AGGTGCACGA | AGCCTTTGAG |     |
| xbmp2  | CTGCCGAGCT | GCGGATTTTT | C...GAGAGC | AGGTCCAAGA | GCCCTTTGAA |     |
| CONS   | C-GC-GA-CT | -C-G-TTTT- | C---G-GA-C | A--T-CA-CA | --C-TT-C-- |     |

|        |            |            |            |            |            |     |
|--------|------------|------------|------------|------------|------------|-----|
|        | 851        |            |            |            |            | 900 |
| hbmp2  | AACAATAGCA | GTTTCCATCA | CCGAATTAAT | ATTTATGAAA | TCATAAAACC |     |
| rtbmp2 | ...AATAGTA | GTTTCCAGCA | CCGAATTAAT | ATTTATGAAA | TTATAAAGCC |     |
| dubmp2 | ...AATAGTA | GTTTCCAGCA | CCGAATTAAT | ATTTATGAAA | TTATAAAGCC |     |
| chbmp2 | AGCAACAGCA | GCTACCATCA | CCGTATTAAT | ATTTATGAAA | TTATGAAGCC |     |
| xbmp2  | AGTGACAGCA | GCAAATTGCA | TCGGATTAAT | ATTTACGACA | TTGTCAAGCC |     |
| CONS   | A---A-AG-A | G-----CA   | -CG--TTAAT | ATTTA-GA-A | T--T-AA-CC |     |

|        |            |            |            |            |            |     |
|--------|------------|------------|------------|------------|------------|-----|
|        | 901        |            |            |            |            | 950 |
| hbmp2  | TGCAACAG.C | CAACTCGAAA | TTCCCCGTGA | CCAGACTTTT | GGACACCAGG |     |
| rtbmp2 | TGCCACAG.C | CAGCTCAAAA | TTTCTGTGTA | CCAGACTATT | GGACACCAGG |     |
| dubmp2 | TGCCACAG.C | CAGCTCAAAA | TTTCTGTGTA | CCAGACTATT | GGACACCAGG |     |
| chbmp2 | AGCCACAG.C | CACCTCCAAG | GACCCTGTCA | CGAGACTTTT | GGACACCAGG |     |
| xbmp2  | AGCGGCGG.C | TGCCTCCCGG | GGCCCTGTTG | TGAGACTATT | GGACACCAGA |     |
| CONS   | -GC----G C | ---CTC---- | ---CC-GT-- | --AGACT-TT | GGACACCAG- |     |

|        |            |            |            |            |            |      |
|--------|------------|------------|------------|------------|------------|------|
|        | 951        |            |            |            |            | 1000 |
| hbmp2  | TTGGT.GAAT | CAGAATGCAA | GCAGGTGGGA | AAG.TTTTGA | TGTCACCCCC |      |
| rtbmp2 | TTAGT.GACT | CAGAACACAA | GTCAGTGGGA | GAG.CTTTGA | TGTCACCCCG |      |
| dubmp2 | TTAGTGGACT | CAGAACACAA | GTCAGTGGGA | GAGCTTTTGT | TGTCAACCCG |      |
| chbmp2 | TTGGTGCA.T | CATAATGCAA | GTAATGGGA  | AAG.TTCTGA | TGTAACGCCA |      |
| xbmp2  | CTGGTACA.T | CATAATGAAA | GCAAATGGGA | AAG.TTTTGA | TGTAACGCCG |      |
| CONS   | -T-GT--A-T | CA-AA---AA | G----TGGGA | -AG--T-TG- | TGT----CC- |      |

|        |            |            |            |            |            |      |
|--------|------------|------------|------------|------------|------------|------|
|        | 1001       |            |            |            |            | 1050 |
| hbmp2  | GCTG.TGATG | CGGTGGACTG | CACAGGGACA | CGCCAACCAT | GGATTCTGTG |      |
| rtbmp2 | GCTG.TGATG | CGATGGACAG | CACAGGGACA | CACCAACCAT | GGGTTTGTGG |      |
| dubmp2 | GTTGTTGATG | CGATGGGACA | GGACAGGGGA | CAACCAACCT | TGGTTTTTGG |      |
| chbmp2 | GCTGTTT.TG | AGGTGGATTG | CACACGGACA | ACCTAACCAT | GGGTTTGTGG |      |
| xbmp2  | GCAATTG.CG | CGGTGGATTG | CACATAAACA | GCCTAACCAT | GGGTTTGTGG |      |
| CONS   | G---TT-A-G | -G-TGG---- | -----A     | -----A-C-T | -GGTT--T-G |      |

|        |            |            |            |            |            |      |
|--------|------------|------------|------------|------------|------------|------|
|        | 1051       |            |            |            |            | 1100 |
| hbmp2  | TGGAAGTGGC | CCACTTGGAG | GAGAAACAAG | GTGTCTCCAA | GAGACATGTT |      |
| rtbmp2 | TGGAAGTGGC | CCACTTAGAG | GAGAAGCCAG | GTGTCTCCAA | GAGACATGTG |      |
| dubmp2 | TGG.....   | .....      | .....      | .....      | .....      |      |
| chbmp2 | TGGAGGTGGT | TCACTTGGAC | AAAGAGAACA | GTGCCTCCAA | GAGGCACGTT |      |
| xbmp2  | TTGAAGTGAA | TCACTTGGAC | AATGACAAAA | ATGTGCCTAA | GAAGCATGTG |      |
| CONS   | T-G        |            |            |            |            |      |

Fig. 49c. Mouse BMP4 probe (mobmp4p) isolates a clone with high homology to human BMP 2 (hubmp2).

```

                                10      20      30
mobmp4p                                GGCACGAGCAGCTTCCATCACGAAGAAGCCATCGA
                                | | | | | | | | | | | | | | | | | | | | | |
hubmp2  GGAGAGGGCAGCCAGCCGAGCCAACACTGTGCGCAGCTTCCACCATGAAGAATCTTTGGA
      620      630      640      650      660      670

      40      50      60      70      80      90
mobmp4p  GGAACCTCCAGAAATGAGTGGGAAAACGTC CCCGACGCTTCTTCTTCAATTTAAGTTCTGT
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  AGAACTACCAGAAACGAGTGGGAAAACAACCCGGAGATTCTTCTTTAATTTAAGTTCTAT
      680      690      700      710      720      730

      100     110     120     130     140     150
mobmp4p  CCCTACTGATGAGTTTCTCACATCTGCGGAG-TCCAGATTTTTTCGGGAACAAATGCAGGA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  CCCACAGGAGGAGTTTATCACCTCAGCAGAGCTTCAGGTTTTCCGAGAACAGATGCAAGA
      740      750      760      770      780      790

      160     170     180     190     200     210
mobmp4p  AGCTTTGGGA---AATAGTAGTTTCCAGCACCGAATTAATATTTATGAAATTATAAAGCC
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  TGCTTTAGGAAACAATAGCAGTTTCCATCACCGAATTAATATTTATGAAATCATAAAACC
      800      810      820      830      840      850

      220     230     240     250     260     270
mobmp4p  TGCCACAGCCAGCTCAAAATTTCCCTGTGACCAGACTATTGGACACCAGGTTAGTGA CTCA
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  TGCAACAGCCAACTCGAAATTTCCCGTGACCAGACTTTTGGACACCAGGTTGGTGAATCA
      860      870      880      890      900      910

      280     290     300     310     320     330
mobmp4p  GAAACACAAGTCAGTGGGAGGAGCTTTGATGTCACCCCGGCTGTGAATGCGATGGACCAG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  G-AATGCAAGCAGGTGGGA-AAGTTTTGATGTCACCCCGCTGTG-ATGCGGTGGA-CTG
      920      930      940      950      960      970

      340     350     360     370
mobmp4p  CAACAGGGGCACATCCACCCTGGGGTTTTTGGTTGGAAGTGG
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
hubmp2  C-ACAGGGACAC-GCCAACCATGGATTCGTGG-TGGAAGTGGCCCACTTGGAGGAGAAAC
      980      990     1000     1010     1020     1030

```

Fig. 49d DuBMP4 comparison with mouse BMP4 sequence

```

                                10      20      30
dubmp4      GGCACGAGTCTGGCCCTCGACCAGGTTCACTGCAGCTG
                                |||
mobmp4  AACCGTCTTGGAGCCTCGACGAGTCCAGTCTCTGGCCCTCGACCAGGTTCAATTGCAGCTT
      120      130      140      150      160      170

      40      50      60      70      80      90
dubmp4  TCTAGAGGTCCCCAGGAGCAGCTGCGGGCGAGCCCGCTTCTGCAGGAACCTATGGAGCCA
      |||
mobmp4  TCTAGAGGTCCCCAGAAGCAGCTGCTGGCGAGCCCGCTTCTGCAGGAACCAATGGAGCCA
      180      190      200      210      220      230

      100     110     120     130     140     150
dubmp4  TTCCGTAGTGCCATTCCGAGCGACGCACTGCCGCAGCTTCTCTGAGCCTTTCCAGCAAGT
      |||
mobmp4  TTCCGTAGTGCCATTCCGAGCGACGCACTGCCGCAGCTTCTCTGAGCCTTTCCAGCAAGT
      240     250     260     270     280     290

      160     170     180     190     200     210
dubmp4  TTGTTCAAGATTGGCTCCCAAGAATCATGGACTGTTATTATATGCCTTGTTTTCTGTCAA
      |||
mobmp4  TTGTTCAAGATTGGCTCCCAAGAATCATGGACTGTTATTATATGCCTTGTTTTCTGTCAA
      300     310     320     330     340     350

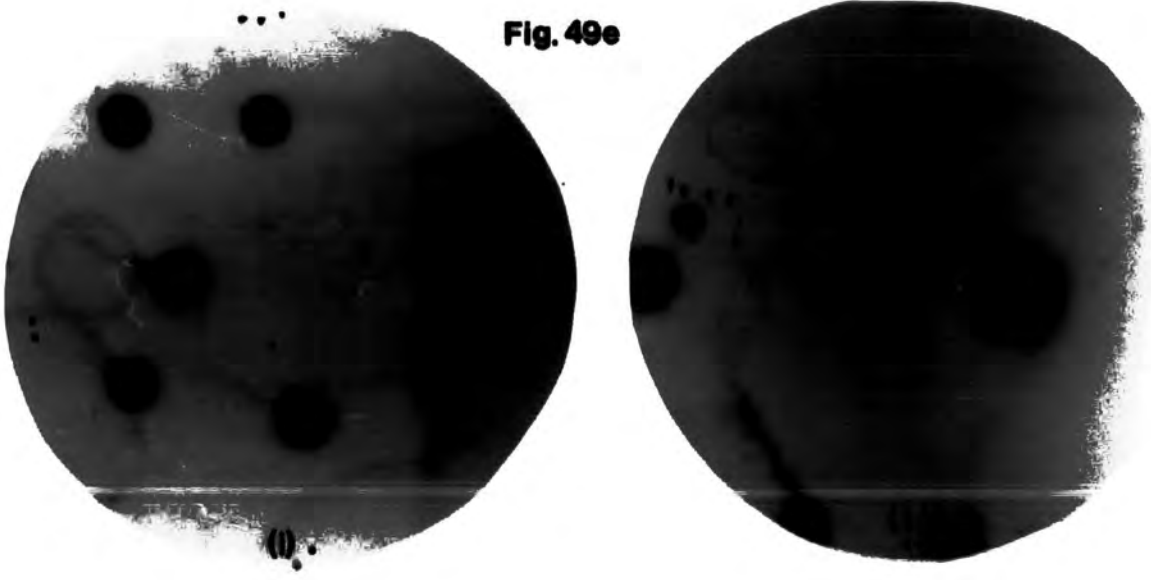
      220     230     240     250     260     270
dubmp4  GACACCATGGATTCCCTGGTAACCGAATGCTGATGGTCGTTTTATTATGCCAAGTCCTGCT
      |||
mobmp4  GACACCAT-GATTCCCTGGTAACCGAATGCTGATGGTCGTTTTATTATGCCAAGTCCTGCT
      360     370     380     390     400

      280     290     300     310     320     330
dubmp4  AGGAGGCGCGAGCATTGCTAGTTTTGATAACTTAGACCGGGAAGGAAAAAAGTTGCCGAG
      |||
mobmp4  AGGAGGCGCGAGCCATGCTAG-TTTGATACCTGAGACCGGGAA-GAAAAAAGTCGCCGA-
      410     420     430     440     450     460

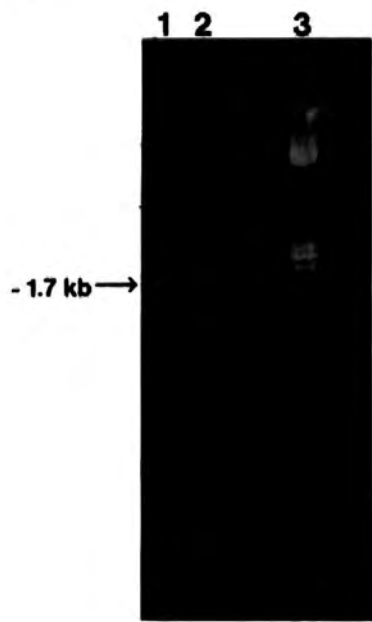
      340     350
dubmp4  GTTTAAAGGCCCCG
      |||
mobmp4  GATTCAGGGCCACGCGGGAGGACGCCGCTCAGGGCAGAGCCATGAGCTCCTGCGGGACTT
      470     480     490     500     510     520

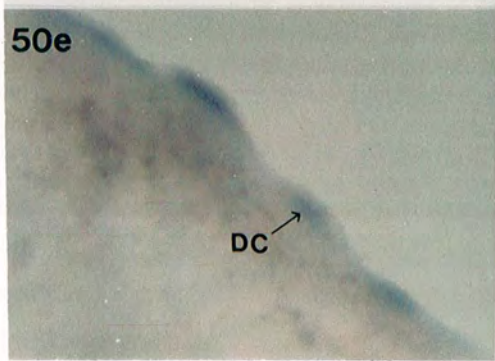
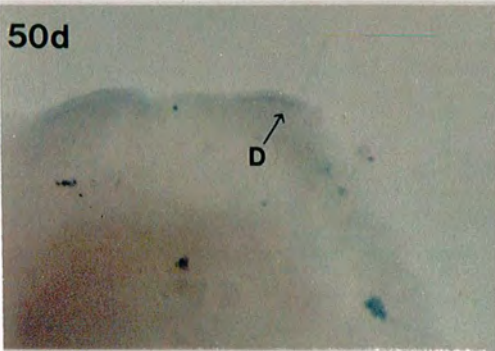
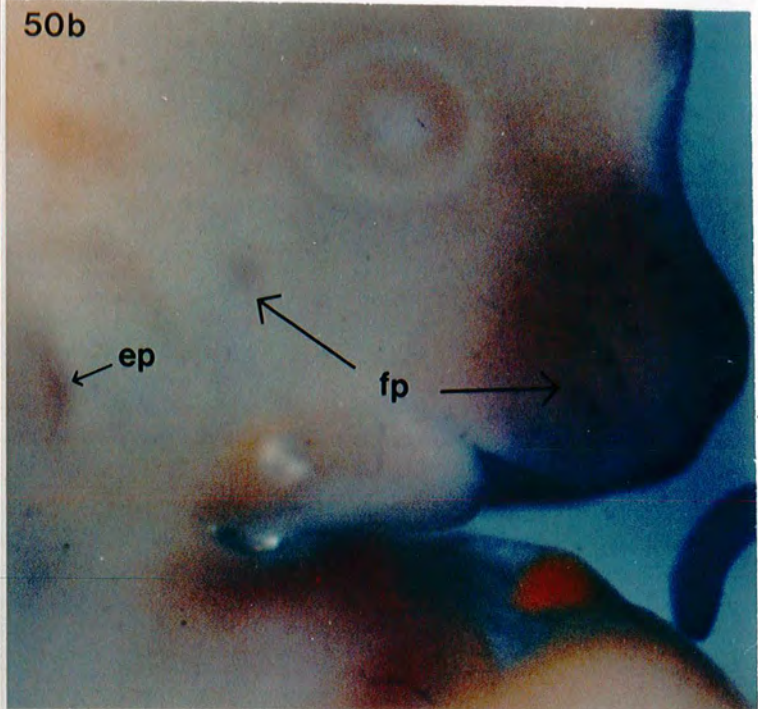
```

**Fig. 49e**



**Fig. 49f**





### **Figure 50a**

In situ hybridization of 14.5D rat embryo in the absence of anti sense BMP4 riboprobe. No background labelling is visible. (Magn. x30)

### **Figure 50b**

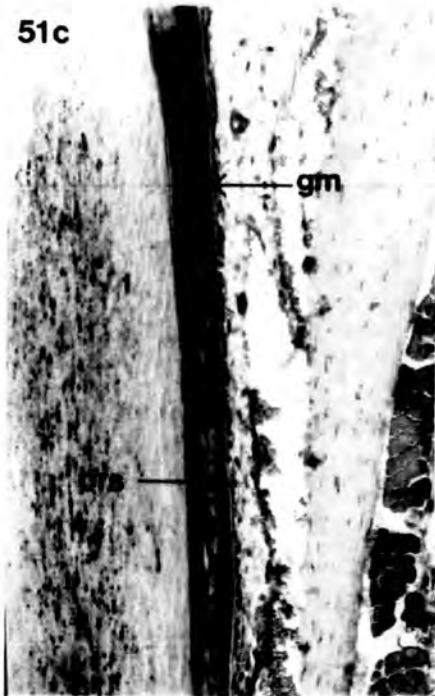
In situ hybridization of 14.5D rat embryo with anti sense BMP4 riboprobe. BMP4 expression occurs within the follicle primordia (**fp**) on the mystacial pad and developing whisker below the eye. Expression is also visible on the developing ear pinna (**ep**). (Magn. x30)

### **Figure 50c**

In situ hybridization of 14.5D rat embryo with anti sense BMP4 riboprobe. Expression is present on the mystacial pad and developing nose of the embryo. Staining was mirrored on either side of the mystacial pad. (Magn. x30)

### **Figure 50d,e**

In situ hybridization of 14.5D rat embryo with anti sense BMP4 riboprobe. Sections of tissue reveal that BMP 4 expression within the developing follicles is restricted to the condensing dermis below the epidermal placode. d) (Magn. x25) , (**D**) dermis and e) (Magn. x25), (**DC**) dermal condensation.



### **Figure 51a,b**

In situ hybridization of rat telogen sinus follicles with anti-sense BMP4 riboprobe. Staining is prominent throughout the outer root sheath cells (**ors**) of the epidermis. **a)** Magn. x36 & **b)** Magn. x147

### **Figure 51c**

In situ hybridization of rat telogen sinus follicles with anti sense BMP4 riboprobe. A line of outer root sheath cells (**ors**) adjacent to the glassy membrane (**gm**) is positive for BMP 4 expression. Magn. x147

### **Figure 51d**

In situ hybridization of rat telogen sinus follicles with anti sense BMP4 riboprobe. Sebaceous gland (**S**) in the upper isthmus of the follicle illustrated strong positive expression for BMP4. Magn. x147

Fig. 52

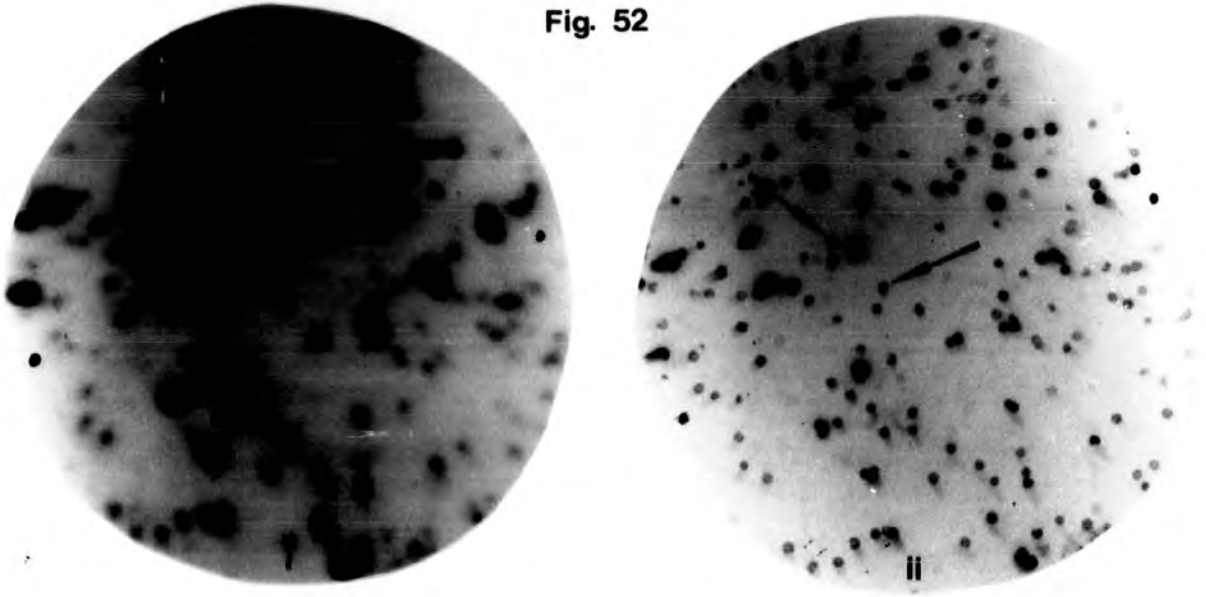


Fig. 53a

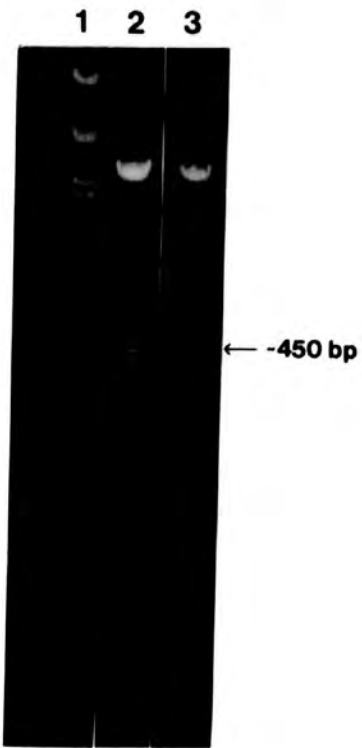


Fig. 54a

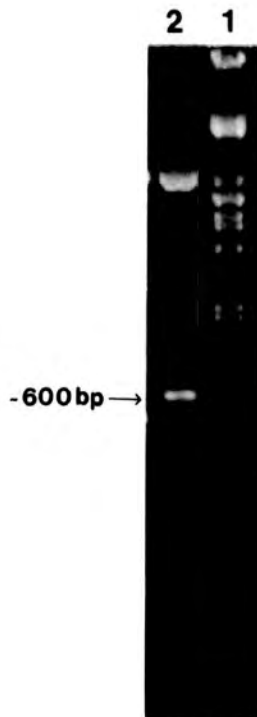


Fig. 52a



## Figure 52

An example of the differential screen described in figure 48. **i)** is the upper film, showing the SC DP expressed cDNA clone population, whereas **ii)** is the lower film from the CL DP and SC DP expressed cDNA populations. The arrows indicate genes which are differentially expressed in the lower film when compared with the upper film.

## Figure 52a

Clone, mas-CL 32, was cut with restriction enzymes Xho I and Pst I. Fragments were run on a 0.8% agarose/TAE gel. Two inserts are released from this digestion, the sum of which give an insert size of approximately 430bp. Lane 1)  $\lambda$ DNA marker cut with PstI Lane 2) mas-CL 32

## Figure 53a

Clones, mas CL-12.1 and mas CL-10.5, were both cut with restriction enzymes Xho I and PstI. Fragments were run on a 0.8% agarose/TAE gel. Inserts were released from this digestion, with both inserts being 450bp in length. Lane 1)  $\lambda$ DNA marker cut with PstI, Lane 2) mas-CL 12.1 and Lane 3) mas-CL 10.5

## Figure 54a

Clone, mas-CL 23, was cut with restriction enzymes Xho I and EcoRI. Fragments were run on a 0.8% agarose/TAE gel. An insert was released of approximately 600bp. Lane 1)  $\lambda$ DNA marker cut with PstI and Lane 2) mas-CL 23.

Fig 52b

|       |            |             |            |             |            |
|-------|------------|-------------|------------|-------------|------------|
|       | 151        |             |            |             | 200        |
| hugif | ACCCAGCAGC | TGGGCGCAGGC | CACCGGCAAG | CCCCCCCAGT  | ACATCGCGGT |
| humif | ACCCAGCAGC | TGGGCGCAGGC | CACCGGCAAG | CCCCCCCAGT  | ACATCGCGGT |
| moder | ACCCAGCAGC | TGGGCGCAGGC | CACCGGCAAG | CCC GCACAGT | ACATCGCAGT |
| momif | ACCCAGCAGC | TGGGCGCAGGC | CACCGGCAAG | CCC GCACAGT | ACATCGCAGT |
| mogif | ACCCAGCAGC | TGGGCGCAGGC | CACCGGCAAG | CCC GCACAGT | ACATCGCAGT |
| chmif | ACCCAGCAGC | TGGCCAAGGC  | CACCGGCAAG | CCC GCAGT   | ACATAGCCGT |
| mas32 | .....C     | TGGGCGCAGGC | CACCGGCAAG | CCC GCACAGT | ACATCGCAGT |
| CONS  | ACCCAGCAGC | TGGC--AGGC  | CACCGGCAAG | CC--C-CAGT  | ACAT-GC-GT |

|       |            |            |            |            |            |
|-------|------------|------------|------------|------------|------------|
|       | 201        |            |            |            | 250        |
| hugif | GCACGTGGTC | CCGGACCAGC | TCATGGCCTT | CGGCGGCTCC | AGCGAGCCGT |
| humif | GCACGTGGTC | CCGGACCAGC | TCATGGCCTT | CGGCGGCTCC | AGCGAGCCGT |
| moder | GCACGTGGTC | CCGGACCAGC | TCATGACTTT | TAGCGGCACG | AACGATCCCT |
| momif | GCACGTGGTC | CCGGACCAGC | TCATGACTTT | TAGCGGCACG | AACGATCCCT |
| mogif | GCACGTGGTC | CCGGACCAGC | TCATGACTTT | TAGCGGCACG | AACGATCCCT |
| chmif | GCACATCGTA | CCTGATCAGA | TGATGTCCTT | CGGGGGCTCC | ACGGATCCTT |
| mas32 | GCACGTGGTC | CCGGACCAGC | TCATGACTTT | TAGTGGCACG | AGCGACCCCT |
| CONS  | GCAC-T-GT- | CC-GA-CAG- | T-ATG-C-TT | --G-GGC-C- | A--GA-CC-T |

|       |            |            |            |            |            |
|-------|------------|------------|------------|------------|------------|
|       | 251        |            |            |            | 300        |
| hugif | GCGCGCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGCGG | CGCGCAGAAC |
| humif | GCGCGCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGCGG | CGCGCAGAAC |
| moder | GCGCCCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGTGG | TGCCCAGAAC |
| momif | GCGCCCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGTGG | TGCCCAGAAC |
| mogif | GCGCCCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGTGG | TGCCCAGAAC |
| chmif | GCGCTCTCTG | CAGCCTCTAC | AGCATTGGCA | AGATTGGAGG | GCAGCAGAAC |
| mas32 | GCGCCCTCTG | CAGCCTGCAC | AGCATCGGCA | AGATCGGTGG | CGCCCAGAAC |
| CONS  | GCGC-CTCTG | CAGCCT--AC | AGCAT-GGCA | AGAT-GG-GG | ----CAGAAC |

|       |            |            |            |            |            |
|-------|------------|------------|------------|------------|------------|
|       | 301        |            |            |            | 350        |
| hugif | CGCTCCTACA | GCAAGCTGCT | GTGCGGCCTG | CTGGCCGAGC | GCCTGCGCAT |
| humif | CGCTCCTACA | GCAAGCTGCT | GTGCGGCCTG | CTGGCCGAGC | GCCTGCGCAT |
| moder | CGCAACTACA | GTAAGCTGCT | GTGTGGCCTG | CTGTCCGATC | GCCTGCACAT |
| momif | CGCAACTACA | GTAAGCTGCT | GTGTGGCCTG | CTGTCCGATC | GCCTGCACAT |
| mogif | CGCAACTACA | GTAAGCTGCT | GTGTGGCCTG | CTGTCCGATC | GCCTGCACAT |
| chmif | AAGACTTACA | CCAAGCTCCT | GTGCGATATG | ATTGCGAAGC | ACTTGCACGT |
| mas32 | CGCAACTACA | GCAAGCTGCT | GTGCGGCCTG | CTGTCCGATC | GCCTGCACAT |
| CONS  | -----CTACA | --AAGCT-CT | GTG-G---TG | -T--C--A-C | -C-TGC-C-T |

|       |            |            |            |            |            |
|-------|------------|------------|------------|------------|------------|
|       | 351        |            |            |            | 400        |
| hugif | CAGCCCGGAC | AGGGTCTACA | TCAACTATTA | CGACATGAAC | GCGGCCAATG |
| humif | CAGCCCGGAC | AGGGTCTACA | TCAACTATTA | CGACATGAAC | GCGGCCAATG |
| moder | CAGCCCGGAC | CGGGTCTACA | TCAACTATTA | CGACATGAAC | GCTGCCAACG |
| momif | CAGCCCGGAC | CGGGTCTACA | TCAACTATTA | CGACATGAAC | GCTGCCAACG |
| mogif | CAGCCCGGAC | CGGGTCTACA | TCAACTATTA | CGACATGAAC | GCTGCCAACG |
| chmif | GTCTGCAGAC | AGGGTATACA | TCAATTACTT | CGACATAAAT | GCTGCCAACG |
| mas32 | CAGCCCGGAC | CGGGTCTACA | TCAACTATTA | CGACATGAAC | GCAGCCAACG |
| CONS  | -----C--AC | -GGGT-TACA | TCAA-TA-T- | CGACAT-AA- | GC-GCCAA-- |

|       |            |              |               |              |            |
|-------|------------|--------------|---------------|--------------|------------|
|       | 401        |              |               |              | 450        |
| hugif | TGGGCTGG.A | ACAAC TCCAC  | CTTCGCCTAA    | GAGCCG....   | ....CAGGGA |
| humif | TGGGCTGG.A | ACAAC TCCAC  | CTTCGCCTAA    | GAGCCG....   | ....CAGGGA |
| moder | TGGGCTGG.A | ACGGT TCCAC  | CTTCGC T TGA  | GTCCTGGCC.   | CCACTTACCT |
| momif | TGGGCTGG.A | ACGGT TCCAC  | CTTCGC T TGA  | GTCCTGGCC.   | CCACTTACCT |
| mogif | TGGGCTGG.A | ACGGT TCCAC  | CTTCGC T TGA  | GTCCTGGCC.   | CCACTTACCT |
| chmif | TGGGCTGG.A | ACGGT TCCAC  | CTT TGCATAG   | AGCTCTCCCT   | CCTGTGCCCA |
| mas32 | TGGGCTGGAA | AACGGT TCCAC | CTTTCGC T TGA | GCCC GGGCCCT | TCACTTACCT |
| CONS  | TGGGCTGG-A | AC---TCCAC   | CTT-GC----    | -----CCT     | -C-----    |



Fig. 53b

```

        651                                     700
mas121  .....GGCA GATTTTACTT GGTGTAATTA
motc130 GGATGGCTGA GCATCAAAGG AGGACAACCA TCTATAACTT GGTGTCATTG
CONS   -----CA --T-T-ACTT GGTGT-ATT-

        701                                     750
mas121  ACAATAATAC TAAGGTTTAT CCAGCTGCCA CATTTTGAAA CATTCTGGGC
motc130 ACAACTATAT TAATGTTTAT CCAGCTGCCG TAGTTTGAAA CATTCTGGGC
CONS   ACAA--ATA- TAA-GTTTAT CCAGCTGCC- -A-TTTGAAA CATTCTGGGC

        751                                     800
mas121  TGGAAGAGGA GGGACCCTGG GGTTCCCTTTT TCACCCCGGA ATGAACATAT
motc130 TGGGAGAAGA GGGACCCCGG GGTTTCTAAT TTC AACCCCA AATGACATGT
CONS   TGG-AGA-GA.GGGACCC-GG GGTT-CT--T T----CC--A A---ACAT-T

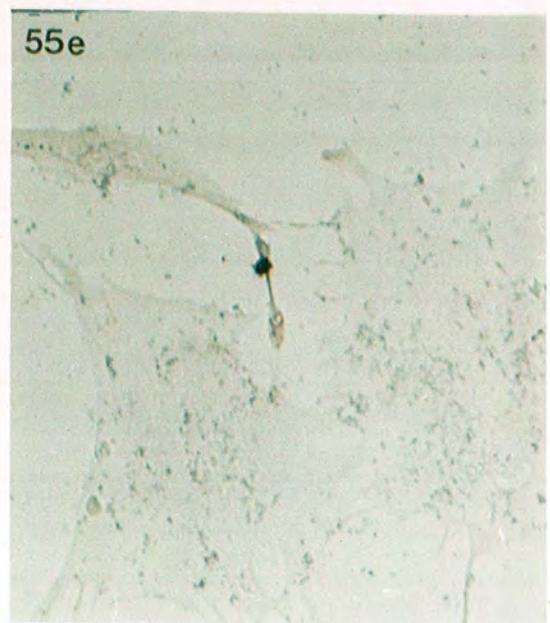
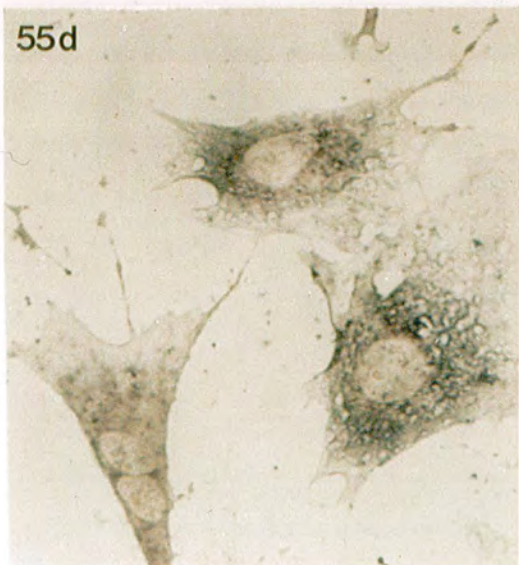
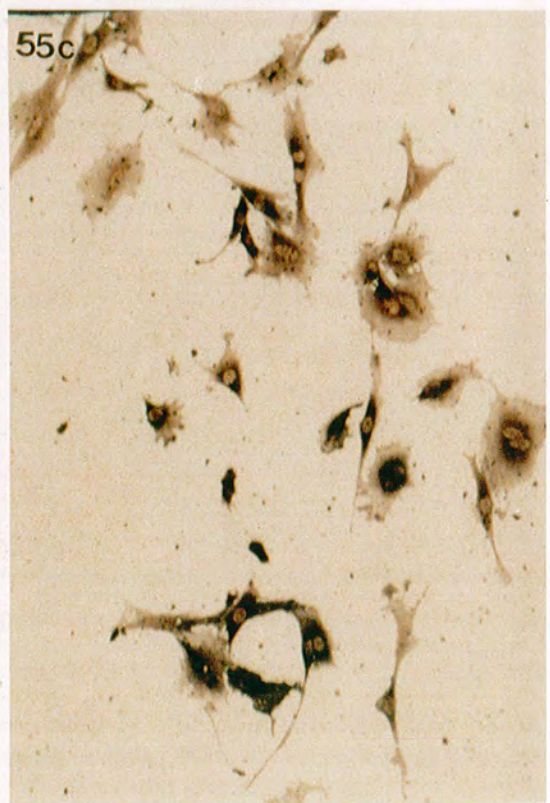
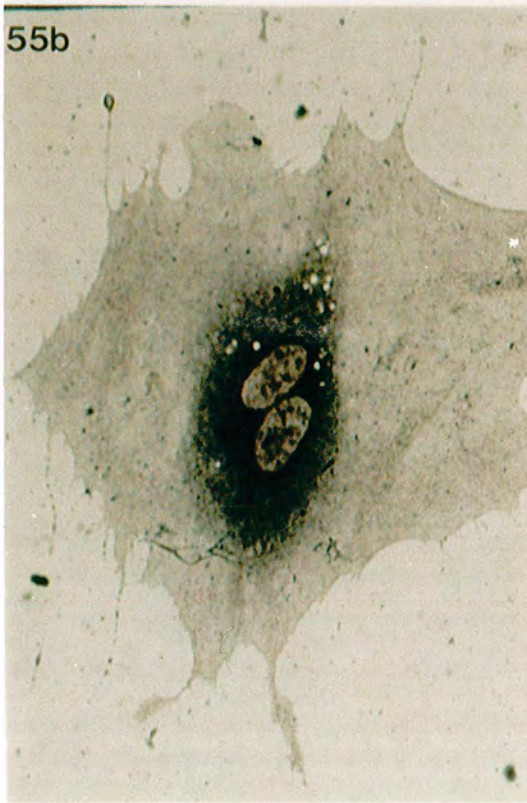
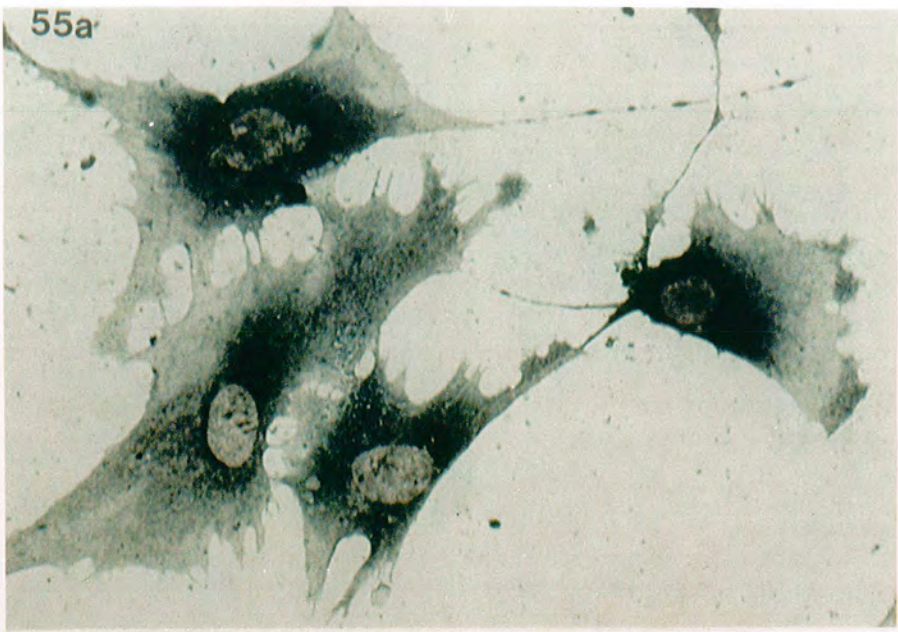
        801                                     850
mas121  TTTTCCCCC CTGAATGTTT AGCAGTCAGA TG.....
motc130 TTATTTGCAC CTG.ATGCTC AGGAGCCAGA TGTGGACCCT GCAGAGCCAG
CONS   TT-T---C-C CTG-ATG-TC AG-AG-CAGA TG-----
```

Fig. 53c

```
GGCAGAGAGCTGGTGGTTCACCTCAGCCTCTTTAATTCCACATCCAGCACCTTAGGGTTCATGG
TCTGGAAGAGGAACAAAGGCCCTAGTGCTGTGGTCTGTTTCTAAGTCCAGGCCCACTGGGCATA
CTTAATCCAGGGCTTCTTTGTGAATCACCCCGATCCT
```

Fig. 54b

```
GTCTCTAGGTATTAAAGAGGTTGCCAATGTATGACAAAGTAGTTCGTCACCTCACACATTCTGTA
TACTCTGTTAGTTCATAGCAAGGTTGTCTTCTGAAGAGTTGTTGAAAGTGTATCATCCGAGTGAC
AGGTGCCCGTGTACAGG
```



**Figure 55 a,b**

In situ hybridization of clone mas-CL 32 on subconfluent dermal papilla cells. Expression was greatest in the perinuclear regions with a marked reduction towards the edges of the cytoplasm (Magn. x430)

**Figure 55c**

In situ hybridization of clone mas-CL 32 on subconfluent dermal papilla cells. A number of subconfluent cells had less overall labelling, and a more diffuse expression throughout the cytoplasm (Magn. x110)

**Figure 55d**

In situ hybridization of 16s, a ribosomal gene expressed within the mitochondria, on subconfluent dermal papilla cells. Cells were positive with a uniform expression throughout the cytoplasm. (Magn. x430)

**Figure 55e**

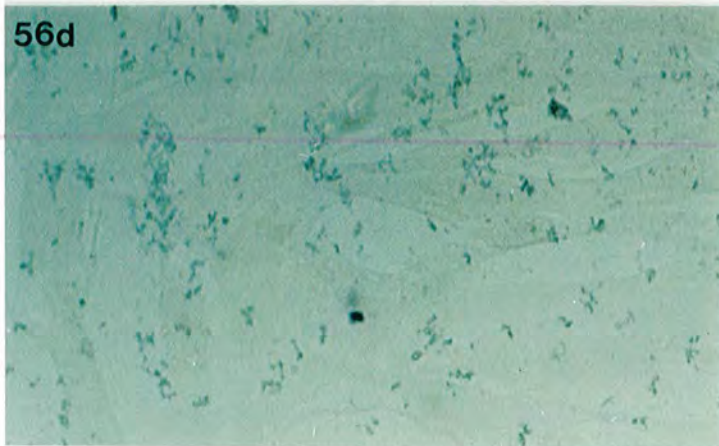
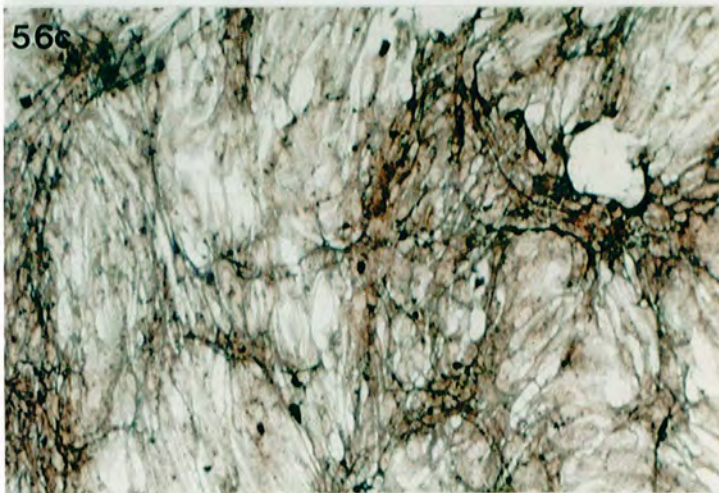
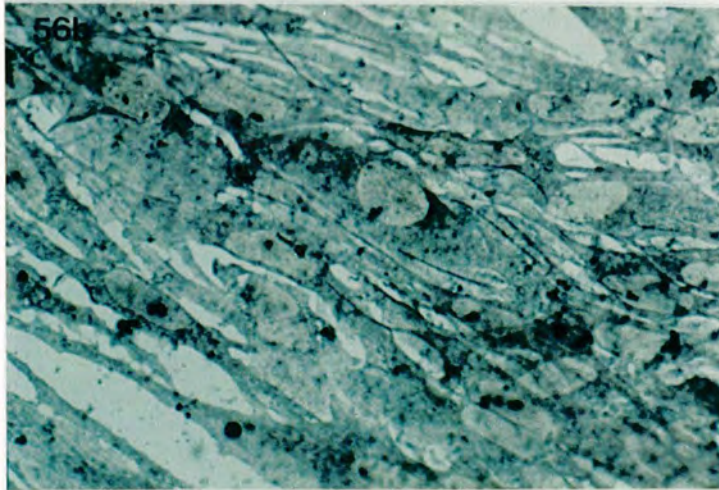
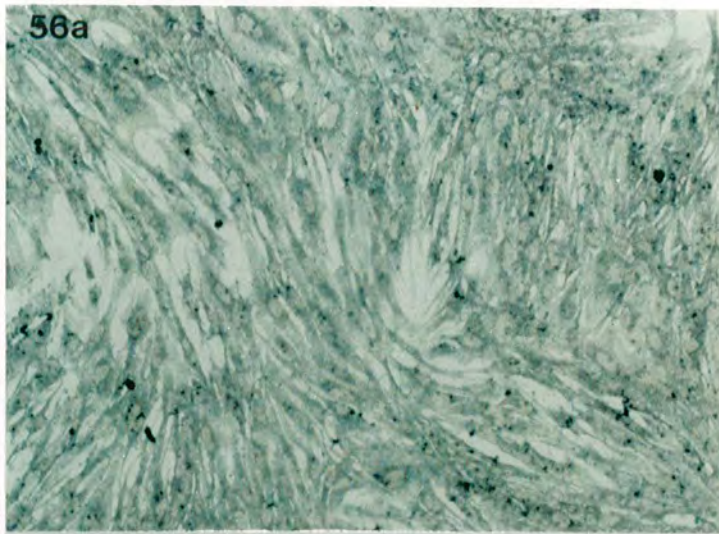
In situ hybridization in the absence of a riboprobe. No labelling of the cell is apparent, with no background stain visible. (Magn. x430)

### **Figure 49e**

End bulb cDNA library screens with mouse probes to BMP2 and BMP4. i) an example of a secondary library screen with mouse BMP2 probe and ii) an example of a secondary library screen with mouse BMP4 probe.

### **Figure 49f**

Dubmp2 and dubmp4 were cut with restriction enzymes Xho I/Pst I and XhoI/EcoRI respectively. Fragments were run on a 0.8% agarose/TAE gel. Both inserts are approximately 1.7kb. Lane 1) duBMP4, Lane 2) du BMP2 and Lane 3)  $\lambda$ DNA marker cut with PstI.



**Figure 56a,b**

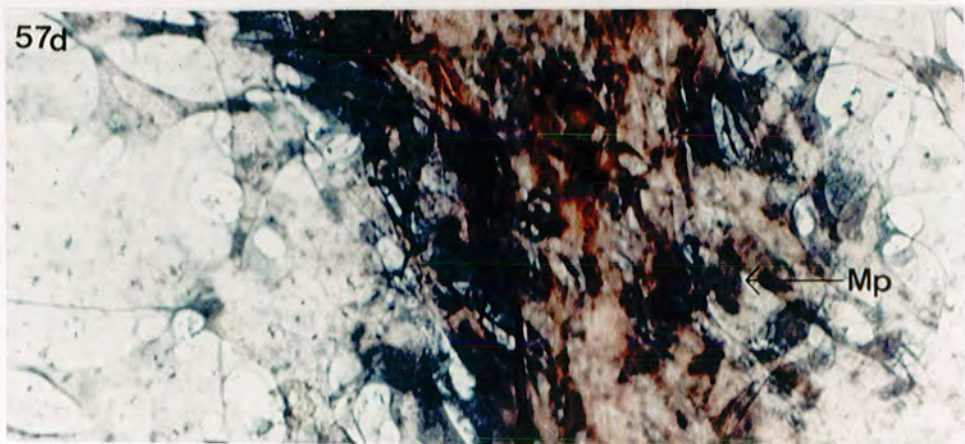
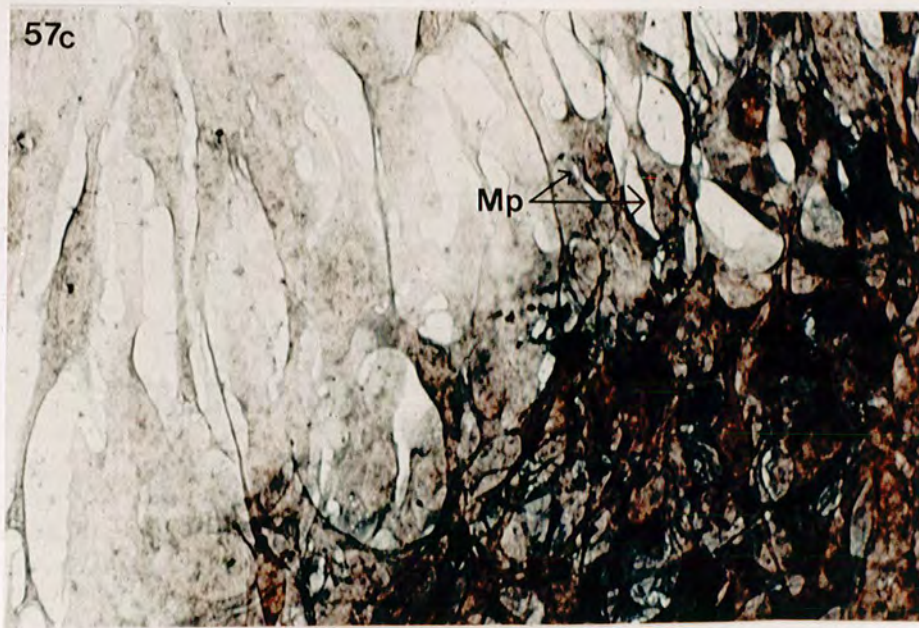
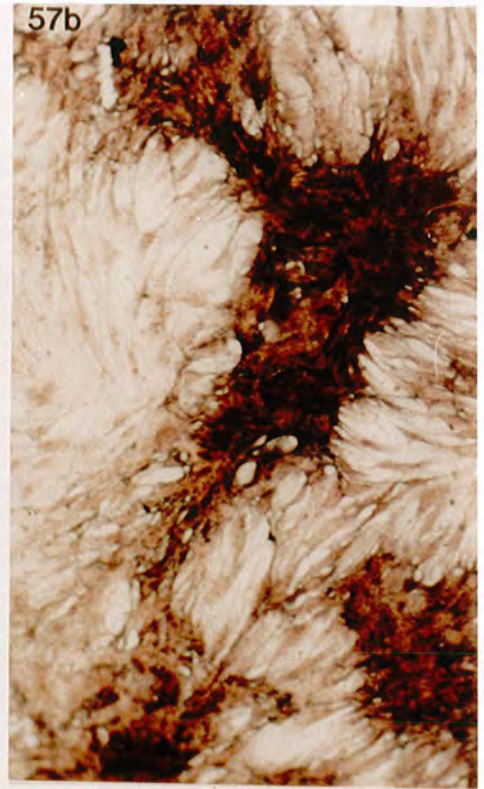
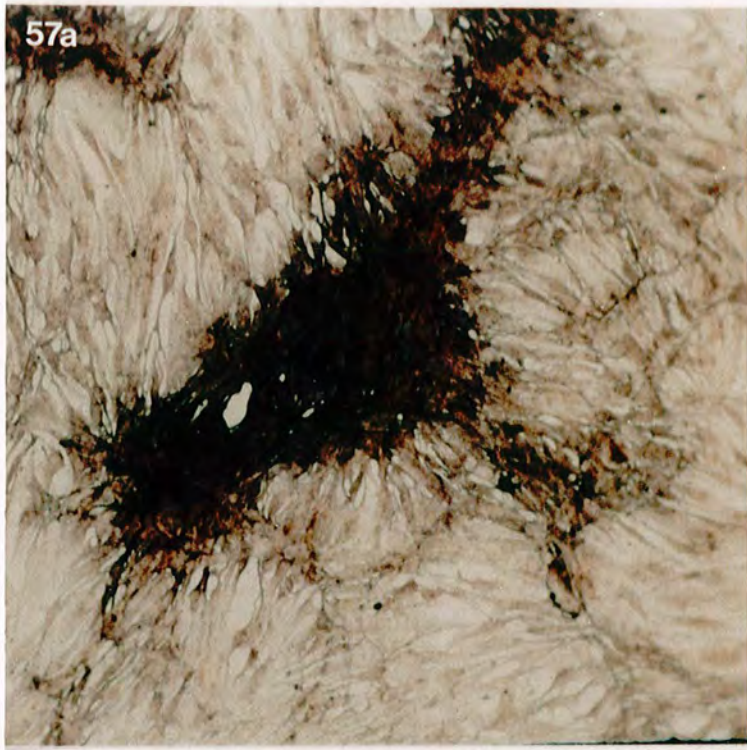
In situ hybridization of clone mas-CL 32 on aggregating dermal papilla cells. Labelling was diffuse throughout the cytoplasm, with no intense labelling in the perinuclear region. (Magn. x217)

**Figure 56c**

In situ hybridization of clone mas-CL 32 on aggregating dermal papilla cells. In pronounced aggregating regions there was an small increase in cytoplasmic labelling. (Magn. x110)

**Figure 56d**

In situ hybridization in the absence of a riboprobe. No labelling of the cells is apparent, with no background stain visible. (Magn. x217)



**Figure 57a,b**

In situ hybridization of clone mas-CL 32 on clumped dermal papilla cells. Intense labelling is seen within the clumped regions, with little or no marking of cells between the clumped structures. Within the clumps, expression of the clone mas-CL 32 was variable among cells. (Magn x110)

**Figure 57c**

In situ hybridization of clone mas-CL 32 on clumped dermal papilla cells. Single cells on the periphery of the clumps were also positive for mas-CL 32 (mas-CL 32 positive (Mp)). This demonstrates that increased expression was not as a result of the piling effect of the dermal papilla cells. (Magn x430)

**Figure 57d**

In situ hybridization of clone mas-CL 32 on clumped dermal papilla cells. In a few clumped regions, mas-CL 32 expression had dropped, however a number of the cells within the clump were still expressing the gene. (mas-CL 32 (Mp)). (Magn x430)

## Discussion

### 5.4.1.1 Rat homologues of bone morphogenetic proteins 2 and 4

Both of the mouse BMP-2 and BMP-4 genes hybridised to their respective rat homologues from the end bulb library constructed from the sinus follicles of PVG inbred rat strains. (see section 5.3.1.1). However, the mouse BMP-4 also hybridised to BMP-2. It has been well documented that BMP-2 and BMP-4 are very similar, particularly with respect to their amino acid sequence (92% homology). Moreover, BMP-4 was first isolated due to a cross hybridization with a probe to BMP-2 (Wozney et al., 1988), therefore it is not surprising that mouse BMP-4 hybridized to rat BMP-2. In the original study of the distribution of BMP 2 and 4 in the mouse embryo, Lyons et al., (1990) and Jones et al., (1991) overcame this problem by removing the highly conserved 3' regions of these genes.

However, it is of even greater interest that prior to this study, BMP-4 had not previously, been shown to be expressed in adult tissue. As the end bulb library was constructed from all the stages in the adult hair cycle, it is unclear whether BMP 4 is universally expressed throughout the anagen and telogen stages or whether it is restricted to the telogen stage alone. So far BMP 4 expression has only been investigated during the telogen stage. Jones et al. (1991) believe that BMP-4 may act as a signalling molecule, instructing the overlying ectoderm during follicle development, however it is now apparent that there may be a further role for this gene within the hair follicle.

### 5.4.1.2 Bone morphogenetic protein 4 expression *in situ*

Consistent with Jones et al., (1991), BMP 4 expression was restricted to the developing whisker pad and nose. Sections of the rat embryos showed that expression was restricted to the dermis in early whisker development and, the epidermis of the nasal

invagination, this reflecting the distribution described in the mouse. (Jones et al., 1991). Due to this similarity between the mouse distribution and rat distribution, it can be assumed that very little cross hybridisation has occurred between BMP 4 and BMP 2. The banded labelling of BMP 4 expression on the mystacial pad reflects the fact that the follicles are developing at different stages. Therefore, the absence of marking in row 'a' corresponds with the fact that the follicles are more developed and past the condensation stage.

Using the same probe, distribution of BMP-4 was examined in the telogen sinus follicle of the rat. Expression was identified throughout the ORS cells of the epidermis and cells of the sebaceous gland. This is in distinct contrast to the mesenchymal distribution in the embryo. In the mature anagen follicle, BMP 2 was previously identified in the precortical cells of the epidermis (Lyons et al., 1989, 1990). However, the section illustrated by Lyons et al., (1990) was cut at an angle, and not through the centre of the follicle and hence BMP-2 distribution within the epidermis may be far greater than is apparent from the illustrated micrograph. The distribution of BMP-2 expression in telogen follicles has not been investigated. It is unusual that BMP-4 was not previously identified in the adult follicle (Jones et al., 1991) when the current work suggests that it has widespread expression in the telogen follicle. It is possible that in the current study there was cross hybridization with the BMP-2 transcript, however without the data for BMP-2 from the respective stage it is unclear whether this is the case. Furthermore, as the embryonic expression of rat BMP 4 is found to be consistent with that described by Jones et al. (1991) for the mouse, it is probable that little cross hybridization had occurred.

Vainio et al. (1993) showed that BMP 4 expression in tooth development occurred first in the epidermis. Subsequently, BMP 4 expression shifted completely to the mesenchymal cells. This shift in BMP 4 expression was attributed to its role in inducing differentiation of the underlying mesenchyme. This alteration of expression from epithelium to mesenchyme, reflects the inductive signaling of the epithelium to induce the oral mesenchyme at the initial stages of tooth formation. Therefore, if a

similar epidermal to dermal signal was required as part of the stimulation of the new anagen cycle, and therefore would support the epidermal distribution of BMP 4 discovered during the resting telogen stage of the vibrissa follicle. However, without the BMP 4 expression data during early anagen, it is unclear whether a shift in expression occurs with reactivation of another hair cycle.

Technically, the benefit of using UTP digoxigenin incorporation in construction of the BMP-4 probe instead of the conventional radiolabelled method is two fold. Firstly, the results are obtained far more rapidly than radiolabelled *in situ* hybridization, and secondly the exact distribution of label can be ascribed to specific cells and tissues. The only problem is that the revelation procedure is very flexible as to when to stop the reaction. It is therefore difficult to establish when the bound label has been revealed or when the background stain is beginning to come through.

#### **5.4.2.1 Differential screen of clumped and subconfluent *in vitro* DP cells**

The conventional method of differential screens was to produce two labelled probe populations from distinct RNA populations. Duplicate plaque lifts were taken, and each probe was then hybridized to separately to a single filter (see Sambrook et al., 1989). When the two films are exposed and then compared one should be able to identify putative differential clones. However, it is virtually impossible to take identical duplicate lifts from a single plated library. Therefore, it is often the case that differentially expressed clones are an artifact of the errors involved in lifting the plaques. Using the dual-labelling method (Olzsweki et al, 1989) one is able to eliminate the problem of duplicate lifts, because two probes are hybridized simultaneously to a single filter.

Another approach to isolate CL specific dermal papilla cell cDNA clones would be to use a subtraction protocol, for example the one described in (Ausubel et al., 1992). The principle behind this method is to subtract out the messages common to two cDNA or messenger RNA populations, thus enriching for the cDNA clones or mRNAs

of interest. However, this second approach was not adopted because it has certain limitations. The clumped phenotype in dermal papilla cells may be as a result of subtle changes in gene expression and the subtractive approach is not sensitive enough to identify discrete changes in gene expression. Also when using a subtraction protocol, homologous sequences are removed. For example, members of gene families or mRNAs that contain repeat sequences. Therefore, genes of interest may be discarded.

Differential screening of the end bulb cDNA library was used to isolate CL dermal papilla cell specific cDNA clones using *in vitro* generated probes to screen an *in vivo* library. The *in vitro* cells were derived from the adult system, however it has been postulated in previous chapters that the *in vitro* aggregation process is a reflection of earlier embryonic dermal condensation events. Even if this is the case, it is still probable that a number of condensation specific genes are not expressed at any time during the adult hair cycle. Therefore, it would be of great interest to repeat this differential screen with an embryonic cDNA library in attempt to further consolidate the variety of genes expressed within the *in vitro* model.

Nevertheless, from the initial differential screen of the end bulb library approximately 40 putative clones were isolated and with further secondary screening, sequencing and characterisation of these clones it is conceivable that the main genes responsible for maintaining the aggregate have already been isolated.

#### **5.4.3.1 What is the differentially expressed cDNA clone, mas-CL 32?**

From the results in section (5.3.2.2), there was very similar homology between three different named nucleotide sequences, *Mus musculus* growth factor-induced delayed early response protein, *Mus musculus* glycosylation inhibiting factor, and *Mus musculus* macrophage migration inhibitory factor. When all of these genes were aligned, there were striking similarities at the nucleotide level that begs the question, are they all the same gene? Considering *M. musculus* growth factor-induced delayed early response protein first, it was discovered that the name used does not reflect a single gene but

rather a group of genes, that were isolated from a differential screen of growth factor induced 3T3 fibroblasts (Lanahan et al., 1992). Furthermore, only a single gene isolated, delayed early response 6 (DER6), was similar to human macrophage migration inhibitory factor (huMIF), and had 88% identity at the amino acid level. Moreover, by *in vitro* transcription and translation, the cDNA DER 6 yielded a protein of approximately 12 KD. Taking into consideration the species difference between human and mouse, and at the time of Lanahans publication, the fact that the mouse MIF protein had not been characterised, it seems apparent that DER6 is merely the mouse homologue of huMIF. By comparing the protein sequence of DER 6 and mouse MIF (Wistow et al., 1993), one finds 100% identity over 111 amino acids with that described in Wistows paper. However, Lanahan et al., (1992) showed that DER 6 hybridized with a large number of mouse, human and rat genomic restriction fragments, implicating this gene as a member of a family of MIF related genes present in the mammalian genome. Furthermore, as DER 6 was identified in a wide variety of tissue types (brain, heart, intestine, kidney, liver, lung, ovary, seminal vesicle, spleen, testis, thymus and uterus), they postulated that the MIF family might have a broad range of cellular activities other than the inhibition of macrophage motility.

Glycosylation inhibiting factor (GIF) was first isolated as a lymphokine that was involved in selective formation of IgE suppressive factor (Ishizaka et al., 1984), by inhibiting N-glycosylation of IgE binding factors, and therefore selectively inhibiting IgE synthesis. Molecular cloning of this gene was first described by Mikayama et al., (1993), isolating a cDNA that encodes for 12.5 KD protein of 115 amino acids. In this paper, the latter author makes reference to its homology with huMIF, stating that " human GIF was almost identical to the sequence of human MIF cDNA." The only difference being that there was an amino acid change, with huMIF containing a serine residue at amino acid 106, whereas the corresponding human GIF residue was an asparagine. Furthermore, this amino acid change was the result of a single nucleotide difference in the genetic sequence. However, this difference of a single nucleotide was due to a comparison with the published hu MIF sequence described by Weiser et al.,

(1989), since then other groups ( Bucala et al., 1993, Paralkar and Wistow, 1994) have shown that this base difference is incorrect, and that according to the numbering in Weiser et al., (1989), nucleotide 367 should read 'a' rather than 'g'. This single base change, then reverts the amino acid in huMIF, described in the latter's author paper, from serine to asparagine. Therefore, on protein sequence and molecular weight, human GIF and hu MIF are identical.

However, Mikayama et al., (1993) went on to describe a functional assay whereby GIF presence was detected by its ability to switch mouse T cell hybridoma 12H5 cells from the formation of glycosylated IgE to the formation of unglycosylated IgE. Using this and a migration assay to illustrate MIF activity, they discovered that MIF activity was absent in the GIF assay, and that GIF activity was absent in the MIF assay. From this Mikayama et al., (1993) deduced that GIF probably belonged to the MIF like family described by Lanahan et al., (1992), but that it was distinct from the MIF gene. What still remains unclear is how MIF and GIF can have identical protein sequences, and yet have different functions? In both of these described assays, each protein used was the result of transfected COS-1 cells. If a mutation occurred in the nucleotide sequence, such as to create an amino acid change, like that of serine to asparagine, then the whole structure and folding of the recombinant protein may alter, and hence its functionality may change. However, there is no evidence for this as yet.

As an in-depth analysis has not been done between these three genes that I am aware of, one cannot be 100% sure that you are dealing with the same gene, particularly in light of this last assay. However, with respect to this thesis, it would be logical to assume that the cDNA clone, mas-CL 32, is in fact MIF, particularly as in chapter 4 migration was inhibited at specific stages in the aggregation behaviour of the *in vitro* dermal papilla cells. In section (5.4.3.4), the *in situ* hybridisation of cDNA clone, mas-CL 32, is discussed. However due to the uncertainty of it being MIF, and the small possibility that the other genes are in fact different, its designated clone number was used throughout the discussion. Moreover, because one cannot assume that they are

identical, one must consider the possibility of cross hybridisation between these so called different genes.

#### **5.4.3.2 The rat homologue of Macrophage Migration Inhibitory Factor**

Macrophage migration inhibitory factor (MIF) was one of the first lymphokines to be isolated, being discovered from crude culture fluids of activated T lymphocytes (Bloom & Bennett, 1966, David, 1966). MIF acted on macrophages to inhibit their migration from capillary tubes *in vitro*. Studies on human MIF have shown it to be heterogeneous, with three isoforms distinguished by gel chromatography or isoelectric focusing (Weiser et al., 1981), however it was not until relatively recently that the first cDNA was isolated encoding for MIF (Weiser et al., 1989). DNA and protein sequence, have now been reported for bovine, chick and murine MIF. The translation of the MIF cDNA predicted a 12 KD protein, consistent with MIF isolated from lymphoid cell lines (Possanza et al., 1979), however, undenatured MIF species from stimulated lymphocytes had molecular weights of between 25-68 KD (Weiser et al., 1981). The possibility then arises that MIF may exist as a dimer or multimer.

Galat et al. (1993) were first to isolate the mouse MIF protein to homogeneity. Using isoelectric focusing they discovered two isoforms which both had high homology at the N-terminus with human MIF. In a follow up paper (Galat et al., 1994), the full amino acid sequence of one of the isoforms was included. In the same study they also report that the bovine MIF had none of the previously documented inhibitory migratory effects on mouse macrophages. However, this is not surprising due to species differences.

Until relatively recently MIF was thought to be purely a product of circulating T-cells, with expression occurring in response to antigens, but it is now apparent that a variety of cells can express MIF. Bernhagen et al., (1993) showed that MIF was produced in hypophysectomized and T-cell deficient nude mice, with expression specific to the pituitary. Stimulated pituitary cells (At T-20) produced a 12.5kDa protein after

stimulation with antigen, this protein being 96% homologous with the N-terminus of the human MIF (Weiser et al., 1989) over 27 amino acids. It was also shown that preformed MIF could be detected within the unstimulated cells. This same group ( Calandra et al., 1994) have also shown that monocytes and macrophages are a source of MIF *in vitro* and *in vivo*, in both stimulated and unstimulated cells. The complexity of MIF expression increases with response to other known cytokines. Bernhagen et al. (1993) illustrated that pituitary derived MIF was increased in the presence of serum, but not released in the presence of the cytokines tumour necrosis factor- $\alpha$ ; interleukin-1 $\beta$ ; interleukin-6; and interferon- $\gamma$ . However, Calandra et al. (1994) indicated that macrophage MIF was stimulated by tumour necrosis factor- $\alpha$  and interferon- $\gamma$ , and not interleukin-1 $\beta$  and interleukin-6. Furthermore, MIF stimulated macrophages were observed to secrete bioactive tumour necrosis factor- $\alpha$ . Therefore, it appears that MIF is under a strict set of feedback controls from a variety of different sources within the immune system. MIF however is not restricted to cells of the immune system and Wistow et al. (1993) showed that it was expressed in the developing chick lens. This is discussed further in the following section.

MIF's role as a cytokine is more complex than at first thought with its expression divided amongst a variety of cell types. What then is its function within *in vitro* dermal papilla cells? In the previous chapter it was shown that the clumped dermal papilla cells had negligible motility. From the earliest sources (David, 1966 and Bloom & Bennett, 1966) it was shown that a molecule produced from sensitised guinea pig T-cells (MIF) could inhibit the outward migration of macrophages from a capillary tube. David (1966) also noted that in some repeat experiments, migration of the macrophages was not significantly inhibited however there was a marked clumping of cells within the migrating macrophages. Therefore it is possible that MIF may be acting in a similar fashion with the *in vitro* dermal papilla cells and in so doing helping to maintain the clumped phenotype. Choung et al. (1991) showed that *in vitro*, chick feather papilla cells expressed N-CAM within the aggregated structures. If a similar expression is present within the rat DP cell cultures then MIF may act in a supporting

role with this cell adhesion molecule. It still remains unclear whether MIF expression *in vitro* is a true reflection of the *in vivo* system. Using whole mount and sectioned *in situ* hybridization with an anti-sense RNA probe to MIF it will be possible to investigate its distribution within the developing embryo, and in particular condensation events. However, if using this technique, great care would have to be taken to separate MIF expression in follicle development from the developing vascularization of the embryo and the possibility of the circulating T-cells producing MIF.

#### **5.4.3.3 The role of MIF in development**

A wealth of data has been obtained relating to the *in vitro* and *in vivo* functions of MIF with respect to its role in the immune system (Bloom and Bennet, 1966, David, 1966, Churchill et al., 1975, Weiser et al., 1991, Pozzi et al., 1992). However, MIF has recently also been postulated to have a specific role in the development of the chick eye (Wistow et al., 1993). It was initially characterised as a 10kDa protein expressed in early embryonic chick lenses. This is not unique, as a 14 kDa protein with an N-terminal equivalent to MIF-related protein-8 (MRP-8) has been isolated from the cow (Odink et al., 1987, Dorin et al., 1987). It was postulated that MIF functioned in the lens either as a differentiation control factor, or as an intercellular messenger, as its expression occurred in the differentiating cells within the lens. In adult mice, MIF is widely expressed in a variety of tissues, from kidney and lung to heart and muscle (Wistow et al., 1993), and this is possibly due to the heavy vascularization of these tissues. However, Wistow et al. (1993) make the point that there is little vascularization in the early stages of lens development, and that blood vessels are absent in the mature system. This, and the fact that MIF distribution is specific to differentiating cells, implies that it has a role in lens development rather than the effect being an immunological one in this system.

The above research poses an interesting question. Was the rat homologue of MIF isolated from the differential screen of the end bulb cDNA library because it was

functioning as gene involved in the cycle of the adult sinus hair or was it merely identified due to the heavy vascularization of the follicle? Current research in this group aims to examine the distribution of MIF within the different stages of the hair cycle to clarify this question.

#### **5.4.3.4 *In vitro* Dermal papilla cell expression of mas-CL 32**

In section (5.3.2.2) it was stated that the predicted amino acid sequence of mas-CL 32 was virtually identical to mouse MIF, and that it was the rat equivalent. However, as shown previously in fig (52b) mas-CL 32 has high homology with a number of very closely related genes, therefore when interpreting expression studies the possibility of cross hybridization cannot be ignored.

As was shown in section (5.3.3.4) dermal papilla cells differentially expressed mas-CL 32 whilst displaying the clumped phenotype (fig 57). However, SC cells also had a high expression of the gene. How then was this clone isolated from the differential screen? In the previous chapter it was noted that SC cell migration was slightly variable, this being due to the fact that the exact onset of the aggregation process was difficult to identify, due to the variations within the culture dishes. Therefore, it is possible that a similar irregularity could have occurred in preparing the SC DP probes. This is supported by the variation of intensity of mas-CL 32 expression within the SC cells, and the overall reduction of expression within the AG DP cells. Expression within the CL DP cultures was clearly differential, with the greatest intensity present within the clumped structures with negligible expression occurring between cultures.

#### **5.4.4.1 Clone mas CL-12, a possible rat homologue of murine Tcl-30**

Complementary DNA clone 12, had relatively high homology with the murine gene Tcl-30 with 79% identity over 155 bp. Furthermore the sequence overlapped in the 3'

untranslated region, therefore homology might be improved if the clone was to be sequenced within the open reading frame. As the cDNA clone isolated was only approximately 450 bp long, the entire sequence from the cDNA clone mas CL-12 would still be outside the open reading frame. Nevertheless, mas CL- 12 could be used as a probe to isolate larger clones from the end bulb library, hopefully giving more information on its sequence and respective homology with the murine Tcl-30 gene.

#### **5.4.4.2 T-cell 30, a gene expressed during apoptosis of T-cells**

Apoptosis can be induced in susceptible cell lines in a number of ways , for example by elevation of internal cAMP levels, irradiation, or glucocorticoid treatment (Daniel et al., 1973, Vedeckis et al., 1983, Sellins and Cohen 1987). Using the glucocorticoid method Harrigan et al. (1989) isolated several genes that were induced during apoptosis of a murine thymoma cell line (WEHI-7TG), and one of these clones was designated as Tcl-30. This gene encodes a 2.4kb mRNA which they report as being specifically expressed in the thymus (Baughman et al., 1992), within a subpopulation of thymocytes called heat stable antigen (+) {HSA<sup>+</sup>} T cells. These cells belong to a class of thymocytes distinguished by their expression of HSA (Scollay and Shortman, 1985, Wilson et al., 1988), which with the expression of CD 4 or CD 8 surface antigens are destined for intrathymic cell death. This elimination of cells via apoptosis is an important physiological process in the differentiation of T-cells within the thymus ( von Boehmer, 1988, Scollay, 1991) .

Harrigan et al. (1989), by using northern analysis, discovered that Tcl-30 was specifically expressed within the murine thymus and not in brain, kidney, liver, lung, heart and spleen. It would therefore seem unusual that it should be expressed within a cDNA library constructed the end bulb of adult sinus follicles.

#### **5.4.4.3 Tcl-30 expression within *in vitro* DP cells**

As discussed in previous chapters the hair follicle is a highly labile tissue that generally fluctuates between long growing phases (anagen) and short resting phases (telogen). During catagen it has been shown that the cells of the differentiated epidermis undergo apoptosis as the follicle 'moves' into the resting phase of the cycle (Weedon and Strutton, 1981). It is interesting however that Tcl-30 should be identified from the library via an aggregated probe isolated from clumped *in vitro* dermal papilla cells. Dermal papilla cells are capable of all the phenotypical traits of apoptosis in culture (section 3.4.1.5), and therefore expression of an induced gene that was discovered within an apoptotic line of T cells does not seem unusual. However, the only region within the hair follicle that is protected from apoptosis, via expression of the Bcl-2 gene (Stenn et al., 1994b), is the dermal papilla. It is therefore possible that the clumped cDNA probe may have hybridized with clones that were derived from apoptotic epidermal cells in the original end bulb cDNA library. Expression studies on the distribution of Tcl-30 within the hair cycle would be very useful in clarifying this question.

The possibility of this hybridization between two different tissue types, however does not detract from the fact that this gene has not been discovered in culture outside the WEHI-7TG T cell line and more importantly it has not, until now, been isolated *in vivo* except within the thymus. The problem now arises, is Tcl-30 really specific to glucocorticoid induced apoptosis or is it a commonly expressed gene within other apoptotic events *in vivo* or *in vitro* ?

#### **5.4.5.1 Unknown putative differentially expressed clones**

Complementary DNA clones, mas CL-10.5 and mas CL-23, have no close homology with any previously described genes. In all cases the cDNA isolated was relatively short, and it is therefore possible that the sequence is within the 3' untranslated region,

this being the least conserved region of a gene. Further sequencing of the clones may identify an open reading frame within these clones. Nevertheless, the possibility arises that these clones maybe differentially expressed novel genes, that are involved in the formation of the DP clumps and, concurrent with this, have a possible role in the *in vivo* cycling of the adult follicle.

In conclusion, although cell adhesion molecules and the more commonly associated extracellular matrix molecules of dermal condensations have not been identified using this differential screen of CL DP cells, the majority of the clones identified from the primary screen have not as yet been characterised further, and the likelihood still remains that more commonly associated molecules may be identified. The differential screen method employed here appears to be a powerful tool, and could be used to identify gene expression by dermal papilla cells at any stage during the aggregation process.

## Chapter 6

### Conclusion

#### 6.1 The hair cycle

In the previous chapters, a wide variety of problems have been addressed with respect to follicle morphogenesis and in particular *in vitro* aspects of the follicular dermal cells. During chapter 2, follicle morphology during the hair cycle was examined with a range of different species. Is it then possible, to draw from this histological evidence, any conclusions on general aspects of the hair cycle?

In the adult mammal, the pilosebaceous unit is one of the few organ systems that constantly redevelops itself throughout the lifespan of the organism on a regular cycle. In chapter 2, the mink vibrissae was used to investigate each stage of the hair cycle from anagen, briefly into catagen and then into resting telogen. This example will be used as an alternative theory to the hair cycle. During catagen, the dermal and epidermal tissue undergoes a vast morphological change, that eventually results in the resting telogen follicle. Of particular interest in the mink vibrissae follicle, is the gradual reduction in size of the papilla to an almost indistinguishable ball of cells within the connective tissue, and a complete removal of epidermal tissue surrounding the papilla. For this to occur, the epidermal matrix cells must migrate proximally, whilst there is a downward migration in outer root sheath cells. Eventually, all the epidermal tissue in the telogen follicle consists of outer root sheath cells and club fibre (Figure. 59). However, what occurs in restimulation of the growing anagen stage is largely unknown. Reynolds and Jahoda (1993) postulate that in rat vibrissal cells, the epidermal germinative cells give rise to the cells of the cuticle, cortex and medulla, whereas the outer root sheath cells give rise to the different layers of the inner root

sheath cells. However, in the mink vibrissae, there is no apparent germinative region, therefore it remains unclear as to how this hypothesis might occur.

For the hair to cycle, it is widely believed that an epidermal stem cell population must exist. However, the location of such cells and the cell lineages that they create have long been an area of controversy. A primary consideration of stem cell behaviour is that their position is protected from injury (Potten and Loeffler, 1990). Historically the germinative cells, in the lower follicle bulb, were credited with the production of all or some of the epidermal layers (Bullough and Laurence, 1958, Montagna and Van Scott, 1958, Montagna and Parakkal, 1974, Powell et al., 1989). However, this is by no means proven. Recently, follicle stem cell research has produced an alternative line of thought, with the new emphasis being on a region of cells in the upper outer root sheath of some follicles, known as the bulge. Evidence in support of the latter idea comes from the position of slow cycling cells (Cotsarelis et al., 1990), and a unique cytokeratin expression (Lane et al., 1991). Furthermore, these cells were clearly visible throughout the hair cycle. In telogen human follicles, the bulge cells are in contact with the papilla, due to the papilla moving up with the upward migration of the follicle. Cells from the bulge are thought to migrate down to replenish the germinative cells, that in turn stimulate the next anagen cycle. However, not all follicle types shorten in this manner (Young and Oliver, 1976) and furthermore unpublished data from our group (Dr A.J. Reynolds pers. comm.) shows evidence of label retaining epidermal germinative cells. Consistent with this, the germinative cells also produce compelling evidence of their pluripotency (Reynolds, 1989). However, their role as a true stem cell is questionable, because what happens to them during the telogen phase of the follicle cycle has not been established. Therefore, it is unclear whether they are a transient or permanent stem cell population. Reynolds and Jahoda (1993) suggest that the germinative cells may be a permanent, with the descending ORS isolating the germinative epidermal cells from the ascending matrix cells (Fig 58). However, this concept of the hair cycle is largely based on the rat vibrissa follicle, which has a very regular growth cycle with a very brief telogen phase. Therefore, the

events involved in systems with a prolonged telogen stage, such as the mink vibrissae, may be considerably different.

In the mink vibrissa late anagen follicle, there was a demarcation between cells of the lower and upper matrix on one side of the endbulb (figure 2b), which during catagen, became more obvious (figure 3a,b), with the lower epidermal matrix population separating. This observation of follicle asymmetry correlated with discoveries by Reynold and Jahoda (1993). However, the mink tissue included more than the postulated germinative region. In telogen, the same authors postulate that germinative epidermal cells remain around the basal stalk, however there was no apparent histological evidence for this in the mink vibrissae. In the mink, the follicle was shown to have extended upwards in telogen, but this proximal migration of the follicle was not as great as that illustrated by human hair follicles and pelage follicles (Chapman, 1990).

In considering the hair cycle further, the action of the dermis must be kept in mind. Appendages form through a series of inductive events between the epidermis and dermis (Sengel 1976). Through an ingenious set of experiments Oliver, (1966a,b, 1967a,b, 1970, 1973), showed that the dermal papilla was the prime controlling factor in the hair cycle, but that interactions or inductive events only occurred when the DP was adjacent to the epidermis. Therefore by inference, the stem cells must remain in contact with the DP for correct morphogenesis of the follicle at the start of each new cycle. If bulge cells are the hair progenitors, then in such cases the dermal papilla, which is generally believed to control the hair cycle (Oliver, 1966a,b) must signal to them through the outer root sheath cells to send down cells to replenish cells of the matrix. However, even in the relatively shortened mink vibrissae telogen follicle, the papilla was not directly adjacent to a bulge region. If the stem cells were opposing the dermal papilla, then they would be ideally placed to initiate follicle growth. As epidermal germinative cells, or indeed any epidermal cells, are not visible around the dermal stalk during telogen, it can be assumed that they have degenerated or moved into a position above the papilla (Fig 59), with the outer root sheath cells.

Nevertheless, there was no morphological evidence of a group of germinative epidermal cells, distinct from the outer root sheath, above the telogen papilla in these follicles.

However, does there have to be a permanent stem cell population or could the cycling occur if stem cells were transient? Lavker et al., (1991) argued that work by Oliver (1966a,b) supported the concept of a permanent stem cell population in the bulge region. Oliver (1966a,b) discovered that an ablated sinus follicle end bulb could regenerate only if the lower third was removed, leaving the upper two thirds with the bulge region. Lavkker et al., (1991) postulated that, as the dermal sheath cells migrate down to replenish the dermal papilla, the bulge daughter cells would also migrate to replenish the epidermal matrix, this mimicking the downward migration of the follicle from telogen to anagen.

Recent results from Reynolds and Jahoda(1992), have shown that dermal papilla cells are capable of stimulating follicle neogenesis in a follicular skin to produce germinative cells and all other follicle components (rat footpad). If DP cells are capable of inducing overlying a follicular epidermis, then by inference DP cells should be able to restimulate the outer root sheath cells of the telogen follicle. Therefore, why should it be necessary to invest in an exclusive mechanism for maintaining a vulnerable stem cell population, particularly during fibre shedding or damage, when there is the means to re-induce the epidermal stem cell properties at the beginning of the anagen cycle?

The lack of stem cell / germinative markers makes it relatively impossible to test this phenomenon. However, current work in our laboratory has generated some interesting expression domains of putative genetic markers for the germinative region, and further work may reveal some interesting insights into this most controversial of questions.

Fig. 58

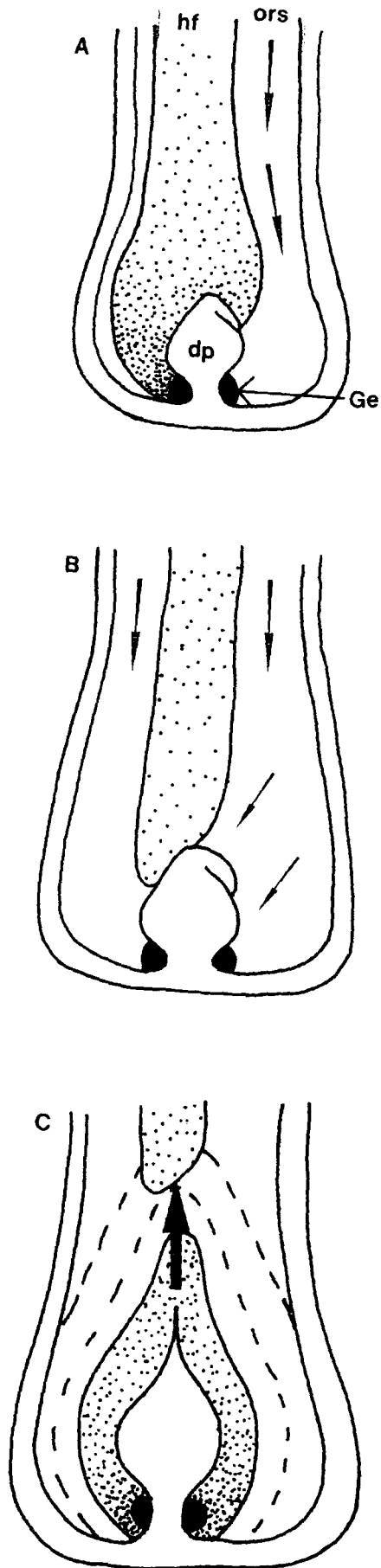
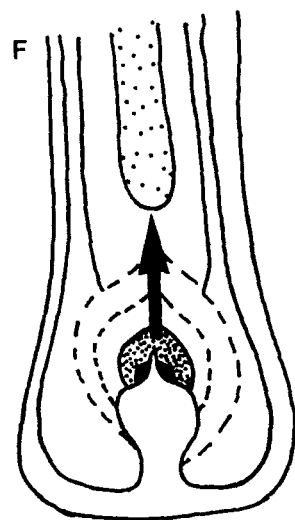
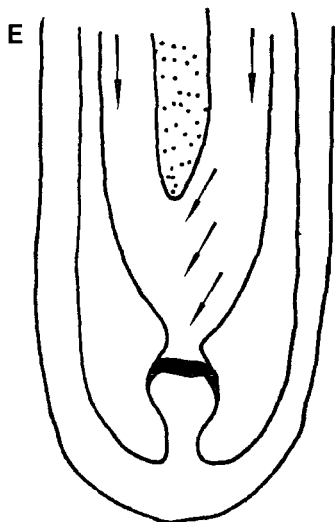
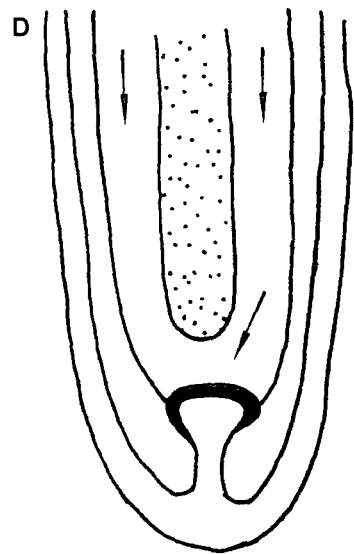
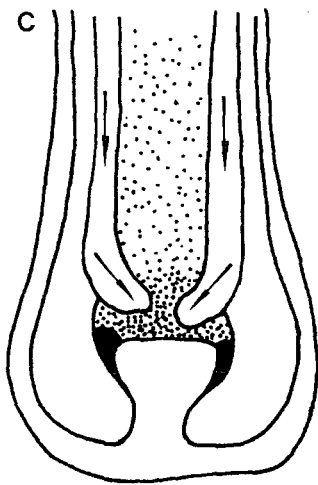
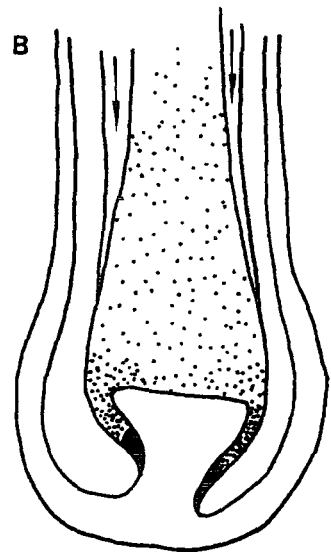
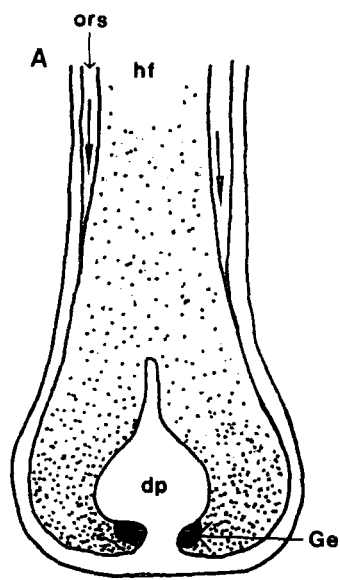


fig.59



## 6.2 Dermal papilla cell *in vitro* aggregation

Throughout this thesis several aspects of *in vitro* dermal papilla cell aggregation have become apparent. Firstly, the aggregations appear not to be the result of focal proliferation; and secondly at a critical time during the aggregation process the DP cells have an enhanced motility, which can be further increased in the presence of the aggregating conditioned medium. Furthermore, once aggregations are formed cell motility is terminated.

This type of phenomenon has been unobserved in mammalian cell culture, however a similar behaviour can be observed in the slime mould *Dictyostelium discoideum*. This slime mold inhabits soil and leaf litter, with their prime source of energy being bacteria and yeasts. During its lifespan it can adopt a range of interesting behaviour patterns (Gerisch, 1987), of which in this context, aggregation phenomenon is probably the most interesting.

When a food source becomes exhausted in any one region, the scattered amoebae stop dividing and migrate to particular focal points. It was discovered (Bonner, 1947, Shaffer, 1953) that these focal migrations were as a result of a signal secreted by the cells themselves. Once formed, the cells in the aggregates remain together by specific cell surface adhesion molecules, called gp24 and gp80, both of which are glycoproteins. (Knecht et al., 1987, Loomis, 1988). The aggregate then grows until it falls over and forms a slug which itself migrates, and moves in response to light and heat. Once migration in this phase has terminated, the slime mold produces and releases spores as the final stages of the life cycle. It is during these early stages of focal migration, that there is a similarity between the DP cells and *Dictyostelium*.

Konijn et al., (1967) and later Bonner et al., (1969) found that the cells signalled to each other with cyclic adenosine 3',5' monophosphate (cAMP). The aggregations are initiated as certain cells begin to produce pulses of cyclic AMP. These focal cells are determined by the distribution of the surrounding amoeba (Keller and

Segal, 1970, Tyson and Murray, 1989). Surrounding cells respond to these pulses of cAMP by moving towards the focal cell, with each movement occurring with each pulse from a focal cell (Robertson et al., 1972, Shaffer, 1975), furthermore the responding cells also produce cAMP and therefore a wave of cAMP is propagated through the population. Therefore, *Dictyostelium* motility is enhanced at a specific stage which results in the focal migration of cells to form a central aggregate, this fluctuation in motility being not dissimilar to events occurring in the DP aggregation process.

### **6.3 Embryonic cell migration**

Although the DP cell *in vitro* aggregation appears to mimic the simple mechanisms of aggregation formation in the slime mold, is there evidence within the advanced embryonic development of the vertebrates, for migration as a cue for aggregate formation and dermal condensation? Cell migration has been documented in a number of different aspects of embryonic morphogenesis. It is involved in shaping gastrulation, the migration of the primordial germ cells, the invasive action of the neural crest cells at neurulation and the setting up of neural networks.

Neural crest migration is one of the best characterised migratory events in the developing embryo. Neural crest cells migrate through a variety of different pathways to reach target sites. The different regions of neural crest are divided along the neural axis, being designated as cranial, vagal, trunk and lumbosacral (Le Douarin, 1982). Within these broad categories, further subcategories occur particularly in the complex cranial region. The specific derivatives of these cells are equally varied, ranging from Schwann cells to melanocytes. How these cells differentiate is largely unknown, however the migratory pathways leading to these differentiated states is beginning to be understood.

Prime candidates in setting up these migration pathways are the extracellular matrix molecules. Fibronectin, laminin, tenascin, collagens and proteoglycans have all been discovered along migratory pathways (Newgreen and Thiery, 1980, Krotoski et

al., 1986, Duband and Thiery, 1987, Bronner-Fraser, 1988, Perris et al., 1991a, Tan et al., 1991). It is thought that these extracellular matrix molecules modulate the relative adhesion of cells to these molecules, and in so doing alter the cells motility. However, a large degree of evidence for this comes from *in vitro* experiments rather than direct *in vivo* investigations (Newgreen, 1984, Perris et al., 1991 a,b, Tan et al., 1991). Nevertheless, it is interesting that many of the molecules that are thought to have a specific function in the migration of neural crest cells, have also been isolated within the condensing dermis of embryonic skin appendages. (see chpt 1). It has already been shown that the developing teeth are derivatives from the neural crest (Lumsden, 1987), therefore the possibility arises that the developing vibrissae dermal papilla may form via a migration and differentiation of the neural crest. Furthermore, as the neural crest cells migrate, they secrete the protease, plasminogen activator, which modifies the local microenvironment, and therefore facilitates cell movement (Valinsky and Le Dourain, 1985, Menoud et al., 1989). How motility in cells is altered by the effect of plasminogen activators and their respective receptors is reviewed by Blasi, (1993). Plasminogen activator production has also been correlated with mammary involution (Ossowaki et al., 1979), a dermal condensation event not dissimilar to that of follicle production. Furthermore, a recent expression study by Carroll et al., (1994), has isolated plasminogen activator within the developing rodent vibrissae follicles, however expression is restricted to the epidermal placode and therefore may just be a reflection of melanocyte differentiation.

Nevertheless, although little is known on the motile activity of cells within the *in vivo* dermal condensation, it appears from circumstantial evidence that cell migration may have a greater role to play in aggregate formation than is currently thought.

## 6.4 Models and theories of dermal condensation

Prevailing ideas about, and models of, dermal cell condensations incorporate cell adhesion and cell traction as the principal mechanisms. Bard (1990) hypothesizes that dermal cell condensation occurs via a focal expression of cell adhesion molecules that are able to induce adjacent dermal cells to express adhesion molecules until the desired structure is reached. At the same time, these cells are exerting specific tractional forces on each other to increase the density of the condensates (the cells are assumed to collide in a random fashion) and when contact is made, cell adhesion expression is induced in the adjacent cell.

Certainly in the embryonic development of skin appendages the early structures are rich in extracellular matrix (Choung et al., 1991, Chiquet-Ehrismann et al., 1986, Vainio & Thesleff, 1992, Trautman et al., 1991), and express many cell adhesion molecules (Choung & Edelman, 1985 a, 1985 b ; Choung et al., 1991; Takeichi, 1988; Hirai et al., 1989). Moreover, monoclonal antibodies against some of the epidermal cell adhesion molecules have been able to disrupt the correct dermal morphogenesis and hence the normal development of these skin appendages (Gallin et al., 1986, Hirai et al., 1992). Adult papilla cells also produce a complex extracellular matrix (Couchman, 1986), particularly when cells are clumped, and papilla cell behaviour supports the idea that there are powerful adhesive forces keeping papilla cell clumps together. However, in early follicle development cell adhesion molecules have been observed more in established condensations (Choung et al., 1991), thus they may not be the only mechanism of producing them.

Concerning tractional forces, Harris et al (1980) illustrated that motile fibroblasts could create considerable forces when attached to various substrates. Harris et al (1984) then simulated mesenchymal condensation, by stabilizing a collagen substrate in a culture vessel, preventing contraction of the gel by fibroblasts. When stabilized, all the contractile forces are transferred directly between cells, and hence aggregates are formed. The above workers were also able to manipulate the size and

number of cells in the condensation purely by increasing the density of the cells. By comparison, the dermal papilla cell model has two more natural elements. First, the dermal papilla cells are able to spontaneously form aggregates on a plastic or glass surface without the aid of added extracellular matrix, and second the size of aggregates is not dependant on the density of the cells. When papilla cell density is increased, the size of the aggregates remains relatively uniform, however it may be that the cells have to reach a certain density in culture before they begin to express the aggregated phenotype. This is indeed the case for chondrogenic aggregation *in vitro*; if cells are maintained at a low density they maintain the fibroblast phenotype, however if cultured at a higher density the cells differentiate, condense and produce cartilage specific molecules. (Von der Mark et al., 1977; Archer et al., 1982)

One of the reasons why it has been considered that migration is unlikely to be important in embryonic condensation behaviour is because precondensation cells are relatively close together (Bard,1990). However, this assumes that all the cells that in the precondensation mesenchyme are identical, and that the condensation is formed only by cells in the immediate vicinity. Leaving aside this question, even with the traction/adhesion mechanism described above, the introduction of increased movement by cells at the time of DP aggregation would accelerate the process of cell contact and hence of condensation. Oster et al. (1983) created a mathematical model of mesenchymal condensation, on the basis that condensations were formed by a traction induced effect from the substrate. These authors also proposed that the events leading up to a complete aggregate utilised motility in the cells although their model also worked if the cells were non-motile Oster et al., (1985). In a later paper Oster and Murray (1989) suggested that aggregation would be enhanced if the cells had a motile property, although they considered the motility only as a process for distorting the extracellular matrix. More recently in another context, Jiang et al., (1993) postulated that active recruitment of cells from surrounding tissue may be involved in dermal condensation prior to bone formation after discovering that an increase in condensation was not due to increased proliferation. Ede et al., (1971) suggested that cell motility

produced the feather dermal condensation, with cells at the centre of the foci being immobile, and being attractive in some chemotactic way to other cells. Furthermore, the rest of the cell population in the dermis is relatively mobile, tending to move around the foci and forming the typical orientation of cells described in this latter paper. In an earlier paper Ede and Agerbak (1968) suggested a similar mechanism in the formation of mesenchymal condensation in the limb bud during chondrogenesis, but at this time they were not sure whether the focal condensation was the result of the foci stimulating chemotaxis or a trapping of the intrinsically motile cells.

Therefore, from the results described in previous chapters it appears that motility of dermal papilla cells may have a greater function in formation of the aggregates, however it is not clear whether the increased motility at the time of aggregate formation is as a result of focal migration in the form of chemotaxis or whether it is a general increase in randomly motile cells.

To overcome this question, Zicha et al., (1991) developed a chamber through which chemotaxis can be observed, with two channels in concentric rings separated by an elevated plateau. The principle behind this is that the chemoattractant is placed in the outer ring, with a non chemoattractive medium and cells in the inner ring. As the chamber is incubated cell position is monitored over time across the plateau and the pathway of movement plotted. Therefore, by using this system chemotaxis, can be monitored. However, this system is more qualitative than quantitative, and thus the modified Boyden chamber, used in chapter 4, is a better starting point in analyzing *in vitro* DP aggregation behaviour, because one can then assay the gross changes in overall motility of the DP cells at different stages in their phenotypes, before examining the exact specificity of these changes, whether they are due to chemotaxis, haptotaxis or contact guidance. Nevertheless, now that a basic understanding of the motile fluctuations of DP cells is understood, it would be of great interest in the future to assess the exact specificity of the different DP conditioned mediums with respect to chamber described by Zicha et al., (1991), particularly as this may directly relate to the Ede's theory of focal migration in dermal condensation formation *in vivo*.

Molecular evidence for motility was discovered in the chapter 5, with the differential expression of macrophage migration inhibitory factor. This molecule was shown to have greater expression during the non motile phases of aggregate formation.

Therefore, assuming a greater role for motility in aggregate formation is it possible to come up with an another working idea for condensation formation? Prior to dermal condensation, the cells are either immobile or have a low level of intrinsic motility. Upon induction of skin appendages, the future dermal condensation cells secrete a molecule or substance, possibly a growth factor such as TGF $\beta$ <sub>1</sub>, bFGF and BMP 4, that increases motility in surrounding pre-condensation cells and stimulates the surrounding cells to further secrete more of this molecule. Subtle changes in the expression of extracellular matrix components, such as tenascin, may also enhance this motility by reducing the strength of cell adhesion to the substrate. Mackie et al., (1987) found that prechondrocytes attachment was greater to a fibronectin substrate, than a tenascin one. When the cells begin to interact, either by moving towards a focal cell or through random increased motility, they begin to express MIF. As the cell to cell interactions increases, so to does the expression of MIF, until all the cells within the aggregate are non-motile. Therefore, there is an antagonistic interaction between non-motility and motility during aggregate formation. Whilst the cells are interacting and becoming non-motile, extracellular matrix molecules and cell adhesion molecules are being laid down as a means to stabilise this newly formed aggregation. Once the condensation is complete, cells may be fixed in a complex array of extracellular matrix and cell adhesion molecules that further inhibits motility. In this way, it is possible to see how the initial condensation might form, due to this fine balance between motility and non motility. Although this type of motile interaction has been hinted at in theoretical models (Oster et al., 1983, Oster et al., 1985, Oster and Murray, 1989), this is the first time that direct experimental evidence has been given for a fluctuation in motility being the driving force behind condensation formation. However, it must be remembered that this concept has been largely derived from *in vitro* experiments on

hair follicle dermal cells, and that it would be of great interest to see if other *in vitro* cells such as prechondrocytes show a similar behaviour.

### **6.5 Could cell motility have a role in androgenically sensitive follicles?**

Hair follicle morphology is basically similar throughout all regions of the human body, however at the onset of puberty a number of follicles alter in a characteristic manner, in response to the hormonal changes that occur. The effect of these androgens on human hair are dependent on the body site of the follicle; with a stimulation of follicle size in the pubic regions in male and females; beard and chest hair stimulation in males; and in some cases a regression of scalp hair in both sexes. Successive experiments, discussed previously, have isolated the dermal papilla as a major factor in controlling the hair cycle. Ibrahim and Wright (1982) discovered, that with an increase in fibre size, there was a resultant increase in the papilla size, and likewise a decrease in fibre size was matched with a decrease in dermal papilla size.

Rather than having an instantaneous effect, androgens act in a gradual manner on the hair follicle. In the production of the beard in males, there is a gradual progression from a short fine unpigmented vellus follicle, to a much enlarged thick pigmented terminal follicle over successive cycles. In this scenario it would appear that androgens have increased the size of the follicle, and hence the size of the dermal papilla. Hodgins et al., (1991) discovered that androgen receptors were present within the dermal papilla cells of androgen sensitive beard follicles, as well as scalp follicles, however high levels of receptors were also present within nonbalding scalp follicles. Murad et al., (1986) were first to discover the presence of androgen receptors in cultured dermal papilla cells. Further investigations by Randall et al., (1991), found that the concentration of receptors was greatest in DP cell cultures derived from androgenic sensitive follicles. However, when grown in the presence of the androgen testosterone, there was no significant increase in proliferation of DP cells. However, androgenic responses occur over a protracted period of time, therefore it is not

surprising that such an obvious response could be noted over the length of time involved in these experiments.

During androgenetic alopecia an inverse of beard formation takes place, with the terminal follicle reducing in size over successive cycles to form a vellus or lanugo follicle. The structural maintenance of the dermal papilla is vital to ensure the correct cycling of the adult hair follicle. In chapter 4 it was shown that DP cells were capable of increased states of motility at specific times during the *in vitro* aggregation process and yet this was terminated during the clumped stage.

If the molecules involved in maintaining the structure of the dermal papilla should alter, would motility be restored to these *in vivo* cells? When passaging clumped *in vitro* dermal papilla cells, the clump is broken down and the aggregation process is started again, therefore restoring motility to the cells. Consequently, could increases and decreases in papilla size in these androgenically sensitive follicles be as a result of changes in the extracellular framework of the papilla, with a respective change in the motility of the cells?

It has been suggested that the terminal to vellus transition in baldness is due to a loss of adhesive properties among the DP cells. From the results in chapter 5, there appeared to be a fluctuation in the expression of MIF, with an increased motility when expression was reduced. It has yet to be ascertained if MIF has a role in the adult hair cycle. However, if MIF was expressed in the adult follicle to maintain the dermal papilla, it may be possible that during androgenic alopecia a reduced expression over time allows cell movement away from the papilla, during successive cycles and hence a reduction in papilla and fibre.

While it is not necessarily the case that MIF is involved in this particular function, if another molecule acted in a similar way, such as N-CAM, it is possible to see how androgenic baldness could occur. If androgens alter expression of these DP maintenance type molecules, the question still remains as to why region specific hair follicles are sensitive to the action of these hormones.

As research groups are still trying to ascertain how a hair follicle cycles in its normal state, few have looked at how the extracellular integrity or growth factor expression alters during androgenetic alopecia. Furthermore, as the integral controlling genes of the normal hair cycle still remain elusive, it will be some time before the events in androgenic alopecia are understood.

## 6.6 Summary

As with most research, this thesis has posed more questions than it has answered, however a number of interesting points have come to light. Firstly, in chapter one, molecular expression between different condensation events was summarized. Although the molecules expressed were wide and variable in function, it became apparent that the same molecules were being expressed at these condensation events. Therefore, are these molecules switched on for the production of the condensation events alone, and if so, what then are the molecules that decide a condensation becomes a tooth or a bone or a hair follicle.

Secondly, *in vitro* DP cells not only resemble early dermal condensation events, but they also express molecules which are capable of supporting follicle production when re-implanted *in vivo*. Furthermore, these cells have illustrated that whilst *in vitro* they behave in an unconventional manner to that of other previously described fibroblast cell lines. Not only do they have fluctuations in motility that correlates with changes in the cells *in vitro* phenotype, but they are also capable of inducing increased motility in non motile SC dermal papilla cells by secretion of a molecule(s) into the medium. This, as described above, being reminiscent of the slime mold characteristics. If the DP aggregates in culture are representative of dermal condensation *in vivo*, then the isolation of this molecule(s) in conjunction with the various motile aspects of the *in vitro* DP cells may be useful in determining the exact sequence of events at this crucial time in organogenesis.

Finally, molecular characterisation of the *in vitro* DP aggregates provided more evidence that motility was a positive function in their formation. Differential expression studies showed that MIF was highly expressed within the clumps of DP cells, a cell phenotype that was earlier proven to have a much reduced motility, when compared to the other previously described DP *in vitro* phenotypes. Only a few clones were characterised further and as 40 putative clones were initially isolated, the potential still remains to isolate many more molecules that may shed light on this *in vitro* aggregation process, and possibly events *in vivo*.

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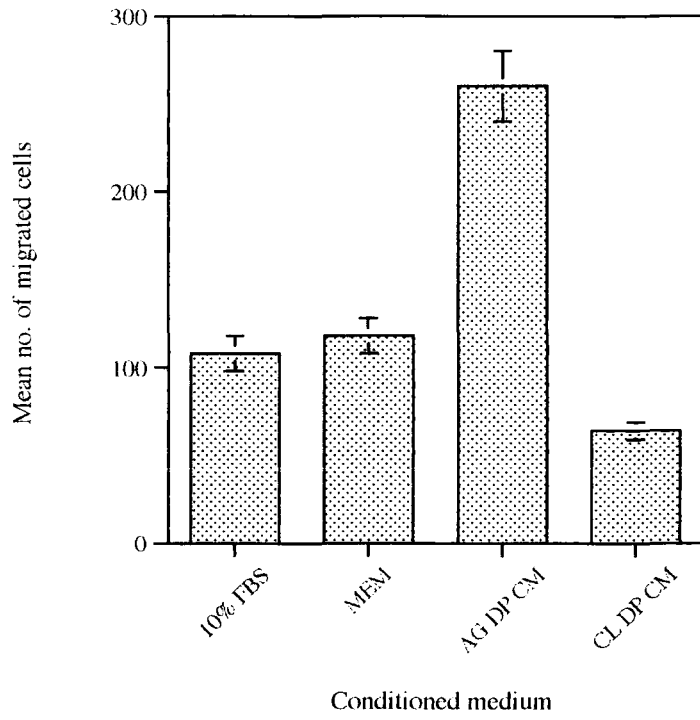
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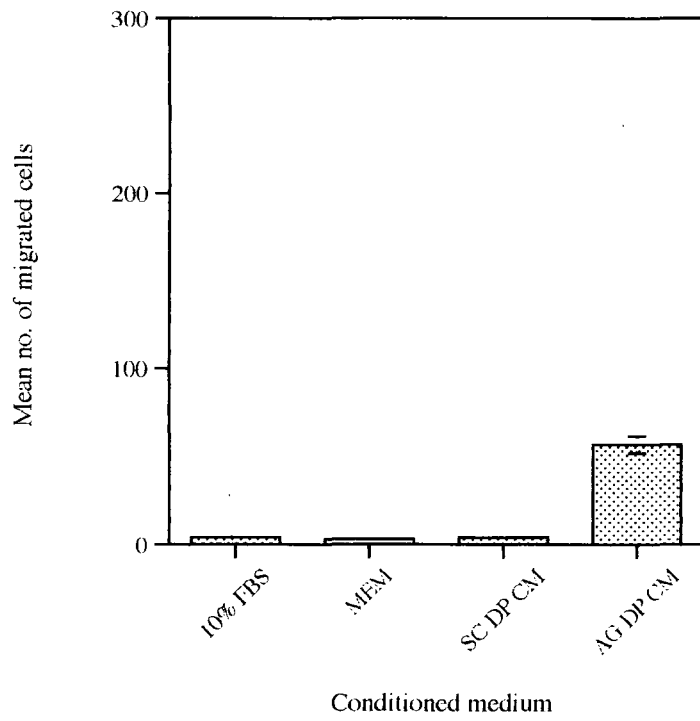
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## Appendices

### Appendix 1a



### Appendix 1b



# Diagrammatic representation of cell migration experiments from chapter 4

## Appendix 2a

|              |                       |             |             |                |
|--------------|-----------------------|-------------|-------------|----------------|
| Top Wells    | SC DP cells           | AG DP cells | CL DP cells | Confl Fi cells |
| Bottom Wells | 10% Foetal Calf Serum |             |             |                |

## Appendix 2b

|                      |   |          |          |          |
|----------------------|---|----------|----------|----------|
| Top Well Combination | i) SC DP cells<br>ii) AG DP cells<br>iii) CL DP cells |          |          |          |
| Bottom Wells         | 10% FCS   | SC DP CM | AG DP CM | CL DP CM |

## Appendix 2c

|                      |                   |          |          |          |
|----------------------|-------------------|----------|----------|----------|
| Top Well Combination | SC/Confl DP cells |          |          |          |
| Bottom Wells         | 10% FCS           | SC DP CM | AG DP CM | CL DP CM |

### Appendix 3a

|              |             |         |              |              |
|--------------|-------------|---------|--------------|--------------|
| Top Well     | SC DP cells |         |              |              |
| Bottom Wells | AG DP CM    | 10% FBS | AG CM > 10KD | AG CM < 10KD |

### Appendix 3b

|              |             |         |              |              |
|--------------|-------------|---------|--------------|--------------|
| Top Well     | SC DP cells |         |              |              |
| Bottom Wells | AG DP CM    | 10% FBS | AG CM > 30KD | AG CM < 30KD |

### Appendix 3c

|              |             |              |             |             |
|--------------|-------------|--------------|-------------|-------------|
| Top Well     | SC DP cells |              |             |             |
| Bottom Wells | AG DP CM    | AG CM > 10KD | AG CM > 3KD | AG CM > 1KD |

### Appendix 3d

|              |             |               |              |              |
|--------------|-------------|---------------|--------------|--------------|
| Top Well     | SC DP cells |               |              |              |
| Bottom Wells | AG DP CM    | AG CM > 100KD | AG CM > 50KD | AG CM > 30KD |

### Appendix 4a

|              |                    |                       |                       |                        |
|--------------|--------------------|-----------------------|-----------------------|------------------------|
| Top Well     | AG DP cells in MEM | AG DP cells in 1% FBS | AG DP cells in 5% FBS | AG DP cells in 10% FBS |
| Bottom Wells | AG DP CM           |                       |                       |                        |

### Appendix 4b

|              |                  |                 |                 |                |
|--------------|------------------|-----------------|-----------------|----------------|
| Top Well     | AG DP cells      |                 |                 |                |
| Bottom Wells | 100%<br>AG DP CM | 50%<br>AG DP CM | 10%<br>AG DP CM | 1%<br>AG DP CM |

### Appendix 5a

|              |             |                |
|--------------|-------------|----------------|
| Top Well     | SC Fi cells | Confl Fi cells |
| Bottom Wells | 10% FBS     |                |

### Appendix 5b

|              |             |          |          |          |
|--------------|-------------|----------|----------|----------|
| Top Well     | SC Fi cells |          |          |          |
| Bottom Wells | 10% FBS     | SC DP CM | AG DP CM | CL DP CM |

### Appendix 6a

|              |                   |                    |                   |                    |
|--------------|-------------------|--------------------|-------------------|--------------------|
| Top Well     | SC DP cells       |                    | SC Fi cells       |                    |
| Bottom Wells | 10% FBS           | 1 ng/ml TGFbeta    | 10% FBS           | 1 ng/ml TGFbeta    |
|              | 0.1 ng/ml TGFbeta | 0.01 ng/ml TGFbeta | 0.1 ng/ml TGFbeta | 0.01 ng/ml TGFbeta |

### Appendix 6b

|              |                 |                  |                 |                  |
|--------------|-----------------|------------------|-----------------|------------------|
| Top Well     | SC DP cells     |                  | SC Fi cells     |                  |
| Bottom Wells | 5 ng/ml bFGF    | 0.5 ng/ml bFGF   | 5 ng/ml bFGF    | 0.5 ng/ml bFGF   |
|              | 0.05 ng/ml bFGF | 0.005 ng/ml bFGF | 0.05 ng/ml bFGF | 0.005 ng/ml bFGF |

### Appendix 6c

|              |                 |                  |                 |                  |
|--------------|-----------------|------------------|-----------------|------------------|
| Top Well     | SC DP cells     |                  | SC Fi cells     |                  |
| Bottom Wells | 5 ng/ml aFGF    | 0.5 ng/ml aFGF   | 5 ng/ml aFGF    | 0.5 ng/ml aFGF   |
|              | 0.05 ng/ml aFGF | 0.005 ng/ml aFGF | 0.05 ng/ml aFGF | 0.005 ng/ml aFGF |

## Appendix 7a

|              |                                    |          |          |          |
|--------------|------------------------------------|----------|----------|----------|
| Top Well     | i) SC DS cells<br>ii) SC tDS cells |          |          |          |
| Bottom Wells | 10% FBS                            | SC DP CM | AG DP CM | CL DP CM |

