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**Signalling Events Controlling Early Cell Fate
Decisions in a Human Embryonal Carcinoma Cell
Line, with Relation to Neural versus Non-Neural
Transitions in Human Ectoderm Development**

Grace Mary Horrocks

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A thesis submitted for the degree of Doctor of Philosophy

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Department of Biological Sciences

Durham University, England



17 APR 2007

ABSTRACT

In this study the human embryonal carcinoma cell line TERA2.cl.sp12 (TERA2.sp12) was utilised as an *in vitro* model in which to study mechanisms governing neural versus non-neural transitions in the human ectoderm. TERA2.sp12 cells have been previously reported to differentiate under adherent culture conditions into both neural and non neural derivatives of the ectoderm in response to treatment with retinoic acid (RA). In this investigation an alternative culture method was also characterised, which markedly modulated the differentiation fate of TERA2.sp12 cells in response to RA. Dissociated cells cultured in the presence of RA under suspension conditions generated aggregates which differentiated rapidly, and as detected by immuno-staining and Western blot analyses, exhibited marked up-regulation of neural markers without evidence of non neural differentiation. Previous work in cultured *Xenopus* animal cap explants show that suspension cultures of dissociated ectoderm differentiate into neural instead of epidermal tissue as a result of the inhibition of Bone morphogenetic protein (BMP) pathway activity. Polymerase chain reaction (PCR) and Western blot analyses demonstrated that RA treated adherent cultures of TERA2.sp12 cells exhibited up-regulated expression of BMP regulated genes in comparison to cultures treated in parallel and cultured under suspension conditions. Correspondingly, addition of the BMP inhibitor Noggin to adherent cultures increased the expression of neural markers (as detected by Western blot analyses) towards that observed in suspension cultures. Both BMP and FGF mediated pathways were also manipulated in the absence of RA exposure; BMP pathway activation or FGF receptor inhibition induced similar differentiation responses, yielding a differentiated heterogeneous population. Correspondingly, FGF receptor inhibitor and BMP treatments were detected to up-regulate the expression of BMP regulated genes by Western blot analysis, supporting the hypothesis that in both treatments differentiation was driven by an up-regulation of BMP pathway activity. Treated cultures displayed positive immuno-staining for trophoblast, smooth muscle and simple epithelial endoderm derivatives, demonstrating for the first time that TERA2.sp12 cells are capable of forming non-ectodermal lineages. To further investigate the full differentiation potential of TERA2.sp12 cells, the differentiation of tumour explants derived from TERA2.sp12 cell injection into immuno-compromised mice was characterised. Although tumours exhibited markedly restricted differentiation complexity when compared with tumours derived from human embryonic stem (ES) cell lines, immuno-staining assays demonstrated that TERA2.sp12 cells formed multiple germ layer derivatives *in vivo*. In conclusion, neural differentiation of TERA2.sp12 cells can be modulated by active manipulation of the BMP signalling pathway, and TERA2.sp12 cells provide a suitable model in which to further investigate the effects of BMP signalling on

neural induction. However, although TERA2.sp12 cells exhibit a marked propensity for ectodermal differentiation, their differentiation potential is not restricted to ectoderm derivatives and is therefore more similar to human ES cells.

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I am grateful to many people for help, both direct and indirect, in producing this thesis. In the first instance, I would like to thank my supervisor Stefan Przyborski, for providing me with the opportunity to undertake this PhD, and for his advice and encouragement along the way. I am also indebted to my second supervisor Colin Jahoda for his valuable and insightful comments, and for taking the time to be involved despite a very hectic schedule. From the wider scientific community, I owe particular thanks to Linda Lako and Steven Lisgo from the University of Newcastle who provided materials for positive controls and Paul Scotting from the University of Nottingham for providing training in RNA detection techniques. This work has been generously funded by the Anatomy Society of Great Britain.

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This thesis is dedicated to my parents and to my new niece Carla. May she stand on the shoulders of all of us.

DECLARATION

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

G. M. Horrocks

March 2007

ABBREVIATIONS

AFP	Alpha-1-fetoprotein
AGG	Aggregate
PBS	Phospho-buffered saline
BMP	Bone morphogenetic protein
BRDU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CNS	Central nervous system
CRAB	Cellular retinoic acid binding protein
CRB	Cellular retinol binding protein
Cy3	Cyanine 3 conjugate
DMEM	Dulbeccos modified Eagle's media
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
EB	Embryoid body
EC Cell	Embryonal carcinoma cell
EDTA	Ethylenediaminetetraacetic acid
EMEM	Ehlich's modified Eagle's media
ES Cell	Embryonic stem cell
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein thiocyanate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent hormone
HCG	Human chorionic gonadotrophin
HCL	Hydrochloric acid
HNF3 β	Hepatic nuclear factor 3 beta
Id-	Inhibitor of differentiation-
Kda	Kilo Daltons
L	Litre
LIF	Leukaemia inhibitory factor
m	Milli-
M	Molar
MAPK	Mitogen activated protein kinase

MON	Monolayer
MAP2	Microtubule associated protein 2
Mins	Minutes
NF-	Neurofilaments-
NSE	Neural specific enolase
Oct4	Octamer binding transcription factor 4
PCR	Polymerase chain reaction
Pen/strep	Penicillin/Streptomycin
PFA	Para formaldehyde
MEM	Minimal essential medium
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RoDH	Retinol dehydrogenase
RXR	Retinoid X receptor
RNA	Ribonucleic acetic acid
RNAse	Ribonuclease
SSEA-	Stage specific embryonic antigen-
Shh	Sonic hedgehog
SCID	Severe combined immune-deficient
TBS	Tris-phosphate buffered saline
Tp63	Tumour protein 63
μ	Micro-
V	volts

CONTENTS

Abstract.....	II
Acknowledgments	IV
Declaration	V
Abbreviations	VI
List of Figures.....	XII
List of Tables	XVI
Chapter 1. Introduction	1
1.1 Stem cells – an overview	2
1.2 From teratomas to embryonic stem cells.....	4
1.2.1 Murine embryonal carcinoma cell lines and their relationship to cells of the early embryo	5
1.2.2 Human embryonal carcinoma cell lines.....	6
1.2.3 Retinoic acid induced differentiation of EC cells.....	8
1.2.4 The derivation of embryonic stem cell lines.....	9
1.3 Utilising embryonic stem cells to study embryogenesis	10
1.3.1 Murine ES cells – models of murine embryogenesis.....	10
1.3.2 A gross comparison of murine and primate embryogenesis	12
1.3.3 A comparison of human and murine ES/EC stem cells	14
1.4 Using TERA2.sp12 cultures as a model of human ectoderm.....	15
1.5 The early stages of neural development in vertebrates – an overview	16
1.5.1 Neural induction and neurulation.....	17
1.5.2 Patterning of the neural tube	17
1.5.3 β Helix-loop-helix proteins and neurogenesis	18
1.6 Aim of this study	19
1.7 Objectives.....	19

Chapter 2. Materials and methods	21
2.1 Cell culture	22
2.1.1 TERA2.sp12 cells	22
2.1.2 Cell lines used as positive controls	25
2.2 Cell count assay	26
2.3 Flow cytometry.....	27
2.4 Western blot analysis.....	28
2.5 Immuno-cytochemistry.....	32
2.5.1 Adherent cultures	32
2.5.2 Suspension cultures.....	34
2.5.3 Tissues	35
2.5.4 Double Immuno-labelling.....	36
2.6 Histological staining.....	37
2.6.1 Preparation of wax embedded sections.....	37
2.6.2 Staining of sections with Haematoxylin and Eosin	38
2.7 Gene expression analysis.....	39
2.8 Generation of tumours in immune-deficient mice.....	41
2.9 Tissues used as positive controls.....	42
Chapter 3. Retinoic acid induced differentiation of TERA2.sp12 cells	43
3.1 Introduction	44
3.1.1 Retinoic acid – a key morphogen <i>in vitro</i> and <i>in vivo</i>	44
3.1.2 Alternative methods of culturing retinoic acid treated TERA2.sp12 cells	48
3.2 Results	49
3.2.1 General observations.....	49
3.2.2 Cell count assay	49
3.2.3 Changes in cell surface marker expression	51
3.2.4 Comparison of neural and non-neural ectoderm protein expression	52
3.2.5 Characterising the EC phenotype.....	54
3.2.6 RA induced differentiation as adherent cultures.....	54
3.2.7 RA induced differentiation as suspension cultures.....	57
3.2.8 Aggregates formed from suspension culture without RA.....	61
3.2.9 Summary of results	64
3.3 Discussion	65
3.3.1 Summary	65
3.3.2 The stem cell phenotype	66
3.3.3 Neural differentiation in RA treated TERA2.sp12 cells	70

3.3.4	Non-neural differentiation in RA treated TERA2.sp12 cells.....	72
3.3.5	Characterisation of aggregates formed from suspension culture	73
3.3.6	Synopsis	78
3.3.7	Conclusions.....	78
Chapter 4. BMP signalling and cell fate determination in TERA2.sp12 cells.....		80
4.1	Introduction	81
4.1.1	Neural versus non-neural fate specification in the ectoderm	81
4.1.2	Bone morphogenetic proteins	83
4.1.3	Neural induction – simply a matter of BMP signalling inhibition?.....	86
4.1.4	Using ES cells as <i>in vitro</i> models for the study of neural induction.....	87
4.2	Results	89
4.2.1	Monitoring the BMP signalling pathway in RA treated cultures.....	89
4.2.2	Manipulating BMP pathway activity in RA treated cultures.....	91
4.2.3	Inhibiting FGF mediated signalling prior to RA treatment.....	92
4.2.4	Manipulation of BMP signalling	94
4.2.5	Inhibition of FGF mediated signalling.....	98
4.2.6	Characterising cultures treated with BMP2, BMP4 or SU5402	99
4.2.7	Monitoring BMP and FGF mediated pathway activity.....	105
4.2.8	Subsequent Noggin and FGF inhibitor treatment	106
4.3	Discussion	107
4.3.1	Summary	107
4.3.2	BMP and FGF mediated signalling in RA treated cultures	108
4.3.3	Manipulation of BMP signalling	113
4.3.4	Similar effects of BMP or FGF inhibitor treatment.....	116
4.3.5	Converging FGF and BMP mediated signalling pathways.....	120
4.3.6	Subsequent inhibition of BMP and FGF signalling.....	122
4.3.7	Conclusions.....	122
Chapter 5. Characterising TERA2.sp12 differentiation <i>in vivo</i>		124
5.1	Introduction	125
5.2	Results	127
5.3	Discussion	134
5.3.1	Summary	134
5.3.2	Histological analysis of TERA2.sp12 derived tumours.....	134
5.3.3	Immuno-staining analysis of TERA2.sp12 derived tumours.....	135
5.3.4	A restricted differentiation potential for TERA2.sp12 cells?	138

5.3.5	Conclusion	140
Chapter 6. General Discussion.....		142
6.1	Summary of the findings from this study	143
6.2	Discussion	145
6.2.1	The effects of dissociation and aggregate formation	145
6.2.2	Investigating the default model of neural induction in humans	146
6.2.3	Maintenance of the stem cell phenotype.....	148
6.2.4	Manipulation of the BMP and FGF mediated signalling pathways.....	149
6.2.5	Working with TERA2.sp12 cells – a useful experimental tool in which to study human development?.....	151
6.2.6	The future of embryonic stem cell research.....	153
6.2.7	Conclusions.....	155
Appendix.....		156
Bibliography		160

LIST OF FIGURES

Chapter 1

- 1.1 Scheme of early development depicting the relationship between early cell populations and the primary germ layers.
- 1.2 Outline of the events leading to the formation of the blastocyst with respect to Oct4 expression.
- 1.3 Schematic representation of a common view of germ cell tumour histogenesis and its parallel with normal development.
- 1.4 Schematic outline of the relationship between mouse teratocarcinoma stem cells and cells of the inner cell mass in a developing blastocyst.
- 1.5 Schematic outline of the method of immuno-magnetic isolation of the clonal cell line TERA2.sp12 from an early TERA2 culture.
- 1.6 Gene expression patterns in developing CNS neurons of the vertebrate embryo and in NTERA2 cells treated with RA.
- 1.7 Schematic depicting the formation of extra-embryonic and embryonic tissues in the murine embryo.

Chapter 2

- 2.1 Outline of well plate plan for flow cytometry showing locations for each antibody tested.

Chapter 3

– Introduction –

- 3.1 Schematic outline of the retinoic acid pathway.
- 3.2 Outline of the two alternate culture methods for generating retinoic acid induced neural differentiation in TERA2.sp12 cells.
- 3.3 Strategy flow chart for investigating the differentiation of retinoic acid treated TERA2.sp12 cells under adherent versus non-adherent conditions.

– Results –

- 3.4 Changes in morphology of adherent cultures of TERA2.sp12 cells treated with retinoic acid.
- 3.5 Aggregate formation of TERA2.sp12 cells cultured under suspension conditions and treated with or without retinoic acid.

- 3.6 Cell count assay of adherent or suspension cultures of TERA2.sp12 cells treated over 14 days with or without retinoic acid.
- 3.7 Flow cytometry comparison of surface antigen regulation in TERA2.sp12 cells treated with retinoic acid under either adherent or suspension conditions.
- 3.8 Western blot detection of neural and non-neural ectoderm proteins in retinoic acid treated adherent or suspension cultures of TERA2.sp12 cells.
- 3.9 Western blot comparison of Keratin 8 and β III tubulin protein expression in RA treated adherent or suspension cultures of TERA2.sp12 cells.
- 3.10 Immuno-staining profile of TERA2.sp12 EC stem cells
- 3.11 Neural differentiation in retinoic acid treated adherent cultures of TERA2.sp12 cells.
- 3.12 Zones of proliferation in 21 day retinoic acid treated adherent cultures of TERA2.sp12 cells.
- 3.13 NF200 and MAP2 immuno-positive aggregate-like structures in 21 day retinoic acid treated adherent cultures of TERA2.sp12.
- 3.14 A distinctive sub-population of retinoic acid treated adherent TERA2.sp12 cells express markers of non-neural ectoderm.
- 3.15 TERA2.sp12 aggregate formation assay.
- 3.16 Histology of sectioned retinoic acid treated aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.17 Down-regulation of stem cell marker expression and proliferation within retinoic acid treated aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.18 Formation of neural tube-like structures within retinoic acid treated aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.19 Regional staining for neurofilaments and MAP2 within retinoic acid treated aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.20 Histology of sectioned aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.21 Pattern of stem cell marker down-regulation within aggregates formed from suspension culture of TERA2.sp12 cells.
- 3.22 Expression of mesoderm, endoderm and ectoderm markers within aggregates formed from suspension culture of TERA2.sp12 cells.

Chapter 4

– Introduction –

- 4.1 Schematic outline of key dissociation experiments and the default neural induction model in *Xenopus* embryos.
- 4.2 Summary of the TGF β signalling pathway.

- 4.3 Outline of the actions of negative regulatory proteins on BMP pathway activity.
- 4.4 Schematic depicting FGF mediated inhibition of BMP pathway activity via ERK1/2 phosphorylation of receptor smad 1.
- 4.5 Strategy chart for Section A: Investigating the role of BMP or FGF mediated pathways in RA induced differentiation of TERA2.sp12 cells under adherent or non-adherent conditions.
- 4.6 Strategy chart for Section B: Investigating the effect of manipulations in BMP and FGF signalling on TERA2.sp12 cells in the absence of RA.

– Results –

- 4.7 PCR detection of Transcripts regulated by BMP signalling in TERA2.sp12 cells treated with RA under either suspension or adherent culture conditions.
- 4.8 Western blot detection of proteins regulated by BMP signalling in TERA2.sp12 cells treated with RA under either suspension or adherent culture conditions.
- 4.9 Changes in morphology of TERA2.sp12 cells cultured under adherent or suspension conditions and treated with RA or additionally with BMP2 or Noggin.
- 4.10 Western blot analysis presenting the effect of BMP2 or Noggin on neural and non-neural ectoderm marker expression in TERA2.sp12 cells treated with RA.
- 4.11 Western blot comparison of neural and non-neural ectoderm protein expression in TERA2.sp12 cells pulsed with SU5402 and cultured under adherent or suspension conditions.
- 4.12 Monitoring MAPK activity in cultures of TERA2.sp12 cells pulsed with the FGF inhibitor SU5402 and cultured under adherent or suspension conditions.
- 4.13 Changes in Morphology of TERA2.sp12 cells treated with RA, BMP2, BMP4 or Noggin.
- 4.14 Histogram depicting mean cell number over 14 days of TERA2.sp12 cells cultured with RA, BMP2, BMP4 or noggin.
- 4.15 Down regulation of stem cell surface marker expression in TERA2.sp12 cells treated with RA, BMP2, BMP4 or noggin.
- 4.16 Western blot detection of neural and non-neural ectoderm proteins in cultures of TERA2.sp12 cells treated with RA, BMP2, BMP4 or noggin.
- 4.17 Neuronal β III tubulin and pan-epithelial keratin immuno-staining of large cells in BMP4 treated cultures of TERA2.sp12 cells.
- 4.18 Nestin and β III tubulin expression in noggin treated cultures of TERA2.sp12 cells.
- 4.19 Developmental regulation of neural, non-neural ectoderm, trophoblast and smooth muscle markers in TERA2.sp12 cells treated with BMP2, BMP4 or the FGF receptor inhibitor SU5402.

- 4.20 Trophoblast marker expression in TERA2.sp12 cells treated with an inhibitor of FGF signalling.
- 4.21 Positive expression of Gata4, HCG and α smooth muscle actin in BMP treated cultures of TERA2.sp12 cells.
- 4.22 Expression of the trophoblast hormone HCG in 17 day BMP treated cultures that were cultured in the last 3 days with or without Insulin.
- 4.23 Regulation of BMP and FGF mediated signalling in cultures treated for 14 days with RA, BMP2, BMP4 or the FGF receptor inhibitor SU5402.
- 4.24 Neural versus non-neural differentiation in TERA2.sp12 cells after sequential inhibition of BMP signalling and FGF mediated signalling.

– Discussion –

- 4.25 Proposed model for the action of BMP and FGF signalling on the differentiation of TERA2.sp12 cells.

Chapter 5

– Introduction –

- 5.1 Flow chart presenting a summary of the aims, objectives and experiments in this chapter.

– Results –

- 5.2 Neuronal and glial differentiation in TERA2.sp12 derived tumours.
- 5.3 Non-neural ectoderm differentiation in TERA2.sp12 derived tumours.
- 5.4 Expression of the endoderm transcription factor HNF3 β in TERA2.sp12 derived tumours.
- 5.5 Expression of the endoderm transcription factor Gata4 in TERA2.sp12 derived tumours.
- 5.6 Detection of Human nuclear antigen within TERA2.sp12 derived tumours.
- 5.7 Detection of a stem cell population within TERA2.sp12 derived tumours.
- 5.8 Varied structures and expression of multiple markers of differentiation in sections of a tumour derived from human embryonic stem cell line HNCL-1.

Chapter 6

- 6.1 Flow chart summarising the main routes of investigation in this study.
- 6.2 Schematic outline of the pathways reported to be involved in the maintenance of the pluripotent stem cell phenotype in human embryonic stem cells.

LIST OF TABLES

Chapter 2

- 2.1** Antibodies utilised in flow cytometry, listing antigen, antibody sub-type, source and dilution factor.
- 2.2** Details of primary and secondary antibodies used in Western Blot analyses.
- 2.3** Details of primary antibodies used in immuno-staining analyses.
- 2.4** Details of secondary antibodies used in immuno-staining analyses.

Chapter 3

- 3.1** List of genes with functional RAREs (adapted from Cytokine & Growth Factor Reviews 11, 2000, Table 1)
- 3.2** Mean number of cells (millions) in adherent or suspension cultures of TERA2.sp12 cells treated with (+RA) or with-out over 14 days.
- 3.3** Combinations of antibodies used to further characterise neural and non-neural ectoderm populations of RA treated TERA2.sp12 cells.
- 3.4** Summary of combinations of individualised TERA2.sp12.GFP and TERA2.sp12 cells seeded into bacteriological dishes.
- 3.5** Details of antibodies used in immuno-staining screen for markers of differentiation
- 3.6** Summary comparison of RA induced differentiation in TERA2.sp12 cells cultured under adherent versus non-adherent conditions.
- 3.7** List of typical characteristics of embryoid bodies derived from the human ES cell line H9 and TERA2.sp12 aggregates formed under similar suspension conditions.

Chapter 4

- 4.1** Details of primers used for PCR analysis.
- 4.2** List of antibodies used in Western Blot analysis of samples from TERA2.sp12 cells treated for 14 days with BMP2, BMP4, SU5402 or RA.
- 4.3** Details of the antibodies and antibody combinations used in multiple immuno-staining and double immuno-staining experiments.
- 4.4** Details of antibodies used in Western Blot analyses to monitor BMP and FGF regulated pathway activity.
- 4.5** Mean number of cells (millions) at 0, 7 and 14 days in cultures of TERA2.sp12 cells seeded at 20 000 cells/cm² and treated with RA, BMP2, BMP4 or noggin.
- 4.6** Scored levels of positive immuno-staining for a range of antibodies in TERA2.sp12 cells treated with BMP2, BMP4, SU5402 or vehicle treated for 14 days.

-
- 4.7** Scored levels of immuno-positivity for trophoblast markers in TERA2.sp12 cells treated with BMP2, BMP4 or SU5402 for 14 days (Treatment 1), prior to continued treatment with or without the addition of Insulin or Forskolin for 3 days (Treatment 2).
- 4.8** Comparison of the materials and methods used in studies by Xu *et al.* and Pera *et al.* with this study, in which BMP 2 or 4 treatment induces either trophoectoderm or extra embryonic endoderm. (Italicised markers are also expressed in other germ layers)

Chapter 5

- 5.1** Details of tumours derived from sub-cutaneous TERA2.sp12 injection into nude mice.
- 5.2** Details of antibodies used in immuno-labelling screen for markers of differentiation.

CHAPTER 1
INTRODUCTION



1.1 STEM CELLS – AN OVERVIEW

Over recent years the rapidly expanding field of stem cell research has gained prominence in both scientific and non-scientific circles, reflecting advances towards the much heralded potential of stem cells as sources for transplantation therapies (Lovell-Badge 2001). A stem cell is classically defined as a cell that has the ability to choose between prolonged self-renewal and differentiation, and under this definition populations exist in both the adult and in the embryo. In adult mammalian tissues such as in epithelia and blood, stem cells function to replace cells lost from normal cellular senescence or damage. Previously unidentified small populations of stem cells have also been characterised in other organs such as the brain (Reynolds & Weiss 1992a), for review (Frisen *et al.* 1998). However, these cells are difficult to isolate and typically undergo limited cellular regeneration or turnover (Andrews 2002). Such adult stem cell populations are defined as *multipotent* – capable of differentiating into a restricted range of cell types (typically within a specific germ layer). Although recent reports indicate that adult stem cell populations may have more plasticity than originally thought (Brazelton *et al.* 2000, Mezey *et al.* 2000, Panchision *et al.* 1998), they typically form a limited number of cell types. In contrast, stem cells of the early mammalian embryo, as the progenitors of the entire range of adult tissues, are termed *pluripotent* – because they retain the remarkable potential to differentiate into any cell type of the body (Smith 2001). An outline of the early germ layers and derivatives in relation to pluripotent embryonic stem cells and multipotent adult stem cells is depicted in Figure 1.1.

A key difference between adult multipotent stem cells and embryonic pluripotent stem cells is manifested in the transient nature of pluripotent stem cells in the embryo. Under steady state conditions adult stem cells typically undergo *asymmetric cell division*, in which a stem cell produces one daughter cell with a stem cell fate and another daughter cell with a differentiated fate, maintaining a stable and self-renewing source of differentiated cells throughout life. In contrast, the pluripotent stem cells in the inner cell mass of a developing blastocyst and early progenitors in embryonic development undergo *symmetric cell division* to generate identical daughter stem cells, effectively and rapidly increasing the size of the stem cell population (for review see Morrison & Kimble 2006). However, these stem cell populations exist only transiently, becoming progressively restricted in their differentiation potential during embryonic development. Pluripotent stem cells in the inner cell mass of the blastocyst promptly differentiate into cells of the primitive ectoderm, which in turn differentiate during gastrulation into cells with increasingly restricted differentiation potentials to form the three embryonic germ layers. This loss of pluripotency is reflected in the

Scheme Of Early Development Depicting Relationship Between Early Cell Populations, The Primary Germ Layers and pluripotent versus multipotent stem cells

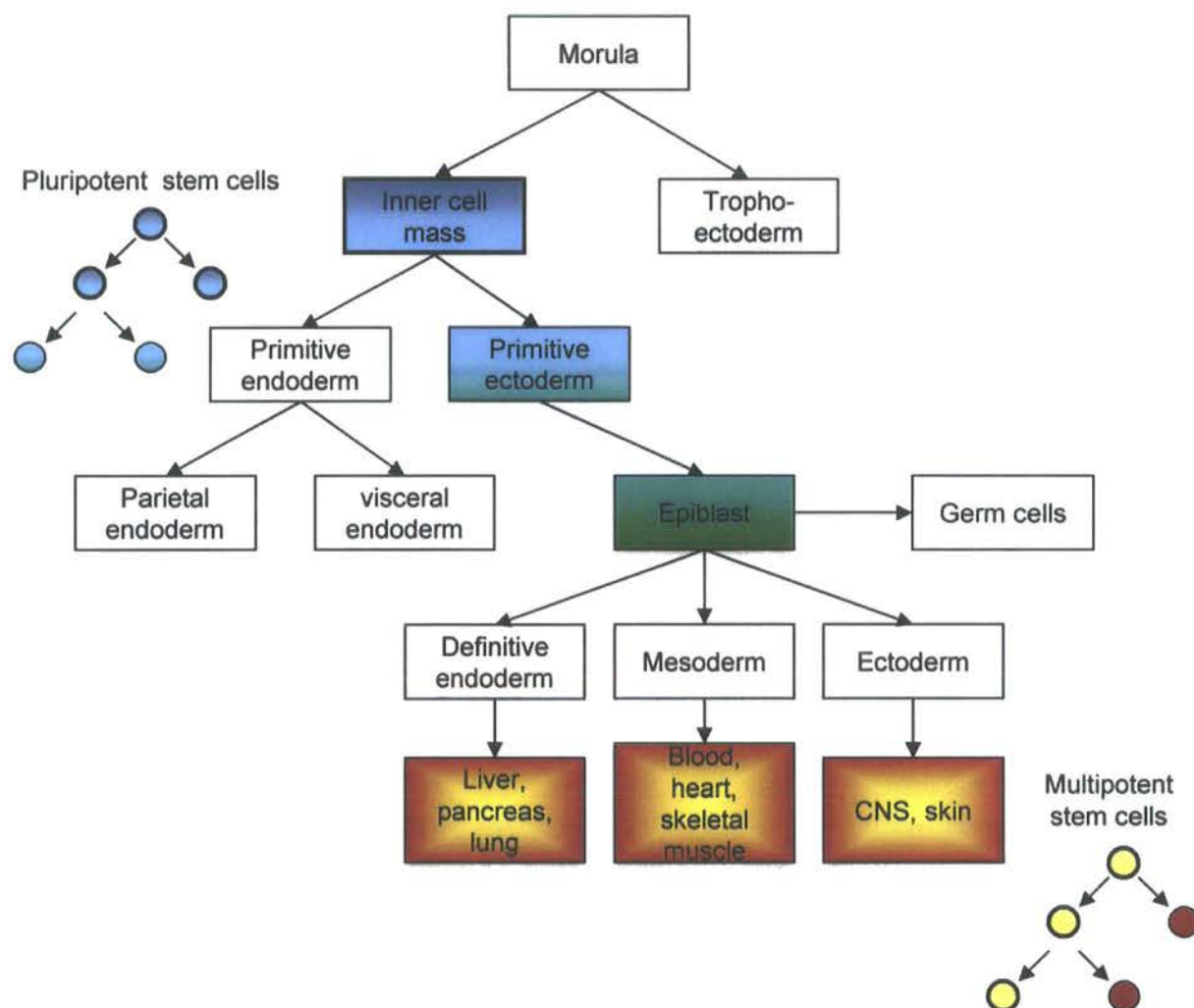


Figure 1.1. Scheme of early development in the mouse, depicting relationship between early cell populations and germ layers. The inner cell mass of the developing blastocyst contains a population of pluripotent stem cells, which have the potential to form derivatives of all germ layers and proliferate by dividing asymmetrically to produce identical daughter cells. This pluripotent population rapidly differentiates into the primitive ectoderm, which in turn will generate the epiblast. The epiblast which will generate all the tissues of the body, including germ cells and the three germ layers – definitive endoderm, mesoderm and ectoderm. Multipotent populations reside in the developing germ layer tissues, which under steady state conditions divide asymmetrically to generate both an identical daughter stem cell and a differentiated precursor cell. In the adult, stem cell populations function to replace differentiated cells lost during injury or normal senescence. Flow chart schematic of germ layers adapted from Keller et al. 2005.

expression of the pluripotency associated transcription factor Octamer binding protein 4 (Oct4), which is first expressed in the developing oocyte and early cleavage embryos, before becoming restricted to the cells of the inner cell mass, such that after gastrulation it is solely expressed by the primordial germ cells, which maintain Oct4 expression until the initiation of sexual differentiation of the gametes (Palmieri *et al.* 1994, for review see Scholer & Pesce 2001). An outline of the events leading to the formation of the murine blastocyst and the corresponding expression pattern of Oct4 is presented in Figure 1.2.

In contrast to the transient nature of pluripotent stem cells in embryonic development, their counterparts *in vitro* – either in the malignant form of embryonal carcinoma cells or as feeder dependent embryonic stem cells – can be maintained under specific culture conditions to self renew through symmetric division, retaining their pluripotency and Oct4 expression (for reviews see Chambers & Smith 2004, Andrews 2002, Scholer & Pesce 2001). As cultured pluripotent embryonic stem cells are capable of forming virtually any cell type, they have been viewed with much excitement as potential sources of cell therapy: In theory, muscle cells could be produced to treat muscular dystrophies and heart disease, insulin producing pancreatic cells could be generated to treat diabetes, haemopoietic cells could be produced to treat leukaemia and there are promising reports in these fields (Donovan & Gearhart 2001, Klug *et al.* 1996, Soria *et al.* 2000). Much research has been directed towards the use of embryonic stem cells as potential sources for neural tissue transplants, partly because of the current limited regenerative potential in traumatised brain tissue, and as a potential therapy for neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. In this respect there are some remarkable demonstrations of the therapeutic potential of embryonic stem cell derivatives in mouse models: Mouse embryonic cell derived glial precursors transplanted into a rat with myelin disease have been demonstrated to interact with host neurons to produce myelin in the brain and spinal cord (Brustle *et al.* 1999), and McDonald *et al.* (1999) report that RA treated mouse embryonic stem cells transplanted into a rat spinal cord after traumatic injury differentiated into astrocytes, oligodendrocytes and neurons, promoting motor recovery. More recently, differentiated dopamine-producing neurons were found to incorporate into the striatum and improve performance in behavioural tests after being grafted into mice that phenotypically model Parkinson's disease (Kim *et al.* 2002, Nishimura *et al.* 2003). Although their potential as sources of cells for transplantation therapies has been the focus of public interest, there is a wide range of potential applications of embryonic stem cells. This includes their use as sources of large amounts of human tissue for drug testing and development, and importantly human ES cells also provide a human experimental model in which to study early development and aspects of embryogenesis in humans (for review Pera & Trounson 2004).

Outline Of Events Leading To The Formation Of The Mouse Blastocyst With Respect To Oct4 Expression Levels.

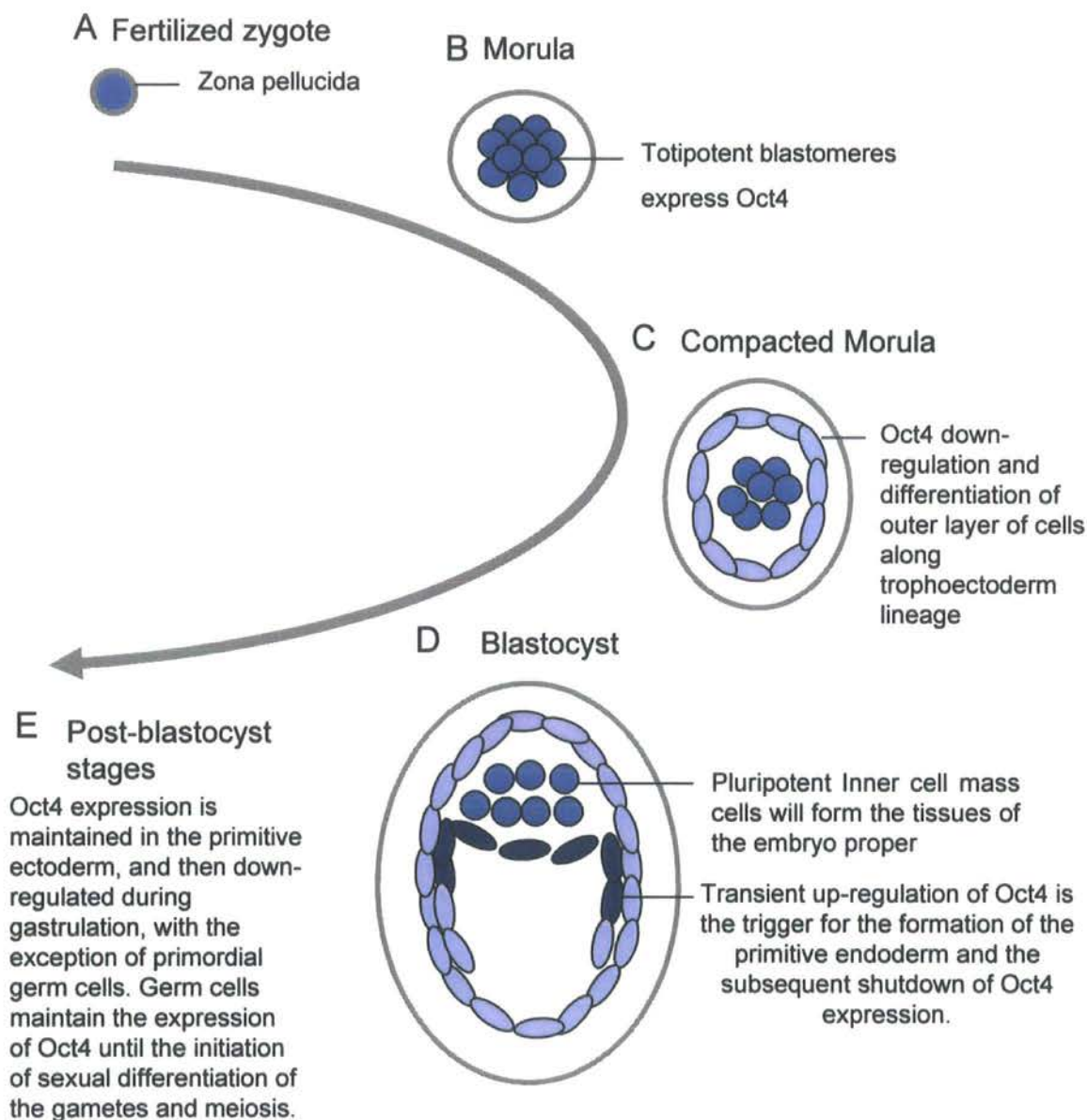


Figure 1.2. Outline of events leading to the formation of the mouse blastocyst with respect to Oct4 expression levels. A. The fertilized zygote expresses Oct4 (blue), and this is maintained in the blastomeres prior to compaction (A,B). The formation of trophoblast tissue in the compacted morula is accompanied by a down-regulation of Oct4 in the outer cells (pale blue). Differentiation of the primitive endoderm in the blastocyst is preceded by a transient up-regulation of Oct4 (dark blue) followed by a subsequent shutdown in Oct4 expression. The primitive endoderm and trophoectoderm lineages will contribute towards the formation of the placenta, whereas the remaining Oct4 positive cells of the inner cell mass will form the tissues of the embryo proper, including the three germ layers (definitive endoderm, ectoderm and mesoderm) and the germ line. Oct4 is down-regulated in these cells during gastrulation such that Oct4 expression becomes restricted to the primordial germ cells, which maintain Oct4 expression until the initiation of sexual differentiation of the gametes. Figure adapted from Figure 1 in Scholer & Pesce 2001.

The first isolation of human embryonic stem cells by Thompson *et al.* (1998) marked a significant advance in human embryonic stem cell research and was consequently accorded with public acclaim. However, this important step was founded on decades of study that began with the characterisation of a class of tumours – the teratomas.

1.2 FROM TERATOMAS TO EMBRYONIC STEM CELLS

The term teratoma is coined from a greek name, roughly translating as ‘monster tumour’, which reflects the properties of these tumours that have fascinated historians, clinicians and biologists since their first observation hundreds of years ago (Andrews *et al.* 2001). Teratomas and teratocarcinomas occur in a range of manifestations and comprise of a variety of differentiated tissues – mimicking normal development but in a grossly disorganised context, such that they present caricatures of embryogenesis. Teratomas typically arise in the gonads as a form of germ cell tumour (Andrews *et al.* 2001), although they are also rarely detected in other sites such as in the spinal chord in adults (Sloof *et al.* 1964) or at the base of the spine in infants (reviewed in Isaacs 2004). In these cases it has been hypothesised that these tumours are generated from multipotential germinal cells that are misplaced in early embryonic development (Bucy *et al.* 1935). The relative proportion of teratomas among the germ cell tumours is quite different in the male and female gonads, with about 95% of ovarian germ cell tumours represented by pure teratoma (Katsube *et al.* 1979, Koonings *et al.* 1989) but only about 4% of testicular germ cell tumours (von Hochstetter & Hedinger 1982). The histopathology and classification of germ cell tumours is complex and controversial, however a simple schematic representation of a commonly held view of germ cell tumour histogenesis in parallel with normal development is presented in Figure 1.3.

The most common teratomas are ovarian dermoid cysts, which form from oocytes that are parthenogenetically activated and begin development, but eventually become disorganised to form a mass of embryonic tissue as depicted in plate A of Figure 1.3. These tumours are generally benign, although they can grow to very large sizes. In contrast, germ cell tumours in the adult male account for almost all testicular cancers and are always malignant, demonstrating a peak incidence in 30-40 year olds (Moller 1993). Testicular germ cell tumours appear to arise from abnormal gonocytes within the seminiferous tubules and as shown in Figure 1.3, invasive testicular germ-cell tumours undergo an initial carcinoma in situ phase (Giwerzman *et al.* 1991, Jacobsen *et al.* 1981, Daugaard *et al.* 1987), lasting for a median period of approximately five years (Skakkebaek *et al.* 1982). Testicular germ cell tumours are categorised as either seminomas (which comprise 35% to 50% of all germ cell tumours), or non-seminomas (Damajanov 1993). Seminomas consist of relatively uniform cells that resemble primordial germ cells, whereas as depicted in plate B of Figure 1.3, non-

Schematic Representation Of A Common View Of Germ Cell Tumour Histogenesis And Its Parallel With Normal Development.

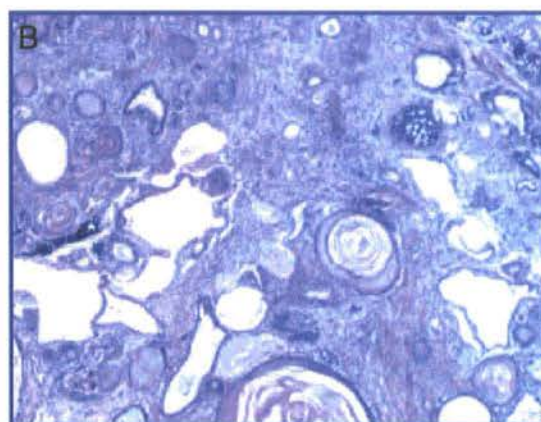
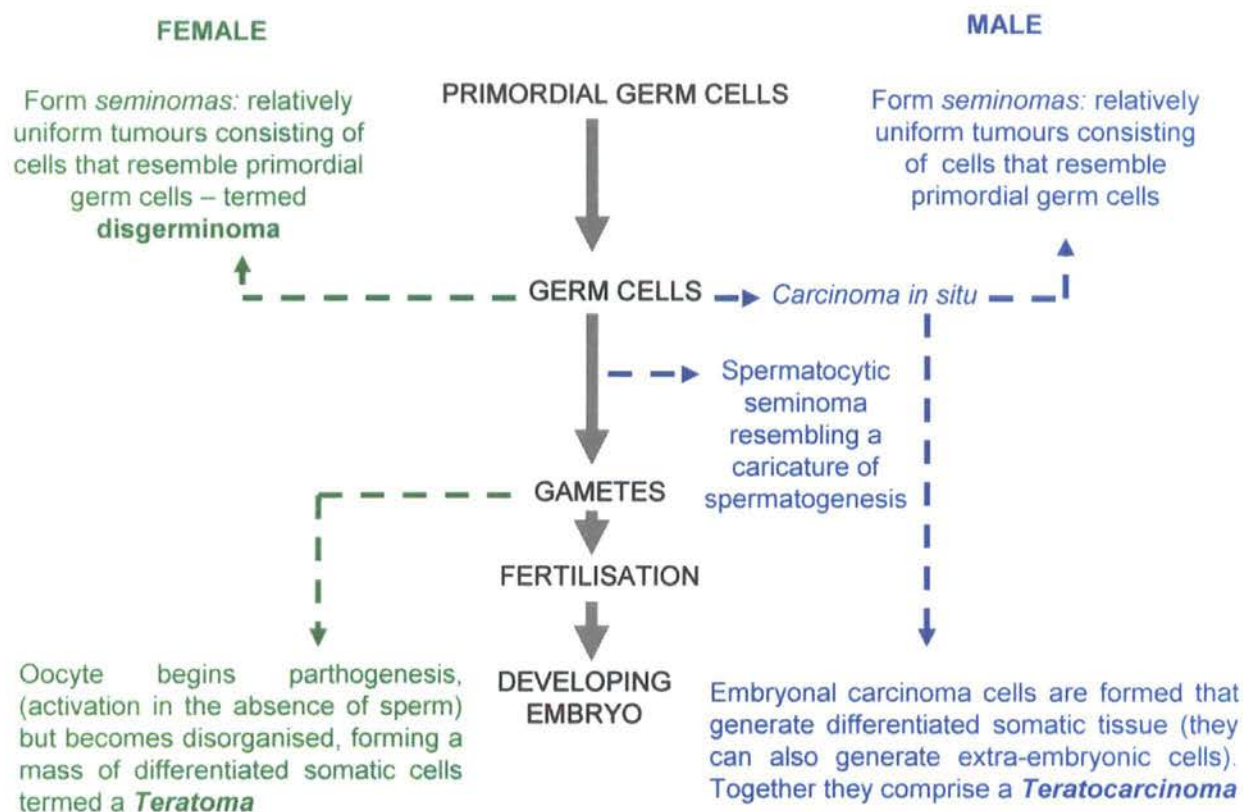


Figure 1.3. Schematic representation of a common view of germ cell tumour histogenesis and its parallel with normal development. The solid arrows represent the progression of events in normal development, whereas the dashed arrows represent the transitions towards tumour formation. In females the most common form of teratoma is generated when an oocyte begins parthogenesis but becomes disorganised, forming a teratoma or dermoid cyst of the ovary. Plate A depicts an example of a mature human teratoma derived from a dermoid cyst of the ovary, showing extensive somatic differentiation including hair and tooth. A malignant germ cell tumour resembling a seminoma can also occur, termed Dysgerminoma, in which the tumour cells resemble primordial germ cells. In the male, testicular germ cell tumours are always malignant, and can occur in the form of seminomas, in which the tumour component resembles primordial germ cells, or in the form of teratocarcinomas. Teratocarcinomas are formed from embryonal carcinoma cells, the stem cell component of this tumour, which generate differentiated somatic tissues (teratoma component), and also can generate extraembryonic cells (yolk sac carcinoma or trophoblast). Plate B depicts a histologically stained section through a well-differentiated mouse teratocarcinoma, note extensive differentiation including keratin pearls and cartilage. In rare cases spermatocytic seminomas occur, a low malignancy tumour which resembles a caricature of spermatogenesis. Figure adapted from Figure 1 in Andrews (2002) and Figure 2 in Andrews et al. (2001).

seminoma germ cell tumours are histologically heterogeneous and frequently contain somatic tissues such as nerve, bone, tooth and muscle (termed the teratoma component), although they can also contain elements of seminoma (Ulbright, 1993, Bosl & Motzer 1997). They also contain histologically undifferentiated cells, termed embryonal carcinoma (EC) cells, which are the key malignant component of these tumours (Damjanov 1990, 1993). The term teratocarcinoma is used to refer to germ cell tumours containing both embryonal carcinoma and teratoma components. Other elements of non-seminoma germ cell tumours include highly malignant cells corresponding to extra embryonic tissues such as the yolk sac (yolk sac carcinoma) and trophoblast (choriocarcinoma), (Ulbright 1993, Mostofi & Sesterhenn 1997).

The first experimental studies of teratomas began with the observation that in the mouse 129 strain teratomas occurred spontaneously in the testes of approximately 1% of males (Stevens & Little 1954). These teratomas could be observed by 15 days of embryonic development, as structures described as embryoid bodies within the seminiferous tubules of the fetal gonad. Stevens (1964) subsequently demonstrated that the incidence of these tumours could be increased by transplanting the genital ridges of early embryos to the testis capsule of adult mice, and furthermore demonstrated that teratomas could successfully be produced using genital ridges transplanted from the embryos of another mouse strain (1970b). Some of these tumours were clearly malignant teratocarcinomas, and could be re-transplanted to successive hosts (Stevens & Hummel 1957). The histologically undifferentiated cells within these tumours – EC cells, had long been hypothesised to comprise the stem cell component of the tumour, acting as the progenitors of all the cell types observed within the teratocarcinoma. This was confirmed by Kliensmith and Pierce (1964), who demonstrated that single EC cells transferred to a new host could reform complex teratocarcinomas with the full range of differentiated derivatives observed in the parental tumour, that could again be re-transplanted to another host (for reviews Andrews 2002, Andrews *et al.* 2001).

1.2.1 Murine embryonal carcinoma cell lines and their relationship to cells of the early embryo

Although testicular tumours could only be induced in a limited number of strains, teratomas could be formed from many strains of mice if early embryos (notably at about 7 days of development) were transplanted to ectopic sites (Solter *et al.* 1970, Stevens 1970a). As in ovarian parthenogenetic tumours, these embryos became disorganised and formed teratomas, or in some cases, retransplantable teratocarcinomas containing EC cells, strengthening a long held hypothesis that EC cells resembled stem cells of the early embryo (for review Andrews *et al.* 2001).

Murine embryonal carcinoma cell lines were first established in culture during the 1970's, allowing these stem cells to be extensively characterised (Evans 1972, Kahn & Ephrussi 1970, Martin & Evans 1974, Nicolas *et al.* 1975). Many of these lines remained pluripotent *in vitro*, exhibiting undifferentiated characteristics and retaining the key ability to form teratocarcinomas when transplanted back into an appropriate mouse host. Numerous murine EC cell lines could also differentiate in culture, although different cell lines required particular culture conditions to do so: Some cells differentiated spontaneously if maintained as confluent cultures for several days (Nicolas *et al.* 1975), whereas others required feeder layers of transformed mouse fibroblasts to maintain an EC phenotype and would differentiate in response to removal from the feeder layer (Martin & Evans 1974). Under suspension conditions these cells frequently formed floating highly differentiated aggregations of cells termed 'embryoid bodies' (Martin & Evans 1975).

On the basis that EC cells resembled early cells of the embryo, antibodies were generated from immunising adult mice of the 129 strain with mouse EC cells (of 129 strain origin) in order to identify specific embryonic cell surface antigens (Artzt *et al.* 1973). To this effect an antigen detected by Anti-F9 sera was demonstrated to be present on EC cells and absent on a range of differentiated cells. Significantly, anti-F9 sera and a subsequently derived monoclonal antibody to stage specific embryonic (SSEA1) antigen were demonstrated to be expressed on both murine EC cells and by the embryonic cells of the blastocyst inner cell mass (ICM), (Artzt 1973, Solter & Knowles 1978). Taken together with a similar expression of other markers such as alkaline phosphatase and their comparable capacity for differentiation, this led to widespread acceptance that EC cells were a malignant counterpart of ICM embryonic cells (Andrews 2001). Further evidence for the close relationship of murine EC cells to the ICM was generated when implanted EC cells were demonstrated to contribute to almost all tissues of the host embryo, and moreover, their malignant character was suppressed (Papaioannou *et al.* 1975), although suppression of malignancy was not always complete (Papaioannou & Rossant 1983). Figure 1.4 depicts an outline of the experimental similarities between murine EC cells and embryonic cells of the blastocyst ICM.

1.2.2 Human embryonal carcinoma cell lines

Human embryonal carcinoma cell lines have also been established *in vitro*. Unlike murine EC cell lines, many human EC cell lines exhibit little capacity for differentiation (termed *nullipotent*), with the exception of a sub-group of human EC cell lines. This may be manifested by the fact that unlike murine EC cells, human EC cells are highly aneuploid (for review Andrews 2001). Cell lines capable of differentiation include GCT27 – a cell line that requires maintenance on fibroblast feeder layers and differentiates upon removal from feeders

Schematic Outline Of The Relationships Between Teratocarcinoma Stem Cells And Cells In The Developing Blastocyst

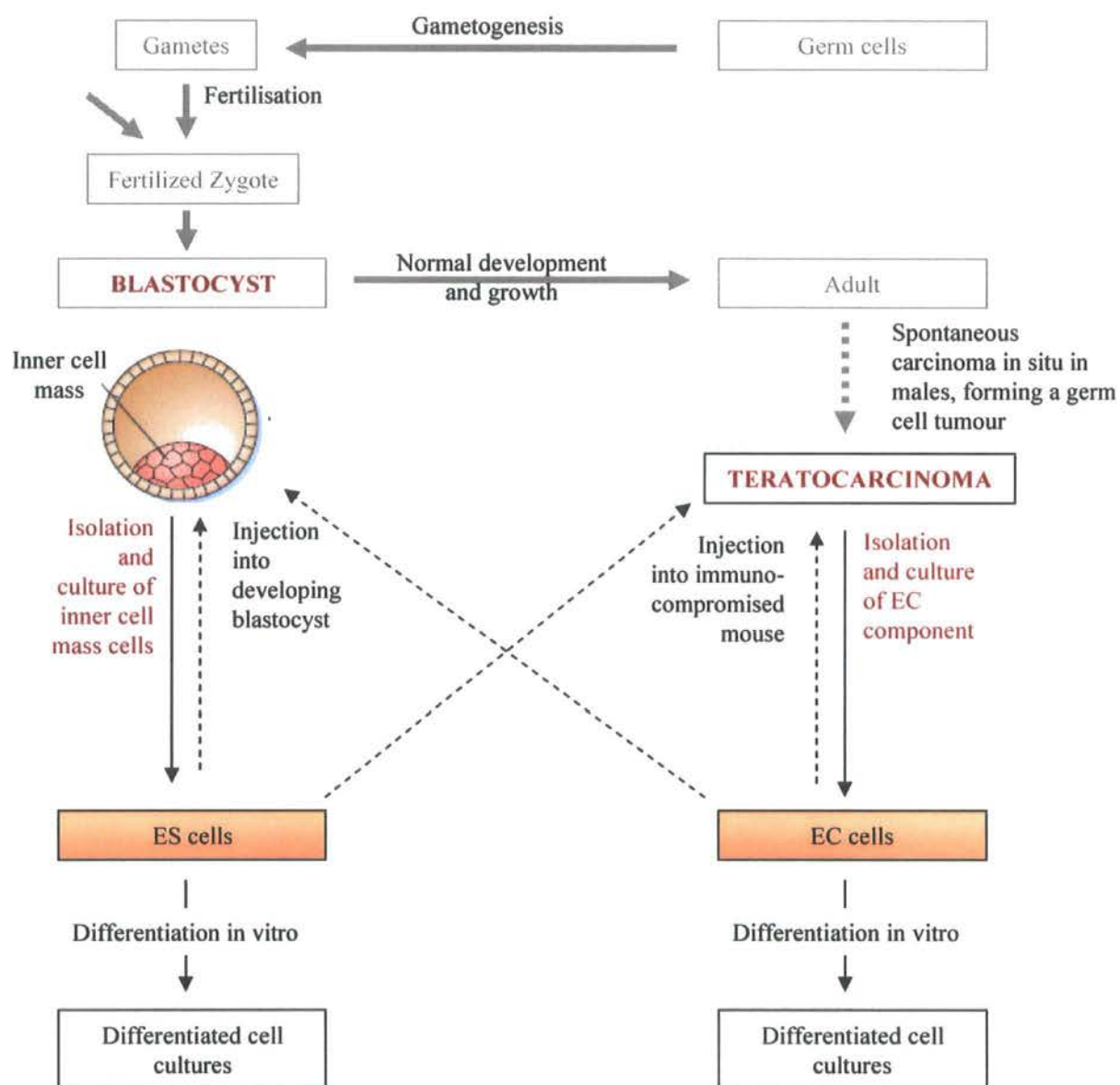


Figure 1.4. Outline of the relationships between teratocarcinoma stem cells and cells in the developing blastocyst of the mouse. A fertilized zygote will form a blastocyst consisting of an outer layer of cells that form trophoectoderm derivatives and an inner cell mass that will generate all the tissues of the embryo. In rare cases a germ cell tumour will arise in the adult male mouse, which can form a teratocarcinoma. The stem cell or embryonic carcinoma (EC) component of teratocarcinomas can be isolated and cultured in vitro, where they can be differentiated into many cell types. They retain their ability to form teratocarcinomas when injected into immuno-compromised adult mice, and can also contribute to the formation of multiple tissues in normal development (except the germ cells) when injected into a developing blastocyst. Conversely, embryonic stem (ES) cells isolated from the inner cell mass of the developing blastocyst can be differentiated in vitro, and can also form teratomas when injected into an adult immuno-compromised mouse, whereas if they are injected back into a developing blastocyst they contribute to all tissues, including the germ cells of the developing embryo. Adapted from Figure 1 in Thompson et al. (1998) and Figure 2 in Andrews et al. (2001).

(Pera *et al.* 1989, Pera & Herszfeld 1998, Roach *et al.* 1993), NCCIT (Damjanov 1993, Teshima *et al.* 1988) and TERA2 (Andrews *et al.* 1980, Andrews *et al.* 1984b, Fogh & Trempe 1975). All of the human EC cell lines generated were characterised by the expression of a distinct set of surface antigens – the globuloseries glycolipid antigens stage specific embryonic antigen 3 (SSEA3), stage specific embryonic antigen 4 (SSEA4), and the keratan sulphate related antigens TRA 160 and TRA 181, and these were down-regulated during differentiation (Andrews 1984a, 1984b, Andrews *et al.* 1984c, Andrews 1980, Andrews *et al.* 1996, Badcock *et al.* 1999).

One of the most established teratocarcinoma cell lines, the TERA2 cell line, was initially derived by Fogh and Trempe (1975) from a lung metastasis in a patient suffering from testicular teratocarcinoma. However, its identity as an EC cell line and pluripotent nature was not recognised until subsequent characterisation by Andrews *et al.* (Andrews 1980, Andrews 1984b) as the specific culture conditions required to maintain pluripotent undifferentiated human EC cells were previously not fully appreciated. In contrast to many mouse EC cell lines, it is necessary to maintain human EC cells at high densities, and TERA2 cells require passaging by scraping rather than using Trypsin. Cultures of TERA2 are frequently heterogeneous, but contain cells that closely resemble the phenotype of EC cells and can form well differentiated teratocarcinomas containing multiple cell types when grown as xenografts in immuno-suppressed mice (Andrews 1980).

Andrews *et al.* (1984b) subsequently demonstrated the stem cell nature of these cells by the isolation of single cell clones from a robust NTERA2 sub-line, generated from a xenograft tumour of the TERA2 explant culture transplanted into an immune-deficient murine host. These clones such as the widely used sub-line NTERA2.cl.D1 (NT2/D1), expressed characteristics common to other human EC cells, such as the expression of SSEA3, SSEA4, TRA160 and high levels of alkaline phosphatase and produced similar teratocarcinomas to the TERA2 cell line upon transplantation into an immune-deficient murine host (Andrews 1984b). Unlike many other EC cell lines; these TERA2 derived lines showed no evidence of trophoblastic differentiation when cultured at low density, although induction of both SSEA1 and Fibronectin occurred (see review Andrews 2001). In 2001 Przyborski established another clonal EC cell line, TERA2.cl.SP12 (TERA2.sp12), derived directly from the earliest available passage (passage 15) of the TERA2 explant culture using immuno-magnetic sorting (Przyborski 2001). Przyborski selected cells that were immuno-positive for the EC stem cell specific antigen SSEA3, by labelling an individualised culture of TERA2 cells with a primary antibody to SSEA3, followed by a secondary antibody that was covalently attached to magnetic particles. As a consequence, cells exhibiting an EC stem cell phenotype and expressing high levels of SSEA3 were bound by high levels of the anti-SSEA3 antibody, and

upon subsequent binding of the secondary antibody acquired a strong magnetic charge. These SSEA3 positive cells could then be isolated from the remaining cells using a direct magnetic separator, and selected single cells cultured in isolation on fibroblast feeder layers to establish clonal EC colonies, one of which was the TERA2.sp12 cell line. Figure 1.5 outlines the method by which SSEA3 immuno-positive cells were isolated from an early culture of TERA2 cells and clonally propagated to form the TERA2.sp12 EC cell line.

1.2.3 Retinoic acid induced differentiation of EC cells

Initial studies of EC cell differentiation focused on their ability to differentiate spontaneously under a variety of circumstances (as previously described). However, the range of cell types produced and the uncontrolled nature of differentiation made study of the underlying processes difficult. A significant advance was made when certain murine EC cell lines were induced to differentiate by exposure to retinoic acid (RA) along specific lineages: An apparently nullipotent murine EC cell line, F9, was the first EC line discovered to differentiate upon RA exposure, generating cells that closely resembled parietal endoderm (Strickland *et al.* 1980). RA was later shown to be capable of inducing the differentiation of a number of murine EC cell lines (for reviews see Alonso *et al.* 1991, McBurney 1993). In particular, the P19 EC cell line differentiates into predominantly neural cells when exposed to RA (Pfeiffer *et al.* 1981).

Although many human EC cell lines did not respond to RA exposure, a number of lines that do differentiate extensively in response to RA have been described: GCT27 EC cells respond to RA by differentiating into cells resembling extra-embryonic endoderm (Roach *et al.* 1994). In contrast RA treated NTERA2 EC cells differentiate towards a predominantly ectodermal lineage: After exposure to 1-10 μ M retinoic acid, these cells rapidly lose their EC phenotype, extinguishing expression of EC markers such as SSEA3, SSEA4 and TRA 160 over a two week period and expressing markers of neural differentiation (Andrews 1984a, Fenderson *et al.* 1987). The differentiation of NTERA2 cells into neurons appears in many ways to recapitulate the steps and molecular events that occur during neural embryonic development: The expression of the proliferating neural progenitor marker Nestin peaks after 3 days of exposure to RA, followed by a peak in expression of Neuro D1 as neural progenitors exit the cell cycle and embark on neuronal differentiation. Transcripts typical of maturing neurons such as neural specific enolase (NSE) appear after 2 weeks of treatment, corresponding with the observation of morphologically distinct neurons, which comprise 2-5% of the population (Przyborski *et al.* 2000). Figure 1.6 outlines the expression pattern of these genes in developing CNS neurons of the vertebrate embryo, and the corresponding expression pattern of NTERA2 cells after treatment with RA.

Schematic Outline Of The Method Of Immuno-magnetic Isolation Of The Clonal Cell Line TERA2.sp12 From An Early TERA2 Culture (Przyborski 2001).

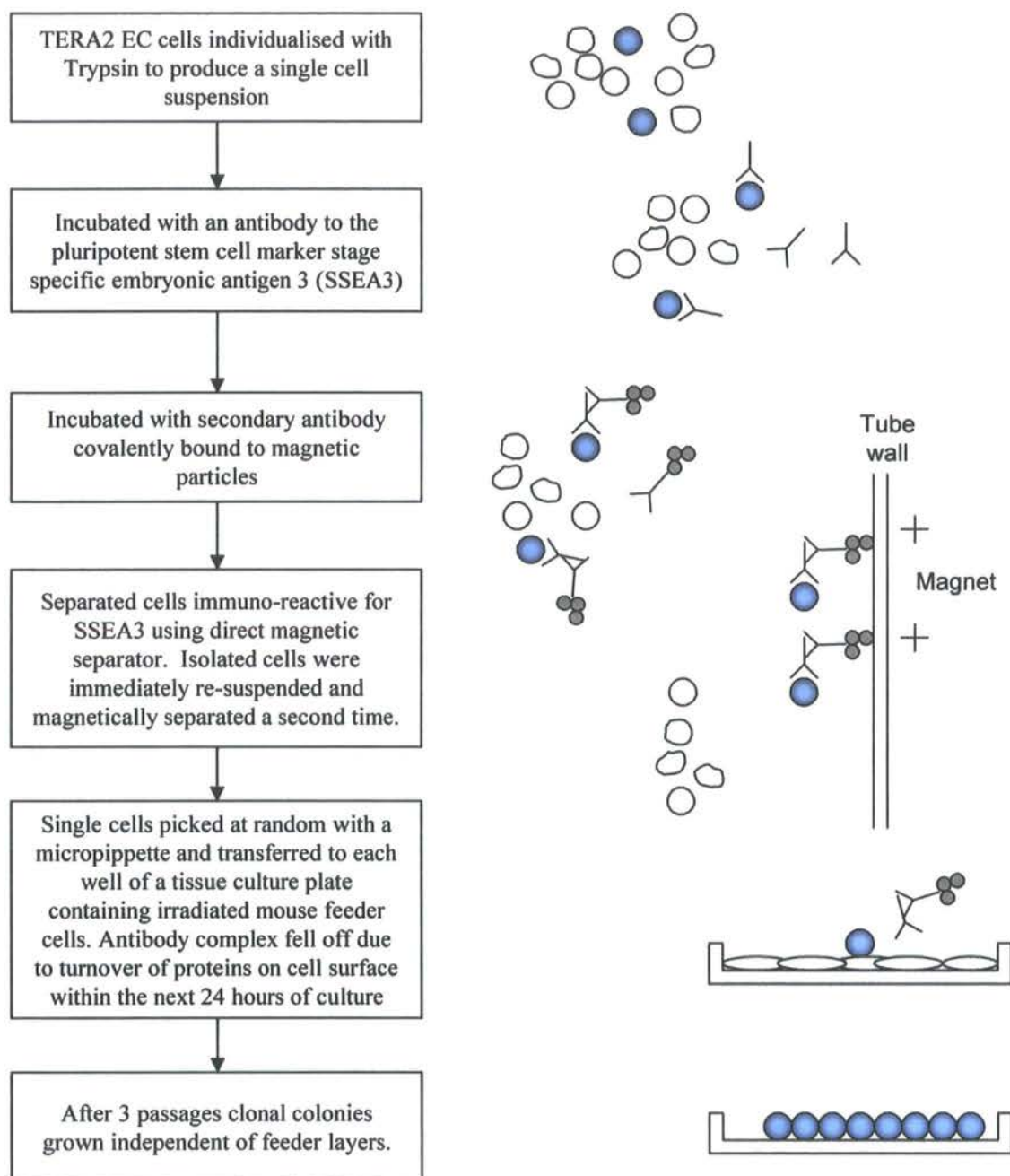


Figure 1.5 Schematic outline of method used for the derivation of the clonal human embryonal carcinoma (EC) cell line TERA2.sp12 from an early culture of TERA2 cells. To isolate cells with an EC phenotype from the heterogeneous population of TERA2 cells, a culture of passage 15 TERA2 cells was dissociated using Trypsin and then incubated with an antibody to the human EC stem cell antigen SSEA3. After subsequent washes in antibody diluent the cells were then incubated with an appropriate secondary antibody that had been covalently bound to magnetic particles. Further washes ensured that SSEA3 expressing EC cells within the TERA2 cultures were the only cells to have acquired a magnetic charge. These cells could then be separated from the remaining solution of cells using a magnetic separator, in which the magnetically charged SSEA3 positive cells would bind to the sides of the tube wall while the remaining cells were flushed away. The SSEA3 positive cells could then be collected and single cells transferred using a micropipette to each well of a tissue culture plate containing irradiated mouse feeder cells. The antibody complexes subsequently fell off the cell surface due to antigen turn over and after 3 passages the single EC cells had established sufficiently large colonies to be cultured in the absence of feeder layers.

Gene Expression Patterns In Developing Central Nervous System Neurons Of The Vertebrate Embryo And In NTERA2 EC Cells Treated With RA

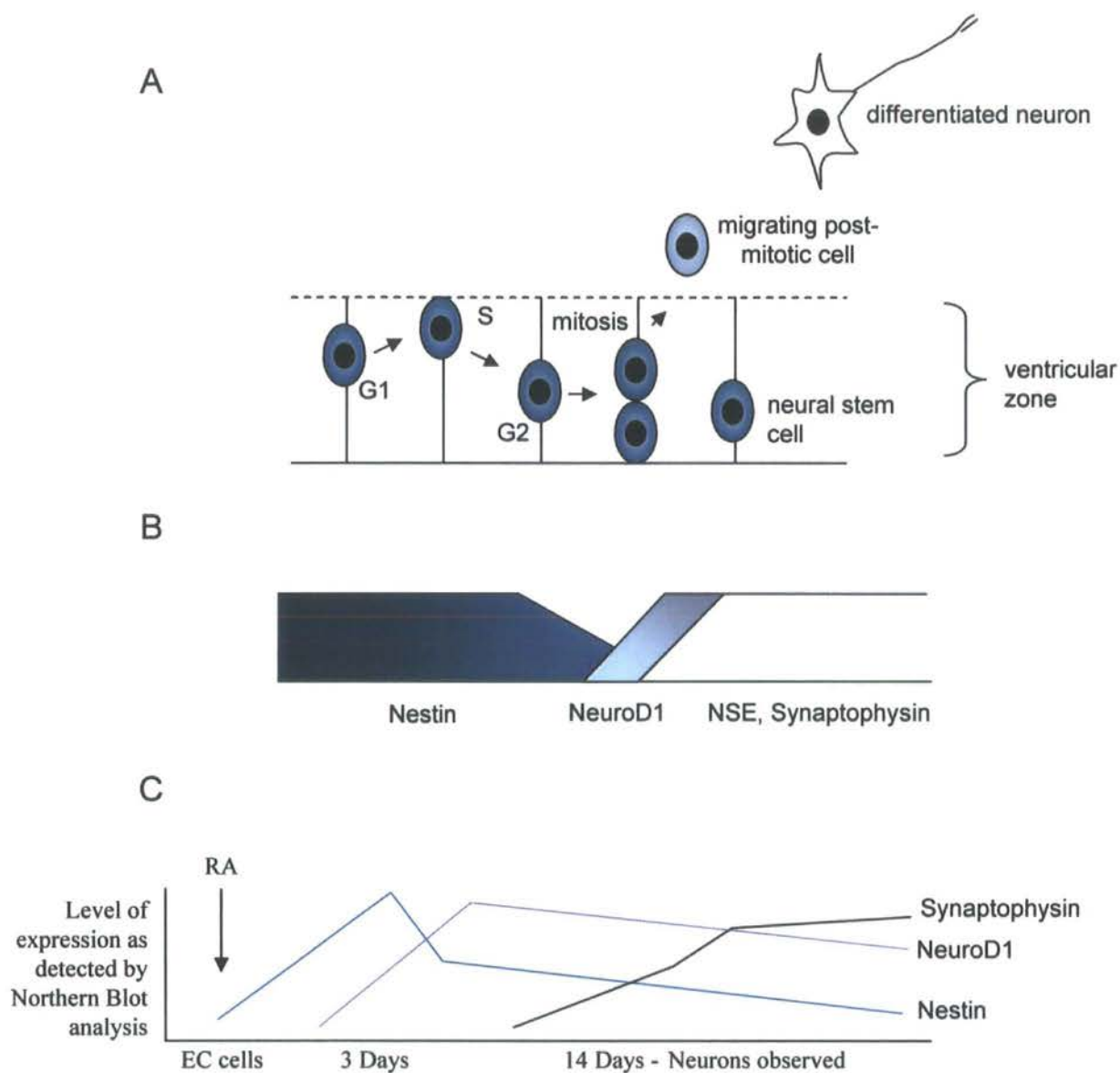


Figure 1.6 Gene expression patterns in the developing CNS neurons of the vertebrate embryo, compared with the expression profile in NTERA2 EC cells after treatment with retinoic acid. (A) Neuronal precursors proliferate in the ventricular zone of the neural tube. As these cells repeatedly divide and pass through the cell cycle, the position of their perikarya oscillates within this germinal epithelium as indicated (G1, S, G2, mitosis). Cells leave the mitotic cell cycle, migrate out of the ventricular zone, and commence differentiation. The levels of previously characterised neural genes change markedly during this process and can be used as indicators of neuronal development. (B) Schematic illustrating the regulated expression of Nestin, NeuroD1, Neuron-specific enolase (NSE) and Synaptophysin in proliferating, postmitotic and maturing embryonic neurons respectively, during the formation of the vertebrate CNS. (C) Depicts a highly representative graph presenting the changes in expression levels of these genes in NTERA2 cells in response to retinoic acid (RA). Nestin expression is rapidly up-regulated, reaching peak levels after 3 days, followed by a peak in NeuroD expression and then the expression of Synaptophysin after 14 days, which corresponds with the observation of neurons in the culture. Adapted from Figure 7 in Andrews et al. (2001) and Figure 2 in Przyborski et al. (2000).

TERA2.sp12 cells have been demonstrated to follow similar developmentally regulated neural differentiation upon RA treatment (Stewart 2003), and can form physiologically active neurons (Stewart *et al.* 2004). They also appear to be able to generate a wider range of neural sub-types compared to NTERA2 cell lines, as they are also capable of differentiating into cells that express astrocytic markers (Przyborski 2001, Stewart *et al.* 2003).

1.2.4 The derivation of embryonic stem cell lines

The recognition of EC cells as the malignant counterparts to cells from the inner cell mass of the blastocyst led to a series of experiments that eventually culminated in the isolation of pluripotent stem cells from very early mouse embryos: In 1981, Evans & Kaufman and Martin derived permanent lines of cells directly from mouse blastocysts, which they termed embryonic stem cells (Evans & Kaufman 1981, Martin 1981). These closely resembled murine EC cells in their expression of SSEA1 and high phosphatase activity, and their ability to form highly differentiated teratomas upon transplantation into immune-deficient murine hosts (for review Andrews 2001). Like some EC lines, these murine embryonic stem (ES) cell lines required feeder layers of mouse fibroblast cells to maintain an undifferentiated phenotype. Additionally they frequently and easily underwent spontaneous differentiation into a range of cell types (Martin 1981). This is consistent with the transience of the corresponding stem cell phenotype in the inner cell mass of the developing embryo, such that the apparent ability of ES cells to grow indefinitely and exhibit an immortal characteristic seems to be a consequence of their removal from the embryo and maintenance in tissue culture (Smith 2001). Conover *et al.* (1993) have since demonstrated that murine ES cells remain undifferentiated and proliferate in the absence of fibroblasts if the cytokine Leukaemia inhibitory factor (LIF) is added to the culture media, and Ying *et al.* (2003) have demonstrated that in the absence of serum, bone morphogenetic proteins (BMPs) are able to act in concert with LIF to maintain self renewal and pluripotency in murine ES cells.

Fourteen years later Thompson *et al.* successfully derived primate ES cells and established their growth on feeder layers of mouse fibroblasts, first establishing ES cells from the embryos of rhesus monkeys, marmosets and finally in 1998 from human embryos (Thompson *et al.* 1998, Thompson *et al.* 1995, Thompson *et al.* 1996, Thompson & Marshall 1998). To summarise, human blastocysts were grown from cleavage-stage embryos produced by *in vitro* fertilization, inner cell mass cells were separated from the trophectoderm by immunosurgery, plated onto a mouse fibroblast feeder layer in the presence of foetal calf serum and colonies sequentially expanded and cloned (Thompson 1998).

1.3 UTILISING EMBRYONIC STEM CELLS TO STUDY EMBRYOGENESIS

Besides being potential sources of differentiated tissues for transplantation therapies, embryonic stem cells provide accessible *in vitro* models in which to study aspects of embryogenesis: To this extent mouse embryonic stem cells have been demonstrated to closely model early developmental events in the embryo (for review see Loebel *et al.* 2003).

1.3.1 Murine ES cells – models of murine embryogenesis

As previously described (see Figure 1.1), after fertilisation a murine zygote undergoes proliferation to form a morula or ball of totipotent cells. This is followed by differentiation events associated with changes in the expression of the pluripotent transcription factor Oct4; such that at the time of implantation, the mouse embryo is composed of three distinct cell types, the trophoectoderm, the primitive endoderm and the inner cell mass. Manipulations of Oct4 expression in murine ES cells have demonstrated that Oct4 regulates these differentiation events, such that repression of Oct4 in murine ES cells generates trophoectoderm differentiation, up-regulation induces primitive endoderm differentiation and sustained Oct4 expression at intermediate levels is required to maintain a pluripotent state (Niwa *et al.* 2000).

As development progresses, the primitive endoderm and trophoectoderm will contribute towards the formation of the placenta, whereas the inner cell mass is organised into a pluripotent epithelial layer (the epiblast), from which the embryonic tissues are derived (Gardner & Beddington 1988). In early post implantation development, gastrulation occurs, a massive migration event involving complex inductive interactions between tissues in which pluripotent epiblast cells are allocated to the three primary germ layers of the embryo; ectoderm, mesoderm and definitive endoderm. In this process, cells ingress from the surface ectoderm into the interior of the embryo to give rise to the mesodermal and endodermal germ layers. In vertebrates, this occurs through a blastopore (in amphibians) or through a primitive streak (as in amniotes such as birds and mammals). These primary germ layers are the progenitors of all foetal tissue lineages; the endoderm will develop into the digestive tract, the ectoderm (consisting of the cells on the outside of the gastrula that played little part in gastrulation) will develop into the skin and CNS, and the mesoderm will form all the other internal organs (as described in Stern 2005).

As previously described, murine ES cells can be differentiated by removal of LIF or feeder layers such that they form a range of differentiated derivatives of all three germ layers *in vitro* (Conover 1993, Martin 1981). This is particularly effective when mouse ES cells are cultured in suspension where they form embryoid bodies. Such murine embryoid bodies first

form a bilayered structure with extraembryonic endoderm on the outside and primitive ectoderm on the inside, and then differentiate into a range of early endoderm, mesoderm, and ectoderm derived progenitors, recapitulating *in vitro* the formation of tissues from all three germ layers – albeit in a physically disorganised fashion (Doetschman *et al.* 1985, for review Loebel 2003). During embryogenesis the processes of germ layer formation and specification of tissue lineages are accompanied by a restriction of developmental potency and the activation of lineage specific gene expression, requiring complex cell-cell interactions that are influenced by the cell surroundings and mediated by the key WNT, Fibroblast growth factor (FGF) and transforming growth factor (TGF) signalling pathways (for review Loebel 2003). A schematic depicting the differentiation of the cells of the murine blastocyst into extra-embryonic and embryonic tissues of the early embryo, accompanied by a depiction of key signals involved in regulating germ layer specification is presented in Figure 1.7. Exposing cultured ES cells to these signals has enabled the differentiation of murine ES cells to be promoted towards particular lineages, thus mimicking inductive interactions of the murine embryo in an *in vitro* model, as described in the following examples:

As part of the TGF β signalling pathway, BMP signalling is essential for the determination of mesoderm and ectoderm cell fates; BMP signalling is required to induce mesoderm differentiation *in vivo*, such that mouse knock out models of BMP4 fail to gastrulate and produce mesoderm (Winnier *et al.* 1995). Correspondingly BMP4 treatment of murine ES cells cultured as embryoid bodies inhibits neural differentiation and up-regulates the expression of mesoderm genes such as Brachyury in a concentration-dependent manner, independent of cell survival or proliferation (Finley *et al.* 1999). BMP4 has also been demonstrated to induce differentiation of murine ES cells towards derivatives resembling posterior–ventral embryonic mesoderm, whereas dorsal–anterior mesodermal cell types are induced by another TGF β related molecule, Activin A (Johansson & Wiles 1995). BMP4 treatment can also promote the differentiation of murine ES cells that would normally be directed towards a neural lineage (such as in the presence of stromal derived inducing factor) to surface ectoderm at the expense of neuroectoderm, underlining the properties of BMP4 as a suppressor of neuroectodermal differentiation (Kawasaki *et al.* 2000). WNT3A signalling induces cells from the epiblast to adopt a paraxial mesodermal rather than neuroectodermal fate after gastrulation (Yoshikawa *et al.* 1997). Correspondingly, antagonism of WNT signalling has been demonstrated to promote neural differentiation in cultured murine ES cells: Transfection of the WNT antagonist Sfrp2 into murine ES cells enhances neuronal differentiation in embryoid bodies (Aubert *et al.* 2002).

For correct patterning and differentiation of the embryo, the influence of BMP signalling must be modulated by antagonistic molecules. In brief, BMP antagonists (such as

Schematic Depicting The Formation Of Extra-embryonic And Embryonic Tissues In The Murine Embryo

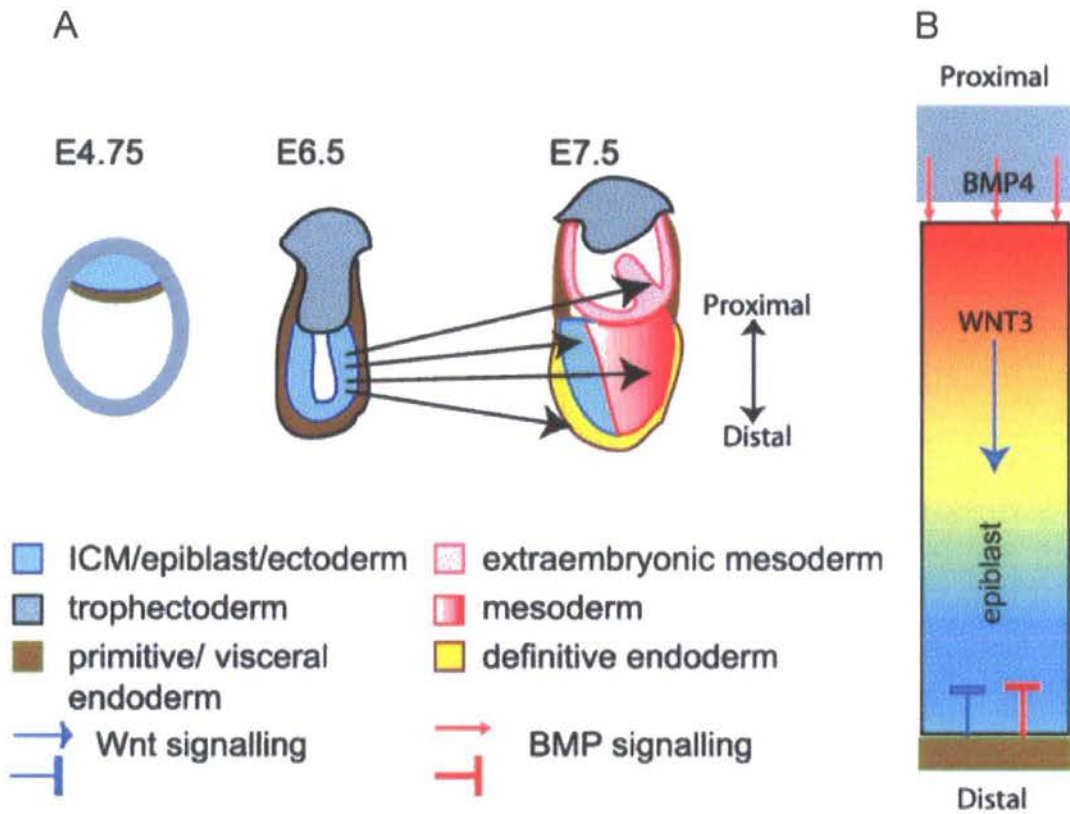


Figure 1.7. (A). The differentiation of trophectoderm (grey), ICM (blue), and primitive endoderm (brown) of the murine E4.75 embryo to the extraembryonic and embryonic tissues of the E7.5 embryo (germ layer tissues are color-coded, see key). In response to complex signalling interactions the cells of the developing epiblast become specified into the progenitors of the three germ layers, the ectoderm (blue), mesoderm (red) and endoderm (yellow). Key signalling pathways regulating these inductive events are Wnt signalling and BMP signalling pathways, who's activity is regulated along a proximal/distal gradient. (B). Proximal and distal polarity of tissue compartments: In the epiblast, progenitor populations are color-coded as previously described. Mesoderm progenitors are generated in proximal regions of high BMP signalling, whereas in the presence of WNT and BMP signalling inhibitors in distal regions ectoderm derivatives are formed. Red arrows and bar, BMP signals and antagonists; blue arrow and bar, WNT3A signals and antagonists. Figures taken from Figure 1, Loebel (2003).

Chordin and Noggin) are expressed in the mouse organizer and are required for anterior neural differentiation in the embryo (Bachiller 2000). Correspondingly, BMP antagonists promote the acquisition of an ectodermal fate by murine ES cells: Exposure of murine ES cells to recombinant Noggin counteracts the effects of BMP4 treatment in embryoid bodies (Finley 1999), and transfection of ES cells with a noggin-encoding plasmid promotes the differentiation of neurectodermal cells (Gratsch & O'Shea 2002).

Together these studies suggest that murine ES cells follow similarly regulated pathways of development to that of the pre-implantation embryo, and can be used as a highly accessible experimental model in which to study aspects of murine embryonic development.

1.3.2 A gross comparison of murine and primate embryogenesis

In comparison to murine embryogenesis and embryogenesis in other vertebrates, very little is known about early stages of human development because of limited access to these developmental stages. Primate studies have demonstrated that there are some key differences between basic stages of murine and primate development, which are extensively reviewed by Pera & Trounson (2004). The main differences between murine and primate embryogenesis (extracted from Box 1 in this review) are listed below:

- Different time scales for development
- High levels of blastomere fragmentation and chromosomal abnormalities in human compared with mouse
- Later transition in human embryos from maternal to zygotic gene expression (this occurs at the 4- to 8-cell stage in humans and at the 2-cell stage in mice).
- Differences in the temporal and spatial patterns of gene expression in the early embryo.
- The mouse blastocyst forms one cleavage stage later than the human.
- Human embryos have two phases of extraembryonic endoderm formation and limited reliance on yolk sac placentation. Mice have one phase of extraembryonic endoderm generation and more reliance on yolk sac placentation.
- The human epiblast is shaped into a disc rather than as a cup, as in the mouse.
- Interstitial implantation occurs in human embryogenesis but not in the mouse.
- Precocious formation of amnion and extraembryonic mesoderm in human embryos compared with in the mouse.

Two key developmental differences between primate and mouse embryos are evident in the formation of extra-embryonic layers and the mode of implantation. The primate embryo devotes its first two weeks almost exclusively to the formation of extraembryonic membranes (Enders & Schlafke 1981, Enders *et al.* 1986, Luckett 1978). Thus, in the time it takes the mouse embryo to develop past midgestation, the primate embryo has just begun germ layer

formation (Pera & Trounson 2004). The formation of a secondary yolk sac between the embryonic disk and the primary yolk sac is a characteristic feature of primate embryos, and undergoes considerable expansion; only near the end of the third week of development is yolk sac hematopoiesis underway (Pera & Trounson 2004).

The mode of implantation as the blastocyst becomes attached to the uterus is another key distinction between murine and human development. A primate blastocyst undergoes interstitial implantation, becoming entirely surrounded by endometrial tissue and eroding into the subepithelial connective tissue, whereas a mouse blastocyst undergoes eccentric implantation, eroding partway into the endometrium on the side of the uterus. Thus the surrounding environment for the blastocyst is markedly different between murine and primate blastocysts.

There is very little information about gene expression patterns and the molecular controls of development during post-implantation development in primates, which makes comparison with the extensive data from the mouse very difficult. However, a study comparing gene expression patterns in rhesus monkey and mouse embryos revealed differences in the abundance of certain transcripts between these species in the oocyte, at the cleavage, morula and blastocyst stages of development (Zheng *et al.* 2004). Unlike the mouse, there is little information on gene expression in the immediate post-implantation period in human development. However, some studies have found differences in the timing and expression patterns of certain developmental regulatory genes between the two species at later stages of development (Bibb *et al.* 2001, Fougousse *et al.* 2000, Hanley *et al.* 2000). For example the spatial expression of the key genes *Wnt7a* and *CAPN3* was detected by Fougousse *et al.* (2000) to be markedly different between murine and human development: *Wnt7a*, a highly conserved gene known to be important in early development, showed significant differences in spatial and temporal expression patterns in the developing brain (midbrain, telencephalon) of man and mice. A human gene encoding for calpain, (*CAPN3*, the locus for LGMD2A limb girdle muscular dystrophy) and its mouse orthologue also differed extensively in expression in embryonic heart, lens and smooth muscle.

Other molecular studies have also highlighted unexpected species differences, questioning the extent to which molecular regulation of human development can be confidently extrapolated from the mouse model: For some human disease genes, rodent models are not available because orthologues have not been identified despite extensive investigation in different rodent lineages, whereas the same approaches have identified clear orthologues in other vertebrates, for example the gene which is responsible for Kallman syndrome whose expression has been studied in human and chick (Lutz *et al.* 1994). In addition, for many human disease genes where rodent orthologues have been found, attempts

to generate mouse models by gene targeting have often produced phenotypes that do not closely resemble the human disorder (Wynshaw-Boris 1996).

1.3.3 A comparison of human and murine ES/EC stem cells

Human and mouse embryonal carcinoma cells share common features, for example their growth patterns and morphology are similar (both tend to grow in clusters of tightly packed cells with relatively little cytoplasm and prominent nucleoli) and both express high levels of alkaline phosphatase (Andrews 2001, Benham *et al.* 1981, Bernstine *et al.* 1973). However, from the first establishment of human EC cells differences between mouse ES/EC cells and human EC cells became evident, such as the expression of a different range of embryonic surface antigens and the ability of human EC cells to differentiate into trophoblast lineages *in vitro* and *in vivo* (Andrews 1980, Andrews *et al.* 1996). These differences could not be confidently attributed to either a distinction between murine and primate species or to the highly aneuploid nature of human EC cells, until the derivation of primate and finally human embryonic stem cells in 1998 (Thompson 1998, Thompson 1995, Thompson 1996, Thompson & Marshall 1998). The subsequent characterisation of human ES cells demonstrated that many of the features of human EC cells which are distinct from those of murine ES and EC cells are maintained in human ES cells:

As previously discussed, the carbohydrate antigen SSEA1 is strongly expressed by murine EC, ES cells and the corresponding inner cell mass (ICM) of the murine developing blastocyst. Two other glycolipid antigens – SSEA3 and SSEA4 – are expressed in early cleavage murine embryos but disappear by the blastocyst stage and are not expressed by cells of the ICM. Correspondingly, they are not expressed by murine EC or ES cells (see review Andrews 2001). This pattern of expression is in marked contrast to that observed initially in human EC cells and also in human ES cells, which do not express SSEA1 and highly express SSEA3 and SSEA4 (Andrews 1996, Damjanov *et al.* 1982, Thompson 1998). Recently, Henderson *et al.* also reported that this pattern of positive SSEA3, SSEA4 and absent SSEA1 expression corresponds with that of cells in the human blastocyst (Henderson *et al.* 2002).

Another important distinction between murine and human ES cells is evidence suggesting that human ES cells require different signalling cues for self renewal. Although both human ES cells and murine ES cells are maintained on fibroblast feeder layers, unlike murine ES cells, the addition of LIF does not prevent human ES cells from differentiating or dying when removed from a fibroblast feeder layer (Reubinoff *et al.* 2000, Thompson & Marshall 1998). This behaviour is reflected in human EC cell lines which are also feeder dependent, as LIF is unable to prevent their differentiation upon removal from feeder layers (Roach 1993).

In addition, human ES cell lines will spontaneously differentiate *in vitro* to extra-embryonic endoderm and trophoblast, evidenced by the secretion of α fetoprotein (AFP) and Human chorionic gonadotrophin (HCG) (Reubinoff 2000, Thompson 1998, Thompson & Marshall 1998). In contrast, mouse ES cells do not spontaneously differentiate into trophoblast lineages *in vitro*, and rarely contribute to trophoblast in chimeras. It is also unclear whether the rare ES derived cells that integrate into the trophoblast are functional (Martin 1981, Beddington 1989). However, mouse ES cells are capable of forming trophoblast *in vitro*, as recently demonstrated by Niwa *et al.* (2000) who showed that repression of Oct4 induces differentiation into trophoectoderm.

In a recent study a direct comparison of the gene expression profiles for human and mouse ES cells under stem cell maintenance and differentiation inducing conditions revealed distinctly different profiles for the expression of the differentiation markers vimentin, β III tubulin, alpha-fetoprotein, eomesodermin, HEB, ARNT, the stem cell marker FoxD3 as well as the expression of the LIF receptor complex LIFR/IL6ST (Ginis *et al.* 2004). Using focused microarray analyses they also identified profound differences in cell cycle regulation, control of apoptosis and cytokine expression, with the overall repertoire of cytokines expressed in mouse ES cells detected as much richer than in human ES cells. Together with the fact that the expression profiles were analysed in multiple independently derived stem cell lines, this provides supporting evidence that marked species-specific differences in gene expression profiles exist between human and mouse ES cells (and that these differences do not arise from differences in culture conditions alone).

These disparate patterns of embryonic antigen expression, gene expression profiles and behavioural differences between human and mouse embryonic stem cells may reflect inherent differences in human and mouse embryogenesis, correlating with differences observed in the gross comparisons of primate and murine early development. Taken together with the evidence from the few molecular studies of human development this suggests that although human embryogenesis resembles that of other mammalian species, there are key differences at not only the morphological level but at cellular and molecular levels. This makes a compelling case for using a human experimental model in which to study human embryogenesis.

1.4 USING TERA2.SP12 CULTURES AS A MODEL OF HUMAN ECTODERM

The characterisation of human ES cells has validated the presumption that human EC cells relate to pluripotent cells of the early embryo, as in the mouse model. Shared patterns of surface antigen expression support the close relationship of human ES and EC cells. However,

some key differences between certain human EC lines and ES cells are also readily apparent: Until recently human ES cells required propagation on an embryonic fibroblast feeder layer or in fibroblast conditioned medium (Thompson 1998, Xu *et al.* 2001, Xu *et al.* 2005) and frequently and easily embarked on spontaneous differentiation into a range of cell types of all germ layers. As a consequence this culture system typically generates a heterogeneous population of cells that has been difficult to consistently characterise and manipulate exclusively towards particular phenotypes. In contrast, certain EC cell lines such as the clonal TERA2.sp12 cell line can be stably and robustly propagated to maintain a stem cell phenotype in the absence of feeder cells, and NTERA2 and TERA2.sp12 cell lines have been demonstrated to undergo regulated differentiation into neural derivatives in response to RA treatment (Przyborski 2001, Stewart 2003).

Another key difference is evident in the remarkable differentiation capacity of human ES cells compared to human EC cells: Upon transplantation into an immune-deficient murine host, human ES cells form highly differentiated tumours containing derivatives of all three embryonic germ layers, including smooth and striated muscle, bone, cartilage, gut and respiratory epithelium, keratinising squamous epithelium, neurons and ganglia (Heins *et al.* 2004, Reubinoff 2000, Stojkovic *et al.* 2004, Thompson 1998). In contrast, histological analysis of tumours generated from transplantation of TERA2.sp12 cells into immunocompromised hosts reveals a markedly reduced range and complexity of differentiation, and solely ectodermal derivatives have been observed (Przyborski *et al.* 2004, Stewart 2004). Together with *in vitro* studies this suggests that like certain other human EC cell lines, TERA2.sp12 cells may have a more restricted differentiation potential than ES cells, such that TERA2.sp12 cells appear to be capable of generating solely neural and non-neural derivatives of the embryonic ectoderm (Przyborski 2004). This may reflect an inherent difference between TERA2.sp12 cells and human ES cell lines, such that TERA2.sp12 cells correspond more closely to the embryonic ectoderm in their behaviour than to cells of the inner cell mass. Correspondingly, the differentiation of TERA2.sp12 cells *in vitro* has been proposed to provide a suitable experimental system in which to study aspects of development in the human ectoderm, in particular the early stages of neural development (Przyborski 2004).

1.5 THE EARLY STAGES OF NEURAL DEVELOPMENT IN VERTEBRATES – AN OVERVIEW

The central nervous system (CNS) is arguably one of the most complex structures to form during embryogenesis, involving the formation and diversification of a wide array of cell types. In describing the early phases of neural development this review has been sub-divided into the following sections, although temporally these are not necessarily distinct:

1.5.1 Neural induction and neurulation

The first step for the formation of the CNS is neural induction, whereby neural epithelium is specified from the ectoderm to form the neural plate. In the vertebrate embryo, ectodermal cells acquire a neural cell fate in response to signals provided by the organizer region, while the remaining ectodermal cells are specified into non-neural progenitors and will form the epidermal cells of the skin (Spemann & Mangold 1924, Beddington 1994, Storey *et al.* 1992). The signalling events regulating this neural induction step in vertebrates have been the subject of intense study and will be reviewed in the introduction to Chapter 4. In brief, experiments in animal models (particularly in *Xenopus laevis*) have demonstrated that the Bone Morphogenetic Protein (BMP) signalling pathway plays a key role in determining whether cells of the naïve ectoderm are specified into neural or epidermal progenitors (Hemmati-Brivanlou & Melton 1994b), although other signalling pathways have also been implicated in neural induction, including the Fibroblast Growth Factor (FGF) signalling and Wnt pathways (reviewed in Wilson & Edlund 2001, Stern 2005).

Subsequent to neural plate formation the neural tube is formed during neurulation, whereby the neural plate folds to produce a neural groove, the folds elevate from the dorsal side of embryo, approach each other medially and initiate fusion to form the a tube. The neuroepithelial cells of the neural tube are the progenitors for all the cell types of the CNS, initially producing neurons in neurogenesis, and glia (including astrocytes and myelin-forming oligodendrocytes) at later stages. As demonstrated in Figure 1.6, during neurogenesis proliferating neuroepithelial cells adjacent to the lumen of the neural tube exit the cell cycle, delaminating out of the epithelium and activating the expression of a large panel of genes indicative of generic neuronal differentiation. Neurons originate from almost all regions of the neuroepithelium, although the onset and period of neurogenesis varies greatly among neuroepithelial cells depending on their location along the neuraxis (Kintner 2002). This reflects the extensive patterning of the neural tube whereby the developing CNS is highly regionalised along both its anteroposterior (AP) and dorsoventral (DV) axes.

1.5.2 Patterning of the neural tube

Anteroposterior regionalisation in the CNS is clearly evident from the localization of the cerebral cortex and eyes at the anterior end and the spinal cord at the posterior end. Similarly, different neurons and neural structures form at specific dorsal ventral positions of the neural tube; for example, motor and sensory neurons form in the ventral and dorsal spinal cord respectively. Studies of anterior-posterior patterning indicate that after neural induction, the cells of the neural plate are anterior in character: In the frog neural induction generates neural tissue with anterior properties, then further posteriorizing signals, including FGFs, Wnts and

retinoic acid, induce midbrain hind brain and spinal chord (Harland 2000). Similarly, early chick neural plate has anterior identity and requires further signals that are provided by both axial and paraxial mesoderm to acquire posterior characteristics (Muhr *et al.* 1999, Dale *et al.* 1999). Dorsal ventral patterning of the CNS has been extensively studied in the spinal chord, where the opposing activities of gradients of the diffusible molecules Sonic Hedgehog (which emanates from the floor plate and notochord), BMPs and Wnts (which originate in the roof plate and adjacent nonneural tissue) activate the expression of several patterning genes in different, partially overlapping domains in a concentration dependent manner (as reviewed in Gomez-Skarmeta *et al.* 2003). These patterning genes encode homeodomain transcription factors, and specific combinations of these patterning genes will define the different progenitor domains in which specific neurons will arise (Lee & Pfaff 2001).

To summarise, this complex organization of the CNS is anticipated by the regionalised expression of specific combinations of patterning transcription factors (Lumsden & Krumlauf 1996) generated in the neural tube in response to dorsoventral and anteroposterior cues from diffusible signalling molecules, such that a unique combination of transcription factors is expressed over time in each progenitor domain (Briscoe *et al.* 2000, Shirasaki & Pfaff 2002). However, regardless of where and when neurons are formed, a genetic program involving a cascade of pro-neural basic helix-loop-helix (bHLH) transcription factors is thought to govern the differentiation of neural epithelial cells, mediating both neuronal determination and differentiation. The regionalisation in different points of the neuroaxes is generated through the means by which these bHLH proteins are activated within neuroepithelial cells by different patterning genes (for review Kintner 2002).

1.5.3 β Helix-loop-helix proteins and neurogenesis

Vertebrate pro-neural bHLH genes are categorised into two families based on their homology to pro-neural bHLH genes in *Drosophila*; those related to the *Drosophila* Achaete-Scute genes such as Mash1, and those related to *Drosophila* atonal, such as the neurogenins, the NeuroD-like, and the ATH genes (Brunet & Ghysen, 1999). As transcriptional activators, these proteins hetero or homo-dimerise and bind to E-box promoters of target genes to induce the expression of both generic neuronal proteins such as β III tubulin and subtype specific proteins (Diez del Corral & Storey 2001).

At the point where cells in the neuroepithelium embark on neurogenesis to form a postmitotic neuron, they express a cascade of transcription factors that is triggered by one or several proneural bHLH genes, and the exact range of genes expressed varies depending on time and place (Kintner 2002). When ectopically expressed, proneural bHLH proteins are potent inducers of neuronal differentiation (Lee *et al.* 1995), activating the expression of a generic set of neuronal genes, and they are key in promoting cell cycle exit as neurons embark

on terminal differentiation (Farah *et al.* 2000). Thus proneural bHLH proteins are thought to be responsible for promoting neuronal differentiation, regardless of when and where a neuron forms in the CNS. Experiments show that when their activity is sufficiently high in neuroepithelial cells, these bHLH proteins activate the expression of a cascade of downstream differentiation bHLH genes, which then act to promote exit from the cell cycle and neuronal differentiation. For example an up-regulation in the expression of bHLH protein Neurogenin precedes the expression of the bHLH protein NeuroD in both the frog and mouse, which is necessary for cell cycle exit and neuronal differentiation (Ma *et al.* 1996). Conversely, other experiments show that when the activity and expression of the determinative bHLH genes is inhibited, neuroepithelial cells exhibit a 'ground state' whereby they can continue to divide and become a neuron at a later time, or serve as the source of progenitor cells for various glia (Nieto *et al.* 2001). Thus the balance of proneural bHLH proteins allows for protracted neurogenesis and the proliferation of neuronal progenitor cells, such that the time of neuron formation is a mechanism of determining neuronal subtype specification (Perron & Harris 2000).

1.6 AIM OF THIS STUDY

As described in Section 1.4, the TERA2.sp12 cell line has been proposed to provide a suitable model in which to study aspects of development in the human ectoderm, particularly the early stages of neural development. In the first stage of neural development, the naïve ectoderm is specified into neural as opposed to epidermal progenitors (termed neural induction). The aim of this study is to investigate whether the signalling mechanisms governing the transition of naïve ectoderm into neural as opposed to non-neural progenitors of the ectoderm are conserved in human development, specifically focussing on the role of the BMP pathway.

1.7 OBJECTIVES

The central objective of the investigations in this study was to determine the role of BMP signalling in neural versus non-neural transitions of TERA2.sp12 cells, as an experimental model of human ectoderm.

1) The objective of the first experimental chapter (Chapter 3) was to characterise the differentiation of TERA2.sp12 cells in response to RA under two alternative culture conditions. These alternative methods had been previously reported to generate different proportions of neurons (Stewart 2003), and the aim of this investigation was to determine if this observation was based on a difference in the specification of neural as opposed to non-neural ectoderm fates. Culture of dissociated TERA2.sp12 cells under suspension conditions

as opposed to adherent conditions was observed to markedly and more rapidly up-regulate RA induced markers of neural differentiation and inhibit the up-regulation of epidermal marker expression.

2) The first objective of the second experimental chapter (Chapter 4 Section A) was to utilise these two systems in which neural versus non-neural ectoderm transitions were differentially regulated, to study the role of the BMP pathway on the specification of TERA2.sp12 cells into neural versus epidermal fates. The activity of the BMP signalling pathway and also the FGF mediated MAPK pathway was compared in these two culture systems, by monitoring the phosphorylation state of selected down-stream signalling proteins which are phosphorylated in response to receptor activation. In the case of the BMP pathway, pathway activity was also monitored by detecting the expression of genes transcriptionally regulated by BMP signalling. The effect of active manipulation of these pathways on neural as opposed to non-neural ectoderm marker expression in these two culture systems was also investigated, through treatments with recombinant activators and inhibitors. The objective of the remaining section of this chapter was to investigate the effect of active manipulation of these pathways on TERA2.sp12 cells in the absence of stimulation by RA, in order to more closely model the signalling events in the development of early neural or non-neural ectoderm progenitors *in vivo*.

4) Since during the course of this study TERA2.sp12 cells were observed under certain conditions to differentiate into non-ectodermal lineages *in vitro*, the objective of the last experimental chapter was to more closely investigate the differentiation potential of TERA2.sp12 cells in an *in vivo* system. Tumours derived from injection of TERA2.sp12 cells into immune-deficient hosts were analysed for the expression of a range of markers associated with lineages of different germ layers and extraembryonic lineages, and compared with a tumour derived from human ES cells.

CHAPTER 2
MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 TERA2.sp12 cells

The human caucasian embryonal carcinoma stem cell line TERA2.sp12 is a subclone derived by Stephan Przyborski (Department of Biological science, University of Durham) from passage 15 of TERA-2 embryonal carcinoma cells (Przyborski 2001), which were originally isolated from a lung metastasis of a 22 year old patient with primary embryonal carcinoma of the testis (Fogh & Trempe 1975).

Maintenance and passaging of TERA2.sp12 cells

TERA2.sp12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose formulation, Sigma) supplemented with 10% foetal calf serum (FCS, Sigma), 2mM L-glutamine (VWR) and 1 μ M penicillin/streptomycin solution (pen/strep, Invitrogen) in NUNC 80cm² T75 filter cap culture flasks (VWR). Cultures were incubated at 37°C under a humidified atmosphere of 3% carbon dioxide in air, with media replacement every 2-3 days. Cultures were passaged when they were over 95% confluent using the following method. The media was removed and 1ml of media was added to the flask, followed by 10-15 small acid washed glass beads (Fisher). The flask was gently rocked such that the rolling beads dislodged the cells, then 8mls of media used to gently re-suspend the cells, which were re-seeded in fresh T75 culture flasks with fresh media at a ratio of 1:3. Cultures were passaged approximately every 6-7 days.

Preparation of TERA2.sp12 cell cultures for storage

Confluent cultures of TERA2.sp12 cells were washed in phospho-buffered saline (PBS, Sigma) then individualised by the addition of 2mls of 1X Trypsin-EDTA, (0.05% Trypsin, 0.53mM EDTA.4Na, GIBCO, Invitrogen) diluted to 0.5X in PBS supplemented with 0.02% EDTA (Sigma), 0.15% phenol red (Sigma) and incubated for 1-2 minutes at 37°C followed by gentle tapping, until the cell layer had dispersed. Individualised cells were then re-suspended and gently titrated in fresh media, then centrifuged at 129g, 4°C for 2 minutes to pellet cells. The media was subsequently removed and cells re-suspended in 3mls of ice-cold 10% dimethyl sulfoxide (DMSO, Sigma) and 90% FCS, transferred to 2ml cryogenic vials, (Nalgene, VWR) and placed in the -80°C freezer overnight, prior to long-term storage at -120°C.

Thawing and culture of TERA2.sp12 cells

Individual vials of TERA2.sp12 at low passage numbers that had been stored at -120°C in 1ml

of 10% DMSO (Sigma) in FCS were rapidly defrosted in a water-bath at 37°C and the contents transferred to a 15 ml centrifuge tube (VWR) containing 9mls of DMEM with 10% FCS, 2mM L-glutamine and 1µM penicillin/streptomycin solution. To pellet the cells the tube was centrifuged at 129g for 2 minutes at 4°C, the supernatant removed and replaced with 10mls of fresh media. The cells were re-suspended in this media by gentle pipetting and then transferred to a NUNC 30cm² T25 filter cap culture flask (VWR), and incubated at 37°C under a humidified atmosphere of 3% carbon dioxide in air.

After 1-2 days, when the culture was over 95% confluent, the cells were passaged using the following procedure: The media was removed and 1ml of media and 10-15 small acid washed glass beads (Fisher) added to the flask, which was then gently rocked such that the rolling beads dislodged the cells. The cells were gently re-suspended in 9mls of media and transferred into a NUNC 80cm² T75 filter cap culture flask (VWR) containing 10mls of media.

RA Differentiation as adherent monolayers

TERA2.sp12 cells were seeded at a differentiation density of 20 000 cells/cm² into electrostatically coated T75 filter cap culture flasks and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep. After 24 hours cells the media was removed and replaced with the same media supplemented with 1µl/ml of 10mM all-trans RA (Sigma) reconstituted in dimethyl sulfoxide (DMSO, Sigma) such that the final concentration was 10µM. In experimental controls the media was replaced with the same media with no supplement or vehicle treated with media supplemented with 1µl/ml of DMSO. Media supplemented with 10µM RA (or with the specified media for experimental controls) was replaced every 2-3 days, and RA treated cultures were maintained for up to 28 days.

RA Differentiation as suspension aggregates

TERA2.sp12 cells were seeded at a differentiation density of 20 000 cells/cm² into uncoated 90mm triple vent bacteriological dishes (VWR) and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep. After 24 hours, media containing suspended cells was transferred to individual 15ml centrifuge tubes (VWR) and centrifuged at 129g for 2 minutes at 4°C to pellet the cells at the base of the tubes. The media was then removed and replaced with the same media supplemented 1µl/ml of 10mM all-trans RA reconstituted in DMSO such that the final concentration was 10µM. Cultures were maintained in media with 10µM RA for up to 14 days, with media and bacteriological dishes replaced every 3 days using the following procedure: Media containing suspended cells was transferred to 15 ml centrifuge tubes, tubes were centrifuged at 129g for 2 minutes at 4°C when up to 3 days old, and 64g for 2 minutes at 4°C after 3 days of culture. The supernatant was then removed and cells re-

suspended in fresh media before being transferred to new bacteriological dishes.

Culture as suspension aggregates without RA

As an experimental control for suspension culture conditions TERA2.sp12 cells were seeded at a differentiation density of 20 000 cells/cm² into uncoated 90mm triple vent bacteriological dishes and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep. After 24 hours, media containing suspended cells was transferred to individual 15ml centrifuge tubes and centrifuged at 129g for 2 minutes at 4°C to pellet the cells at the base of the tubes. The media was then removed and replaced with the fresh media. Cultures were maintained for up to 14 days, with media and bacteriological dishes replaced every 3 days as follows: Media containing suspended cells was transferred to 15 ml centrifuge tubes, tubes were centrifuged at 129g for 2 minutes at 4°C when up to 3 days old, and 64g for 2 minutes at 4°C after 3 days of culture. The supernatant was then removed and cells re-suspended in fresh media before being transferred to new bacteriological dishes.

Manipulation of BMP signalling in adherent cultures

Individualised TERA2.sp12 cells were seeded at a differentiation density of 20 000 cells/cm² into electro-statically coated NUNC tissue culture flasks and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep supplemented with either 25ng/ml BMP2, 25ng/ml BMP4, 100ng/ml Noggin, 10µM RA or mock treated with no additional supplement. Media was replaced every 2-3 days.

Inhibition of FGF signalling in adherent cultures

To investigate the effect of continuous inhibition of FGF mediated signalling in TERA2.sp12 cells, individualised cells were seeded at a differentiation density of 20 000 cells/cm² into a NUNC 12 well dish and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep supplemented with either 20µM of the pharmacological inhibitor of FGF receptor kinases, SU5402 which specifically interacts with intracellular catalytic domain of FGF receptors (Mohammadi *et al.* 1997), or vehicle treated with DMSO. Media was replenished every 3-4 days, (or every 1-3 days as required for vehicle treated cultures).

TERA2.sp12.GFP cells

TERA2.sp12.GFP cells, TERA2.sp12 cells which have been transfected with the GFP (green fluorescent protein) expression vector pTP6 and selected for their resistance to puromycin, constitutively express GFP (Stewart *et al.* 2004). These cells were cultured as described for TERA2.sp12 cells.

2.1.2 Cell lines used as positive controls

The pharynx carcinoma derived cell line FaDU

FaDU cells were used as a positive control for the detection of simple epithelial keratins and the epidermal stem cell transcription factor tumour protein 63 (Tp63) in both immuno-staining and Western Blot analyses. They were also used as a negative control for markers of neural differentiation.

A frozen vial of FaDU cells was obtained from the ATCC bank, (code HTB-43, lot 381 6051), and handled according to instructions in the accompanying data sheet as described briefly below. The vial of cells was thawed for 2 minutes at 37°C in a water-bath and then the contents transferred directly into a T75 flask containing 11mls of Eagles minimal essential medium with Earle's BSS (MEM from Sigma), 2mM L-glutamine and 10% FCS media at 37°C. Media was replaced every 2-3 days, and when over 90% confluent, cells were sub-cultured at a ratio of 1:6 using the following procedure. The media was removed, and cells washed in 5mls of PBS. Then cells were incubated for 5-10 minutes with Trypsin at 37°C with minimal agitation and observed under the microscope until the cell layer had dispersed. Then 8mls of growth medium was added and gentle pipetting used to aspirate cells, before transfer into fresh T75 flasks. After an initial 2 passages, 1 flask of FaDU cells was maintained and the cells from superfluous flasks trypsinised and stored as frozen cell pellets at -120°C.

The neuroblastoma derived cell line IMR 32

IMR 32 cells were used as a positive control for the detection of neuronal markers in both immuno-staining and Western Blot analyses, and also used as a negative control for the detection of endoderm and non-neural ectoderm markers.

The human Caucasian neuroblastoma cell line IMR 32 was obtained from ECACC (number 86041809) and cultured in EMEM with 2mM L-glutamine, 1% non essential amino acids and 10% FCS. Sub-confluent cultures (70-80%) were split using 0.25% Trypsin, and reseeded at a ratio of 1:3 in fresh T75 culture flasks (extra care was taken as cells have a low plating efficiency and easily detach).

The hepatocarcinoma derived cell line Hep G2

HepG2 cells were used as a positive control for the detection of endoderm expressed markers hepatic nuclear factor β (HNF3 β) and alpha-1-fetoprotein (AFP) in both immuno-staining and Western Blot analyses, and as a negative control for the detection of neural markers.

A confluent flask of the human Caucasian hepatocyte carcinoma cell line HepG2 was obtained from Dr Maria Bakari (sourced from ECACC, number 85011430) and cultured in EMEM, (EBSS) with 2mM Glutamine, 1% non essential amino acids (Sigma) supplemented with 10

% FCS and 1ml pen/strep. Media was replaced every 6 days and cells were sub-cultured at a ratio of 1:4 using the following method. The media was removed, and cells washed in 5mls of PBS prior to incubation for 5-10 minutes with Trypsin/EDTA and observation under the microscope until the cell layer had dispersed. Then 8mls of growth medium was added and gentle pipetting used to aspirate cells, before transfer into fresh T75 flasks. In the first few days after sub-culture the cells grew as islands, which formed a confluent monolayer after 6-7 days of culture.

The human embryonic stem cell line H7

As an additional positive control for markers of germ layer differentiation, spontaneously differentiated human H7 embryonic stem cells were kindly donated by Linda Lako in Newcastle. In summary, cells were cultured on mouse embryonic feeder layers with embryonic stem cell medium containing knock-out DMEM (Invitrogen), 100 μ M β -mercaptoethanol (Sigma), 1mM L-glutamine (Invitrogen), 100mM non-essential amino acids, 10% serum replacement (SR, Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 4ng/ml bFGF (Invitrogen). Colonies were passaged by incubation in 1mg/ml collagenase IV (Invitrogen) for 5-8 minutes at 37°C and then grown in feeder-free conditions in ES medium.

2.2 CELL COUNT ASSAY

Individualisation of cultures using Trypsin

At the specified time-points the selected adherent cultures were washed gently in PBS and incubated at 37°C for 2-3 minutes with 2mls of 0.5X Trypsin in EDTA with gentle tapping used until the cell layer had dispersed. Once cells had individualised 9mls of media was added to inactivate the Trypsin, and the cells gently aspirated with a pipette.

For non-adherent (suspension) cultures, the contents of each dish was transferred to individual 15ml centrifuge tubes and centrifuged for 2 minutes at 64g, 4°C. The media was removed and to was the aggregates they were re-suspended in 10mls of PBS, pelleted by centrifugation as previously described, and the PBS removed. To dissociate the aggregates into individual cells 1ml of 0.5X Trypsin in EDTA was added to the centrifuge tubes and these were incubated at 37°C, for 3-5 minutes, with periodic gentle aspiration using a pipette. When the aggregates were no longer visible and a suspension of single cells was formed, the Trypsin was inactivated by adding 9mls of media.

Cell counts

To count the individualised cells in each sample, 10 μ l of solution was loaded onto a haemocytometer, and cell counts performed using a phase contrast light microscope (Nikon

eclipse TS100). Three different replicates of each sample were assayed using this method, and if any unusual cell counts were collected the counts were repeated. These counts were then used to estimate the concentration of cells in each sample (count multiplied by 10,000), and the total cell number estimated by multiplying this value by the total volume of solution (10mls). These counts were also compared to the size of the pellets when centrifuged at 129g, to ensure as much as possible that they were representative.

2.3 FLOW CYTOMETRY

The remaining cells from the cell count assay were analysed using live flow cytometry to determine the level of expression of surface antigens associated with the stem cell phenotype (TRA 160, SSEA 3) or with the neural phenotype (VINIS 53, A2B5). In each experiment confluent flasks of undifferentiated TERA2.sp12 were used for the EC samples. Three replicates of cells from the same passage number were used for each treatment, and processed in parallel. For the duration of the following procedure cells were stored on ice and re-suspended in solutions maintained at 4°C to ensure cell viability.

Cell samples suspended in media were centrifuged for 2mins at 129g, 4°C and the media removed. To wash off any remaining media the pelleted cells were re-suspended in PBS, centrifuged for 2mins at 129g, 4°C and the PBS removed. The remaining pelleted cells were then re-suspended in ice-cold flow cytometry buffer consisting of PBS supplemented with 1% normal goat serum (Invitrogen) and 0.1% sodium azide (Sigma) at an appropriate volume such that the concentration was (1000 cells/ μ l). The cell solutions were then dispensed in 200 μ l volumes into the appropriate number of wells in a 96 ml round-bottomed plate with lid (VWR), according to the well plate plan outlined in Figure 2.1. Each plate was then centrifuged at 129g, 4°C for 2mins to pellet the cells, and the supernatant removed by quickly and briefly turning the plates upside down. Cell pellets were then re-suspended in 25 μ l of the appropriate primary antibody diluted in flow cytometry buffer, as detailed in Table 2.1 and following the well plate plan outlined in Figure 2.1.

Table 2.1. Antibodies utilised in flow cytometry, listing antigen, antibody sub-type, source, and dilution factor.

ANTIGEN	ANTIBODY SUB-TYPE	SOURCE	DILUTION FACTOR
SSEA3	Mouse IgM	Hybridoma bank	1:5
TRA 160	Mouse IgM	Hybridoma bank	1:5
A2B5	Mouse IgM	Chemicon	1:100
VINIS 53	Mouse IgG	Hybridoma bank	1:5
VINIS 56	Mouse IgM	Hybridoma bank	1:2
Mouse P3X	Negative control antibody to mouse antigen	Hybridoma bank	1:5
Mouse IgM (μ chain)	Fluorescein-conjugated goat IgG fraction	Cappel	1:100
Mouse IgG	Fluorescein-conjugated goat IgG fraction	Cappel	1:100

Schematic Of Well Plate Plan For Flow Cytometry Experiments

		IgM						IgG					
		1	2	3	4	5	6	7	8	9	10	11	12
A		P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53	
B			P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53
C		P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53	
D													
E		P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53	
F			P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53
G		P3x		SSEA 3		TRA 160		A2B5		P3X		VINIS 53	
H													

TREATMENT I

TREATMENT II

Figure 2.1 Outline of well plate plan for flow cytometry showing locations for each antibody tested. Treatment I and treatment II represent different cell culture treatments (for example RA treated adherent cultures and RA treated suspension cultures at 14 days), and within each culture treatment each row (i.e. row A) was loaded with cells from one of the three replicate cultures at the same passage number. Separate pipette tips were used during washes between each treatment, and the well spacer between each antibody minimised transfer during washes.

Following 30 minutes of incubation on ice, cells were washed 4 times with buffer to remove unbound antibody using the following method. One hundred and fifty microlitres of buffer was added to each well and the cells suspended using a multi-pipette, then the plate centrifuged at 129g, 4°C for 2mins, and the supernatant removed as previously described. Cells were then re-suspended in 25µl of the appropriate secondary antibody diluted in flow cytometry buffer, as described in Table 2.1 and Figure 2.1 and incubated for 30 minutes in the dark. To remove unbound secondary antibody cells in each well were then washed 3 times with buffer as described previously, and finally re-suspended in 200µl buffer. The suspended cells of each well were then transferred to individual 5ml glass test-tubes (Invitrogen). To maximise cell collection the wells were washed with a further 200µl of buffer and this was also transferred to the corresponding test-tubes such that each tube contained 400µl of buffer and suspended cells.

Immediately prior to loading a sample into the flow cytometer (EPICS XL from Beckmann and Coulter), 1µl of propidium iodine (Sigma) was added to each sample. All flow cytometry data was processed using WINMIDI 2.8, and statistics analysed using Windows Excel software.

2.4 WESTERN BLOT ANALYSIS

The recipes for the solutions in the following protocol are listed in Section 7.2 of the appendix.

Collection of samples as cell pellets

The cells in each culture treatment were individualised with Trypsin in EDTA and collected in centrifuge tubes with 10mls of media as described within Section 2.2. Following centrifugation for 2mins at 64g, 4°C the media was removed and the pelleted cells re-suspended in PBS. To remove any remaining media the cells were pelleted by centrifugation as described, the PBS removed and replaced with 3-4mls of fresh PBS, depending on the size of the cell pellet. For each sample, 1ml of re-suspended cells in PBS was then transferred to 1.5ml PCR clean eppendorf tubes (VWR), such that approximately three eppendorf tubes were used to collect cells from each flask. The eppendorf tubes were then centrifuged for 5 minutes at 1500g and the supernatant removed. To maximise supernatant removal the eppendorf tubes were centrifuged for a further 10 seconds at 1500g, and any remaining supernatant removed using a fine pipette tip. Pelleted cells were then rapidly frozen by placing in liquid nitrogen, before storage at -80°C, or used immediately in the following procedure.

Lysis and extraction of protein from cell pellets

In the following procedure the eppendorf tubes containing the pellets of cells and the solutions used were all maintained on ice. To lyse the cells in each sample 100-200 μ l of freshly prepared protein lysis buffer was added to each tube of pelleted cells and agitated using a vortex every 5 minutes for 30 minutes. To isolate the protein fraction the samples were then centrifuged at 15,000rpm in a microcentrifuge for 5 minutes at room temperature, and the supernatant containing the protein suspension transferred to a fresh eppendorf tube.

Measurement of protein concentration

To measure the protein concentration of each sample solution, disposable semi micro cuvettes suitable for absorbance at 595 nm (Fisher) were loaded with the following:

1. 798 μ l distilled water
2. 200 μ l BIO-RAD protein assay dye reagent (BIO-RAD laboratories)
3. 2 μ l sample solution

As a loading control an additional cuvette was prepared with the above except 2 μ l of lysis buffer was loaded instead of 2 μ l of sample solution, and categorised as a blank control using Vision software.

A calibration curve was previously prepared by measuring the absorbance at 595nm of known concentrations of protein (BSA standards) with a Helios β mass spectrometer and then saved using Vision software. The absorbance of the sample solutions at 595nm was then measured with the mass spectrometer and the corresponding protein concentration calculated from the calibration curve using Vision software. The measured concentration of the loaded samples was then used to calculate the concentration of the corresponding protein solutions.

Preparation of protein samples for electrophoresis

For each sample, a solution of protein in loading buffer was prepared such that each sample contained 10 μ g of protein, with the following composition, (where a= calculated volume of protein solution required for 10 μ g).

- 3 μ l 5X loading dye
- a μ l sample
- 15-a μ l H₂O

Each loading sample was then boiled at 90°C for 3 minutes to denature the proteins within these samples.

Electrophoresis of protein samples and transfer to PVDF membrane

Protein electrophoresis gel kits (BIO RAD) were used following the manufacturers instructions. To summarise, 10% agarose gel was freshly prepared and loaded into casts of glass plates clamped into position, followed by a small quantity of isopropanol (Fisher) to

level the top of the gel, and allowed to set for 20 minutes. The isopropanol was then washed off with distilled water, excess water removed carefully with filter paper, and 4% agarose gel loaded above the 10% gel. The well mould was subsequently added and the gel allowed to set for 20 minutes.

Once the gels had set, the glass moulds were removed from the clamps, and placed in the electrophoresis box with running buffer. SDS was flushed out of wells using a syringe; the protein samples in loading dye were loaded into the wells. To separate the proteins within each loaded sample according to their size the samples were then electrophoresed at 200v for approximately 1 hour, or until the dye reached the base of the gel.

The gels containing electrophoresed protein were washed in ice cold transfer buffer at 4°C for 10 minutes to remove any remaining SDS. The separated proteins on each gel were then transferred to a PVDF membrane using the following procedure. Pieces of PVDF membrane, (Amersham biosciences) cut to the corresponding gel size were activated by placing in methanol for 10 seconds, followed by subsequent 10 minute washes in water and transfer buffer. The gel and membrane were then placed in the transfer cassette, sandwiched by suitably sized filter paper and fibropads following the order listed:

1. fibropad
2. 2mm X 3mm filter paper
3. PVDF membrane
4. gel
5. 2mm X 3mm filter paper
6. fibropad

The cassette was then placed in an electrophoresis box with ice cold transfer buffer and subjected to 100v for 1 hour and 20 minutes at 4°C, or for 30v overnight at 4°C.

Using molecular markers to map protein size to distance travelled.

During Western blot analysis protein standards were used as a reference to determine the molecular weight of proteins identified by antibody probes. Electrophoresis gels were loaded with precision plus all blue pre-stained standards (Bio Rad) and the protein bands detected through subsequent immuno-blotting mapped to the corresponding ladder of molecular standards to estimate the molecular weight of the protein. The expected molecular weight of each protein detected is listed in Table 2.2. This method was used for the detection of novel proteins. For proteins in which detection was standard in the lab, molecular markers were only used initially.

Ponceau staining of protein bound PVDF membrane

To determine that equal levels of total protein were present for each sample on the PVDF membrane, the membrane was washed once in Tris-buffered saline (TBS) for 10 minutes, and then washed in Ponceau stain for two minutes, followed by three five minute washes in 5% acetic acid, and a final wash in TBS.

Immuno-detection of PVDF bound protein

To detect specific proteins on the protein bound PVDF membranes, the Amersham biosciences protocol was used unless otherwise specified. To summarise, protein bound PVDF membranes were treated with blocking buffer overnight at 4°C in a rolling 50ml centrifuge tube (VWR), and subsequently incubated with primary antibody diluted in 4mls of blocking buffer to the specified concentration (see Table 2.2) for 1hr in a rolling 50ml centrifuge tube at room temperature.

Table 2.2 Details of primary and secondary antibodies used in Western blot analyses.

ANTIGEN	MOLECULAR WEIGHT (KDa)	ANTIBODY ISOTYPE	SOURCE	DILUTION	CATEGORY
β actin	42	Mouse IgG	Sigma	1: 4000	Constitutively expressed
Nestin	220-240	Mouse IgG	Chemicon	1:200	Ectoderm- neural
β III Tubulin	50-54	Rabbit IgG	Covance	1:4000	"
β III Tubulin	50-54	Mouse IgG	Sigma	1:1000	"
NF200 or NFH	200	Rabbit IgG	Chemicon	1:1000	"
MAP2	280, 70	Mouse IgG	Sigma	1:400	"
Vimentin	58	Mouse IgG	Sigma	1: 400	"
TP63 / TP73L	73	Goat IgG	R&D systems	1:200	Ectoderm – epidermist
Keratin 8	55,45	Mouse IgG	Sigma	1:400	"
Keratin 8	55,45	Rabbit IgG	Abcam	1:400	"
α smooth muscle actin	42	Mouse IgG	Sigma	1:400	Smooth muscle
HCG	40	Mouse IgG	Abcam	1:200	Trophoblast
P-Smad 1	60	Rabbit IgG	Chemicon	1:100	BMP pathway
Smad 1	60	Rabbit IgG	Santa Cruz	1:100	"
ID2	15	Rabbit IgG	Santa Cruz	1:200	"
ID3	15	Mouse IgG	Santa Cruz	1:200	"
ERK 1/2	43, 41	Rabbit IgG	Santa Cruz	1:500	MAPK pathway
P ERK 1/2	43, 41	Mouse IgG	Santa Cruz	1:500	"
Mouse IgG	-	horse radish peroxidase conjugate (from sheep)	Amersham	1:1000	Secondary antibodies
Rabbit IgG	-	horse radish peroxidase conjugate (from donkey)	Amersham	1:1000	"
Goat IgG	-	horse radish peroxidase conjugate	Sigma	1:1000	"

Unbound primary antibody was subsequently removed by washing the blots three times for 10 minutes in blocking buffer, and then the blots were incubated with the corresponding secondary antibody conjugated to horse radish peroxidase diluted in blocking buffer at a

concentration of 1:1000 (unless specified otherwise) for 1 hour at room temperature. Details of the secondary antibodies used are listed in Table 2.2. To remove unbound secondary antibody the blots were then washed 3 times for 10 minutes in TBS. To visualise the bound secondary antibody each blot was then exposed to freshly mixed developing solutions 1 and 2 in the dark, placed in a photo-casette, exposed to hyperfilm (Amersham) and the film developed using an X-ograph imaging system compact X-4 automatic X-ray film processor developer or manually.

2.5 IMMUNO-CYTOCHEMISTRY

2.5.1 Adherent cultures

TERA2.sp12 cells were seeded into electrostatically coated NUNC 12 well dishes (VWR) and treated as specifically described in each experiment. At the selected time-points, the media was removed and cultures were fixed in 4% paraformaldehyde, PFA (Sigma) in PBS for 20 minutes at room temperature (unless specified otherwise), then washed three times in PBS for 10 minutes. To reduce non-specific antibody interactions the cells were then incubated for 30 minutes with blocking buffer consisting of 5% goat serum (Invitrogen) or 5% horse serum (Invitrogen), 0.2% triton X 100, (Sigma) and 0.1% bovine serum albumin (BSA, Sigma). After this blocking step the buffer solution was then removed and the cells incubated with 200 μ l of primary antibody diluted in antibody diluent (1% goat or horse serum, 0.2% triton X100, 0.01% BSA) for 1 hour at room temperature or in specified cases at 4°C overnight in a sealed chamber. Table 2.3 lists the details and concentrations at which the primary antibodies were used, and Table 2.4 lists the details and concentrations at which the secondary antibodies were used. In blocking and antibody diluent solutions, goat serum was used except when using antibodies which had been raised in goat, whereupon horse serum was substituted. After the primary antibody incubation step, to remove unbound primary antibodies cells were washed three times in PBS for 10 minutes, and then incubated for 1 hour in the dark at room temperature with 200 μ l of the appropriate secondary antibody diluted in antibody diluent (to the concentration described in Table 2.4). After incubation with the secondary antibody, unbound secondary antibody was removed with four sets of 10 minute washes in PBS and the stained cells were mounted under 16mm diameter round glass coverslips (Fisher) in Vectashield mounting medium (Vector laboratories) with 0.1% of the nuclear stain Hoescht 33342 (Molecular probes).

Table 2.3 Details of primary antibodies used for immuno-staining analyses

ANTIGEN	ANTIBODY	SOURCE	DILUTION	FIXATIVE	CATEGORY	REFERENCE
Oct4	Rat IgG	R&D systems	1:40	PFA	Stem cell	(Niwa <i>et al.</i> 2000, Okamoto <i>et al.</i> 1990, Scholer <i>et al.</i> 1990)
Nanog	Goat IgG	R&D systems	1:50	PFA	"	(Mitsui <i>et al.</i> 2003)
Tra 160	Mouse IgM	Hybridoma	1:5	PFA no TX100	"	(Andrews <i>et al.</i> 1984, Badcock <i>et al.</i> 1999)
SSEA 4	Mouse	Hybridoma	1:5	PFA no TX100	"	(Shevinsky <i>et al.</i> 1982)
BrDu	Mouse IgG	Hybridoma	1:40	PFA then treat with 4MHCL	Dividing cell	
Nestin	Mouse IgG	Chemicon	1:400	PFA	Ectoderm-neural progenitor	(Lendahl <i>et al.</i> 1990)
Musashi	Rat IgG	GIFT	1:200	PFA	"	(Kaneko <i>et al.</i> 2000)
β III Tubulin (TUJ1)	Rabbit IgG	Covance	1:400	PFA	Ectoderm – neuronal	
β III Tubulin	Mouse IgG	Sigma	1:200	PFA	"	
NF160 or NFM	Mouse IgG	Chemicon	1:100	PFA	"	
NF200 or NFH	Rabbit IgG	Chemicon	1:400	PFA	"	
NF68 or NFL	Mouse IgG	sigma	1:100	PFA	"	
MAP2	Mouse IgG	Sigma	1:200	PFA	"	
GFAP	Mouse IgG	Sigma	1:100	Methanol	Ectoderm-glial	
S100	Rabbit IgG	Abcam	1:200	PFA	Ectoderm-astrocytes	
TP63 / TP73L	Goat IgG	R&D systems	1:50	PFA	Ectoderm – epidermis	(Pellegrini <i>et al.</i> 2001)
Keratin 8	Mouse IgG	Sigma	1:200	PFA	"	
Keratin 8	Rabbit IgG	Abcam	1:100	PFA	"	
Keratin epithelial	Mouse IgG	Chemicon	1:400	PFA	"	
Fibronectin	Mouse IgG	Sigma	1:100	PFA	"	
Gata 4	Goat IgG	R&D systems	1:50	PFA	Endoderm	(Arceci <i>et al.</i> 1993)
AFP	Mouse IgG	Sigma	1:100	PFA then treat for 1 minute with ethanol	"	
HNF3 β	Goat IgG	R&D systems	1:50	PFA	"	(Ang <i>et al.</i> 1993, Heins <i>et al.</i> 2004)
Brachyury	Goat IgG	R&D systems	1:50	PFA	Mesoderm	(Hermann <i>et al.</i> 1990)
Alpha smooth muscle actin	mouse IgG	Sigma	1:100	Methanol with acetic acid	developing muscle	
BMP 2/4	Goat IgG	R&D systems	1:50	PFA		
HCG	mouse IgG	Abcam	1:50	PFA	Trophoblast	(Muyan & Boime 1997, Xu <i>et al.</i> 2002)
Keratin 8	Rabbit IgG	Abcam	1:100	PFA	"	

To control for non-specific binding of the secondary antibodies, some culture wells were incubated in antibody diluent without a primary antibody, and subsequently incubated with secondary antibodies as described above. In the case of antibodies that were not routinely used, to control for non-specific binding of the primary antibody cultures of other human cell

lines such as FaDU, HepG2 and IMR32 cells were immuno-stained in parallel, with HepG2 and FaDU cells used as a negative control for neural antibodies, IMR32 cells used as a negative control for non-neural ectoderm associated antibodies and so on. Positive controls for each antibody are described in specific experiments. Stained cultures were examined under a Nikon diaphot 30 fluorescence microscope with filters suitable for the detection of fluorescence of fluorescein thiocyanate (FITC), Alexa flour 488, Cy3 and hoescht dyes. Pictures were taken with a Nikon digital camera DXM1200 using ACT-1 software and overlay images created and processed using Adobe Photoshop software.

Table 2.4 Details of secondary antibodies used for immuno-staining analyses

ANTIGEN	ANTIBODY	SOURCE	DILUTION
Rat IgG	Rabbit conjugated to Fluorescein isothiocyanate (green)	Sigma	1:100
Goat IgG	Rabbit conjugated to Fluorescein isothiocyanate (green)	Sigma	1:100
Mouse IgG	Donkey Conjugated to Cy3 (red)	Jackson Immuno-Research Labs	1:600
Rabbit IgG	Goat Conjugated to Cy3 (red)	Jackson Immuno-Research Labs	1:600
Mouse IgG	Donkey conjugated to Alexa flourophore 488 (green)	Molecular probes	1:600

Methanol based fixation methods

Where indicated, instead of PFA fixation, the following methods of methanol based fixation were used:

1. Methanol fixation: Cells were rinsed in PBS and then fixed in ice-cold methanol, (Fisher) for 10 minutes.
2. Methanol-acetic acid fixation: Cells were rinsed in PBS and then fixed in ice-cold methanol with 5% acetic acid (Fisher) for 10 minutes.
3. Methanol-acetone fixation: Cells were rinsed in PBS, then fixed in 50:50 cold Methanol/Acetone (Fisher) for 10-15 minutes, then allowed to air-dry for 10 minutes. (Cells could then be stored at -20°C and hydrated with PBS 5 minutes prior to staining).

2.5.2 Suspension cultures

Fixing and sectioning aggregates

TERA2.sp12 cells were seeded into uncoated 90mm triple vent Bacteriological Petri dishes, (VWR) at a density of 20 000 cells/cm² and treated as specifically described in each experiment. At the specified time points, the contents of the Petri dish for each treatment was transferred into individual 15ml centrifuge tubes and centrifuged for 2 minutes at 64g, 4°C to pellet the aggregates. The supernatant was then removed, and the cells re-suspended in PBS, before being centrifuged once more as above. To fix the aggregates the PBS was removed, the

aggregates re-suspended in 4% PFA and incubated with gentle rolling for 20-30 minutes at room temperature. After fixation, aggregates were washed 3 times for 10 minutes in PBS by consecutive centrifuging, supernatant removal and re-suspension steps, before being cryo-protected by incubation in 30% sucrose (Invitrogen) solution in PBS on a roller at 4°C overnight. After centrifugation for 2mins at 64g, 4°C, the sucrose solution was removed and replaced with OCT embedding medium (RA lamb), and incubated at room temperature on a roller. After 20 minutes aggregates were transferred to a small mould made from aluminium foil and frozen slowly at -20°C, before being transferred to -80°C. Ten micrometer sections were made on a Leica CM3050 S cryostat, using DB80L disposable microtome blades (Leica), collected on histobond glass slides (RA Lamb) and stored at -80°C.

Immuno-staining of sections

Immuno-staining of slides of aggregate sections was carried out as described for adherent cultures, except prior to processing slides were air dried for up to 30 minutes, and to contain the antibody solutions on the slides a wax PAP pen was used to outline the cryostat sections on each slide. Each slide was then gently washed in PBS before being incubated in blocking buffer, and in all cases slides were incubated with primary antibodies overnight in a humidified chamber. Stained sections were mounted under 24mm X 50mm glass coverslips (VWR) in Vectashield mounting medium with 0.1% Hoescht, and the edges sealed with clear nail varnish.

To control for non-specific binding of the secondary antibodies some slides were incubated in antibody diluent without primary antibody, and subsequently incubated with secondary antibodies as described above.

2.5.3 Tissues

Tissues were either perfusion fixed in PFA prior to extraction and placed in PBS in a 50ml centrifuge tube, or transferred into a 50ml centrifuge tube, washed in PBS and then fixed in 4% PFA for 40 minutes or overnight at 4°C depending on the specific tissue (as described for each experiment).

OCT embedding and sectioning

The solution of 4% PFA was carefully removed, replaced with PBS and the tissue incubated on a roller for 30 minutes, with the PBS replaced with fresh PBS every 10 minutes. The tissue was then cryo-protected by incubation in 30% sucrose solution in PBS on a roller at 4°C overnight. Fifty percent of the sucrose solution was then removed and replaced with OCT embedding medium, and incubated on a roller for 20 minutes. Then the solution was replaced with 100% OCT embedding medium, and the tissue incubated for 20 minutes on a roller. The

tissues were then transferred to a small aluminium foil mould filled with fresh OCT medium and frozen at -20°C , before being transferred to -80°C . Ten micrometer sections were made on a Leica CM3050 S cryostat using disposable microtome blades, collected on histobond glass slides and stored at -80°C .

Paraffin wax embedding and sectioning

The solution of 4% PFA was carefully removed, replaced with PBS and the tissue incubated on a roller for 30 minutes, with the PBS replaced with fresh PBS every 10 minutes. Tissues were incubated for 30-50 minutes in the following dehydration series, 25%, 50%, 75% and 100% ethanol, (depending on the size of the tissue). The ethanol was subsequently replaced with histoclear (National diagnostics). After approximately 1 hour (depending on the size of the tissue) the histoclear was removed and replaced with fresh histoclear, and the tubes transferred to an oven at 55°C .

After 30 minutes 50% of the histoclear was removed and replaced with filtered molten wax (55°C) and after an hour the supernatant was removed and replaced with fresh filtered molten wax. This step was repeated after another hour, (or longer depending on the size of the tissue) and then the tissue was transferred into a mould containing fresh molten wax, allowed to cool and stored at 4°C . Eight micrometer sections of aggregates were cut on an RM2125 microtome (Leica), using disposable microtome blades. Paraffin ribbons of sections were then floated on $40-43^{\circ}\text{C}$ water to smooth out and eliminate folds and wrinkles, mounted on histobond slides and allowed to air dry overnight or for up to 8 hours at 40°C .

Immuno-staining of tissue sections

OCT embedded tissue sections were immuno-stained using the same protocol described for aggregate sections. Paraffin wax embedded tissue sections were first processed for antigen retrieval prior to immuno-staining as described in the following protocol.

Rehydration and antigen retrieval of wax embedded sections

The slides were placed in the specified solutions for the specified times as listed below.

1. Histoclear – 4 minutes
2. Histoclear – 4 minutes
3. 100% ethanol – 1 minute
4. 95% ethanol – 1 minute
5. 70% ethanol – 1 minute
6. Distilled water – 5 minutes
7. Distilled water – 5 minutes

Following rehydration, the slides were placed in 10mM citrate buffer (pH 6), boiled for 5 minutes in a microwave oven, allowed to rest for 30 seconds, then boiled for a further 5

minutes, and allowed to cool for 20 minutes. The slides were then washed three times in PBS for 10 minutes, prior to proceeding with the immuno-staining protocol described for aggregate sections in Section 2.5.2.

2.5.4 Double Immuno-labelling

To further characterise cell phenotypes, many cell cultures and tissue sections were double immuno-labelled. In this case the slides or well plates were treated following the described immuno-staining method except both primary antibodies were added at the recommended dilutions to the same antibody diluent prior to staining. Secondary antibodies conjugated to either green or red fluorescence emitting constructs (a combination of Cy3-conjugated secondary and either FITC or Alexiflour conjugated secondary antibodies) were also mixed in the same antibody diluent prior to application. Care was taken to only use combinations of primary antibodies that were raised in different hosts, and also to ensure that the secondary antibodies were not raised in the same host as either of the primary antibodies. In effect only mouse-rabbit or mouse-goat double immuno-labelling was possible with the antibodies available. All antibodies were previously used alone to control for changes in antibody staining behaviour in double-immunolabelling preparations. To control for non-specific binding of the secondary antibodies, cells were processed in parallel but with no antibodies used in the primary incubation step.

2.6 HISTOLOGICAL STAINING

Haematoxylin and eosin staining was used to investigate the structure of aggregates formed under suspension conditions or the structure of tumours derived from injection of TERA2.sp12 cells or HNCL-1 human ES cells into immune deficient mice. Haematoxylin is a basic dye that stains acidic structures, (RNA/DNA containing nuclei, ribosomes and rough endoplasmic reticulum). Eosin is an acidic dye that stains basic structures red or pink (as most cytoplasmic proteins are basic the cytoplasm stains pink). Aggregates were collected, fixed in 4% PFA and embedded in OCT embedding medium as described in Section 2.5.2, or embedded in filtered wax, (RA lamb) using the method described below. Tissues were fixed in 4% PFA, embedded in paraffin wax and sectioned as described previously in Section 2.5.3.

2.6.1 Preparation of wax embedded sections

Aggregates were transferred to 15 ml centrifuge tubes and washed in PBS as previously described, then centrifuged for 2mins at 64g, 4°C and the PBS removed, and incubated for 20 minutes in with the following dehydration series, 25%, 50%, 75% and 100% ethanol, (Fisher) using consecutive centrifugation and supernatant replacement. The ethanol was subsequently

replaced with histoclear, (National diagnostics). After 20 minutes the histoclear was removed and replaced with fresh histoclear, and the tubes transferred to an oven at 55°C.

After a further 20 minutes 50% of the histoclear was removed and replaced with filtered molten wax (55°C) and after 30 minutes the supernatant was removed and replaced with fresh filtered molten wax. After another 30 minutes the aggregates in wax were transferred into a pre-warmed glass embryo dish and the wax allowed to cool, before being stored at 4°C. The wax was then trimmed to fit a mounting block and 8µm sections of aggregates were cut on an RM2125 microtome (Leica), using disposable microtome blades. Paraffin ribbons of sections were then floated on 40-43°C water to smooth out and eliminate folds and wrinkles, mounted on histobond slides and allowed to air dry overnight or for up to 8 hours at 40°C.

2.6.2 Staining of sections with Haematoxylin and Eosin

Wax embedded sections

Prior to haematoxylin and eosin staining, the paraffin was removed from sections and the slides re-hydrated following by placing the slides in following solutions for the specified times listed below:

1. Histoclear – 5 minutes
2. Histoclear – 5 minutes
3. 100% ethanol – 1 minute
4. 95% ethanol – 1 minute
5. 70% ethanol – 1 minute

OCT embedded sections

OCT sections were rapidly removed from -80°C storage and without allowing the section to dry or thaw, immersed in 70% ethanol for 30 seconds. Then the slides were processed following the standard haematoxylin and eosin staining protocol below.

Staining protocol

The slides containing sections were placed in the following solutions for the specified times listed below:

1. Distilled water – 1 minute
2. Mayer's Haematoxylin -Ehrlich- naturally ripened, (RA lamb) – 10 seconds
3. Distilled water – 30 seconds
4. Blueing reagent (alkaline alcohol) – 5-10 seconds
5. 70% ethanol – 30 seconds
6. 1% aqueous eosin (RA lamb) – 10 seconds

7. 95% ethanol – 30 seconds
8. 95% ethanol – 30 seconds
9. 100% ethanol – 30 seconds
10. 100% ethanol – 30 seconds
11. HistoClear – 4 minutes

Stained sections were mounted under 24mm X 50mm glass coverslips using high viscosity formula DPX (RA Lamb) and viewed with phase contrast microscopy using a Nikon Diaphot 30 microscope. Pictures were taken with a Nikon DXM1200 digital camera using ACT-1 software.

2.7 GENE EXPRESSION ANALYSIS

Polymerase chain reaction (PCR) analysis was used to determine the expression of specific genes in TERA2.sp12 cultures. Culture samples were collected as pellets as for Western blot analysis in Section 2.4 except gloves, RNase free filter barrier pipette tips (Thistle) and PCR clean eppendorf tubes were used at all times.

Isolation of RNA

RNA was isolated from cell pellets following the manufacturer's instructions for isolation of RNA with Tri-reagent (Sigma), as outlined in the steps below.

1. 1ml of Tri-Reagent added to each pellet (approximately 1 million cells).
2. Contents pipetted repeatedly to lyse pellet.
3. Allowed to stand for 5mins (room temperature).
4. 200ul Chloroform (Fisher) per ml of Tri-Reagent added to each tube.
5. Tubes shaken vigorously for 15 seconds.
6. Allowed to stand for 15mins at room temperature.
7. Tubes centrifuged at 12,000g, 4°C for 15 minutes to separate the solution into 3 phases: A red base layer (the organic phase – containing mainly proteins), a white inter-phase layer (containing DNA), and the uppermost phase- a colourless aqueous layer (containing RNA).
8. The aqueous uppermost layer was transferred into a fresh 1.5ml eppendorf tube.
To remove DNA contaminants, the following additional steps were carried out
9. Mixed with one tenth of the total volume of isopropanol to be added to the aqueous RNA solution (50µl).
10. Allowed to stand for 5 minutes at room temperature.
11. Samples centrifuged 12,000g for 10 minutes at 4°C to precipitate any contaminating DNA at the base of the eppendorf tube.

12. The supernatant was transferred to a fresh tube.
13. For each tube the remaining amount of isopropanol ($500\mu\text{l}$ minus $5\mu\text{l}$ = $450\mu\text{l}$) was added to precipitate the RNA, and the tubes allowed to stand for 5-10 minutes at room temperature or overnight at -20°C .
14. To pellet the RNA samples were centrifuged at $12,000g$, 4°C for 10 minutes.
15. The supernatant was removed and the pellet washed by adding approximately 1ml of 75% RNase free ethanol and vortexing.
16. The samples centrifuged at $7,500g$, 4°C for 5 minutes, and the supernatant removed.
17. An additional brief centrifuge (10 seconds) used to allow the collection of any remaining supernatant that had collected on the sides of the tube.
18. The pellet was allowed to dry by allowing the tube to stand for 5 minutes (depending on the size of the pellet) at room temperature with an open lid (care was taken not to over-dry the pellet)
19. Approximately $50\mu\text{l}$ of RNase free water was added to each pellet (depending on the pellet size), and the solution stored at -20°C .

Determining the concentration of sample RNA solutions and assaying for RNA degradation

For each sample disposable semi micro-cuvettes suitable for wavelength absorbance at 260-280nm (Fisher) were loaded with $995\mu\text{l}$ water and $5\mu\text{l}$ sample. As a blank an additional cuvette was loaded with $1000\mu\text{l}$ of water. Sample absorbance was then measured at 260nm using a Helios β mass spectrometer and the sample concentration calculated on the basis that $40\mu\text{g}$ of RNA/ml will have an absorbance at 260nm of one. (For each cuvette sample the absorbance reading was multiplied by 40 and by the dilution factor to calculate original RNA concentration in the corresponding sample). To assay for RNA degradation, $5\mu\text{g}$ of each sample was loaded with blue juice loading buffer (Promega) into a 1% agarose gel prepared following the recipe listed in the appendix, and electrophoresed for 40 minutes at 80 volts. To visualise the bands of RNA the agarose gel was then viewed under UV light.

Reverse transcription and PCR analysis

To convert the RNA to DNA and detect selected gene transcripts by primer specific amplification the access quick RT-PCR system (Promega) was used according to the manufacturer's instructions, as described in the following list.

1. For each 50 μ l reverse transcription reaction, combine:
 - a. 25 μ l Master mix.
 - b. 10 μ M upstream primer.
 - c. 10 μ M downstream primer.
 - d. RNA template, (0.1-1 μ g)
 - e. Nuclease free water to a final volume of 50 μ l.
2. Vortex samples.
3. Add 1 μ l AMV Reverse Transcriptase as the final component and mix by gentle vortexing.
4. Incubate tubes at 45°C for 40-45 minutes.
5. Proceed with PCR cycling as below:
6. Initial denaturation at 95°C, (2mins)
7. Then 30 cycles of annealing, (55-65°C as detailed for each primer), 1min for extension at 70°C, 1min for denaturation at 95°C.
8. Soak at 4°C overnight.

Primer sequences were obtained from published papers or designed using the Primer DNASTar package, and primers were ordered from MWG-Biotech. Details of primers and the annealing temperature used are specified in each experiment.

To detect the PCR products 1 μ l of blue juice dye was added to 15 μ l of each sample and the mixture subsequently loaded onto a 1 % agarose gel and electrophoresed at 80 volts for 40-60 minutes, according to the predicted size of the PCR product. The PCR products in the agarose gel were subsequently visualised using a transilluminator (Gel Doc 2000, Bio Rad).

2.8 GENERATION OF TUMOURS IN IMMUNE-DEFICIENT MICE

Preparation of TERA2.sp12 cells for injection

For each batch of injections, a confluent flask generated from TERA2.sp12 cells cultured under maintenance conditions was used and cells prepared for injection using the following procedure: A confluent T75 flask of TERA2.sp12 cells was washed in PBS then individualised using Trypsin, and counted using a haemocytometer as described in Section 2.3. In summary, cells were incubated at 37°C for 2-3 minutes with 2mls of 0.5X Trypsin in EDTA, and gentle tapping used until the cell layer had dispersed. Once cells were individualised 9mls of media was added to inactivate the Trypsin, and gentle pipetting used to aspirate cells. Ten micro-litres of solution was then loaded onto a haemocytometer, and cell counts performed using a phase contrast light microscope (Nikon eclipse TS100). The cells were counted twice, and if any unusual cell counts were collected the counts were repeated.

Haemocytometer counts were then used to estimate the concentration of cells in each sample (count multiplied by 10,000), and the total cell number estimated by multiplying this value by the total volume of solution (10mls).

The suspension of cells was then centrifuged at 129g 4°C for 2 minutes, the supernatant removed, the cells gently re-suspended in PBS and then this step was repeated. Care was taken to ensure the cells were thoroughly washed as it is essential that no media is injected into the mouse host. After the final wash the cells were suspended in a defined amount of PBS such that 1ml was added for every million cells. The cells were gently aspirated to ensure an even distribution and then 50µl samples of cell suspension (for each mouse to be injected) were pipetted to form individual drops in a sterile bacteriological dish, such that each formed a meniscus of cell suspension with 50,000 cells in 50µl. Then for each meniscus, using a sterile 1 ml disposable syringe (VWR) and sterile gauge 21 syringe needle (Fisher), the solution of cells was taken up into the syringe, the sterile needle cap replaced and the syringe placed upright in ice ready for injection.

Subcutaneous injection of TERA2.sp12 cells into immune-deficient mice

All procedures involving mice were carried out in accordance with institution guidelines and institution permission, using licensed procedures and according to Home Office guidelines. The cells were injected subcutaneously on the left flank into nude (*C57BL/6J-Hfh1/nu*) adult male mice. Injections were performed by Stefan Przyborski or Alison Ritchie (manager of the Biological Sciences animal unit).

2.9 TISSUES USED AS POSITIVE CONTROLS

Human placental tissue

As a positive control for trophoblast markers, day 19 human placenta villous tissue was kindly donated by Dr Lisgo from University of Newcastle. Pieces of villous tissue were dissected and rapidly frozen in liquid nitrogen prior to storage at -80°C for use in Western blot analysis. The remaining placental villous tissue was washed in PBS before being fixed in 4% PFA for 40 minutes. After fixation, the placental tissue was washed 3 times for 10 minutes in PBS and then cryo-protected in 30% sucrose at 4°C overnight. The sucrose solution was then removed and replaced in stages with OCT embedding medium from RA lamb. After 30 minutes the tissue was transferred to a small mould made from aluminium foil, frozen slowly at -20°C, and transferred to -80°C. Ten micrometer sections were made on a Leika cryostat, collected on Histobond slides and stored at -80°C.

CHAPTER 3
RETINOIC ACID INDUCED DIFFERENTIATION OF
TERA2.SP12 CELLS

3.1 INTRODUCTION

The following introduction reviews the retinoic acid (RA) signalling pathway, discussing the key roles of RA in early development and also its use as a potent inducer of stem cell differentiation. Subsequently RA induced differentiation in the context of TERA2.sp12 cells is described, and two alternative culture methods of RA induced differentiation are introduced which are reported to generate markedly different proportions of neurons from TERA2.sp12 cells. Finally the objective of the investigations in this chapter is proposed, namely to compare and characterise the differentiation of TERA2.sp12 cells under these alternate culture conditions.

3.1.1 Retinoic acid – a key morphogen *in vitro* and *in vivo*

Retinol (vitamin A) and its retinoid derivative retinoic acid (RA) play a fundamental role in the development of mammalian embryos as well as other chordate embryos: Mouse and quail retinoid knockout models have linked vitamin A function to the development of the heart, the embryonal circulation and to the central nervous system (Maden *et al.* 1996, for review Ross *et al.* 2000), and most remarkably, in the absence of sufficient vitamin A the embryo is eventually reabsorbed (Wellik & Deluca 1995).

Retinol is obtained by cells of the embryo or adult from the blood, where it circulates bound to retinol binding protein (Maden 2002). Upon entering the cell, retinol is bound to cellular retinol binding protein (CRBP) and then metabolised into RA in two oxidation steps, as described in Figure 3.1.A. Retinol is converted initially by retinol dehydrogenases into retinal, and subsequently by the retinal dehydrogenases (RALDHs) into RA. There are several members of each of these enzyme classes, and the most important ones for the embryo are RALDH1, RALDH2 and RALDH3. Cells that require RA also contain cellular RA-binding proteins (CRAB1 and CRAB2), which bind to RA in the cytoplasm (for review see Maden 2002). Upon entering the nucleus RA serves as a ligand that controls the action of nuclear RA receptors (Mangelsdorf *et al.* 1994) either in the cells where it is generated or in neighbouring cells via paracrine signalling (Mic *et al.* 2002). These nuclear receptors are heterodimers and function as RA activated transcription factors, recognising consensus sequences termed retinoic acid response elements (RAREs) in the control elements of RA responsive genes. A summary of the cellular mechanism of retinoid action is depicted in Figure 3.1.B. Alternatively, RA can be further metabolised into inactive products by two cytochrome p450 enzymes (CYP261A CYP261B) as shown in Figure 3.1.A, although there is evidence that some of these retinoids are also bioactive modulators of development (Pijnappel *et al.* 1993).

RA can exist in two isoforms, as either all-*trans* RA or 9-*cis* RA, which act through

Pathways For The Synthesis And Mechanism Of Action Of Retinoic Acid

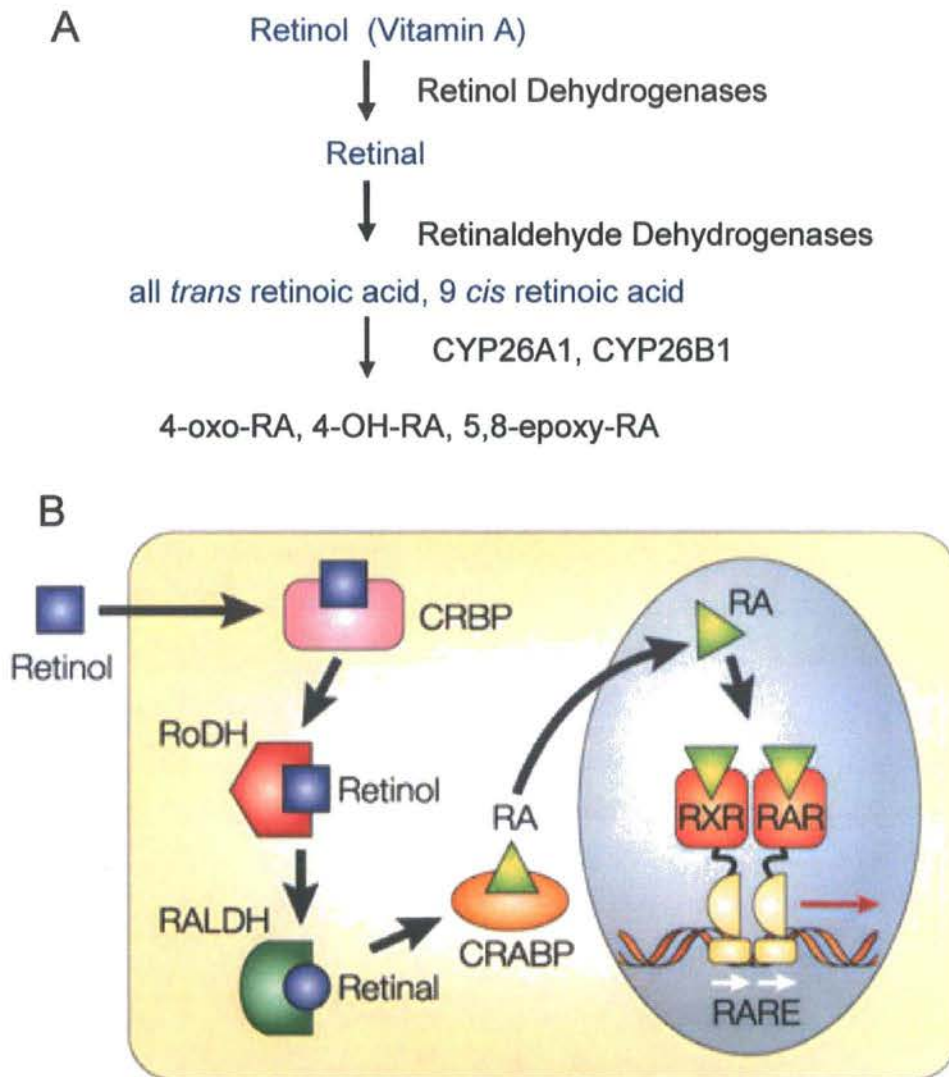


Figure 3.1 Pathways for the synthesis and mechanism of action of retinoic acid.

A. The metabolic pathway that converts vitamin A (retinol) into various isoforms of retinoic acid (RA), which involves three classes of enzymes, the retinol dehydrogenases, the retinaldehyde dehydrogenases and the cytochrome p450 enzymes CYP26A1 and CYP26B1.

B. The cellular mechanism of retinoid action. Retinol is taken up from the blood and bound to CRBP (cellular retinol-binding protein) in the cytoplasm. The retinol dehydrogenase enzymes (RoDH) metabolise retinol to retinal then retinal is metabolised to RA by the retinaldehyde dehydrogenases (RALDHs). RA is bound in the cytoplasm by CRABP (cellular RA-binding protein). RA enters the nucleus and binds to the RA receptors (RARs) and the retinoid X receptors (RXRs), which themselves heterodimerise and bind to a sequence of DNA known as the retinoic acid response element (RARE). This activates transcription of the target genes. Figure and legend copied from Figure 1 in (Maden 2002).

different nuclear receptors. In human, rat and mouse, there are two classes of these RA receptors, the retinoic acid receptors (RARs) RAR α , RAR β RAR γ , which are activated by all-trans RA and 9-cis RA, and the retinoid X receptors (RXRs) RXR α , RXR β RXR γ which are only activated by 9-cis RA (Heyman *et al.* 1992, Levin *et al.* 1992, for review Maden 2000). All *trans*-RA has been reported to be the main biologically active retinoid occurring during embryogenesis in zebra fish (Costaridis *et al.* 1996), and most studies to date use the readily available all-*trans* RA isoform. Correspondingly an embryonic lethal mutation in which RA synthesis is inhibited can be rescued by retinoid activation of RARs but not RXRs (Mic *et al.* 2003).

In early studies, ectopic addition of RA was demonstrated to have a marked teratogenic effect on normal embryonic development, with embryos presenting abnormalities remarkably similar to that of vitamin A deprivation (Fantel *et al.* 1977, Kochhar 1973, Shenfelt 1972). This is an important demonstration that although RA is necessary for normal embryogenesis to occur, it is required at precisely controlled levels within the developing embryo. Correspondingly, the pathway of retinol metabolism and mechanism of RA mediated gene regulation as summarised in Figure 3.1, presents several points at which a cells response to retinol can be regulated: Through the regulation of retinol uptake from the blood, the presence of enzymes to convert retinol to RA or to degrade RA, the presence of RARs and RXRs and the presence of further co-activators and repressors that interact with the retinoic acid nuclear receptors (Maden 2002).

Retinoic acid – a universal differentiation agent?

RA promotes the differentiation of a variety of progenitor cell lines (Mogi *et al.* 2005, Takahashi 1999) and *in vitro* studies demonstrate that RA can induce an inordinate number of genes (reviewed in Sporn *et al.* 1995). To this effect it has been described as a universal differentiation agent (McCaffery & Draeger 2000). However, the extensive range of genes induced by RA is typically a result of secondary or even tertiary transcriptional responses, and the number of genes containing retinoic acid response elements in their promoters and that can be directly transcriptionally regulated by RA is much lower. Table 3.1 lists some of the genes reported to contain RARE in their promoters.

In the embryo, genes directly regulated by RA include key transcription factors (such as the homeobox hox genes), signalling molecules (Sonic hedge hog) and enzymes (Protein kinase C), each of which can activate multiple pathways of development (for review McCaffery & Draeger 2000). This contributes to the powerful morphogenetic effects of RA and its attributes as a key trigger of differentiation. The range of genes activated directly and indirectly by RA also depends on the transcriptional co-activators and inhibitors present in

each cell, and the concentration of RA (McCaffery & Draeger 2000). As a consequence, the response of a cell to RA is highly dependent on the context of the cell type and the level of exposure to RA.

Table 3.1 List of genes with functional RAREs (Taken from Table 1 of McCaffery & Draeger 2000)

RA SIGNALLING COMPONENTS	RAR α 2 RAR β 2 CRBP II CRABP II
TRANSCRIPTION FACTORS AND CO-FACTORS	Hepatocyte Nuclear Factor -3 α Hox genes a1,b1,b4,d4 Oct3/4 (repression) Stat1
ENZYMES	Cholesteryl ester transfer Phosphoenolpyruvate carboxykinase Protein kinase C 17 beta-hydroxysteroid dehydrogenase type 1 Tissue-type plasminogen activator Medium chain acyl-coenzyme A dehydrogenase
NEUROTRANSMITTER RECEPTORS	Beta 1-adrenergic receptor Dopamine D2 receptor
HORMONE RELATED/SIGNALLING FACTORS	Gonadotropin-releasing hormone Thyroid stimulating hormone-beta Oxytocin Sonic hedgehog
ADHESION PROTEINS	Intercellular adhesion molecule-1 Laminin B1 Intercellular adhesion molecule-1 Laminin B1
OTHER	α fetoprotein

Retinoic acid in embryonic development – patterning the CNS system

In the developing mouse and chick embryo all the main regions of the embryo were detected to contain endogenous RA, but at varying levels (Horton & Maden 1995, Maden *et al.* 1998). In both cases the highest levels of RA were detectable in the neural tube, with decreasing levels found in the somites, eye, tail bud, fronto-nasal mass, branchial arches, limb buds and heart (see review Maden 2000). Within the neural tube, the levels of RA detected were not uniform – the region which would form the spinal chord had the highest levels, whereas the forebrain and midbrain had virtually undetectable levels, and the hindbrain intermediate levels of RA.

RA has since been demonstrated to play key roles in neurogenesis and the patterning of both the anterior posterior and dorsal-ventral axes of the neural tube (for review Maden 2002). Proneural genes that control the process of primary neurogenesis have been found to be controlled by RA in vertebrates, including prepattern genes (X-ngnr-1, X-MyT1), neurogenic genes (X-delta-1) and RA is also responsible for down-regulating genes that inhibit

neurogenesis (*Zic2*, *X-shh*) (Franco *et al.* 1999, Papalopulu & Kintner 1996, Sharpe & Goldstone 2000, Sharpe & Goldstone 1997). Correspondingly manipulations in the level of RA in *Xenopus* embryos regulate the number of primary neurons formed (for review Maden 2002).

The potent effect of a RA gradient on the anterior-posterior patterning of the neural tube is demonstrated by the concentration dependent effect of RA on the expression of different classes of Hox genes, each of which activates a different sub-set of cell-type specific genes. Hox homeobox genes exhibit segmental expression in the developing hindbrain and are intimately involved in the formation and identity of the eight rhombomeres that constitute the mouse hindbrain (Krumlauf 1993, Wilkinson 1993), and several members of the mammalian Hox gene family are direct targets of RA signaling during hindbrain development, including *Hox b1* (Simeone *et al.* 1990). Complexes of homeobox hox genes respond differently to RA, following a trend whereby Hox genes positioned at the 3' end of the complex are activated more rapidly and to lower doses of RA than genes positioned more 5' within the complex (for reviews Lufkin 1996, 2006).

Retinoic acid induced differentiation in embryonic stem cells

The differentiation of pluripotent stem cells has proved an invaluable tool in characterising early lineage decisions in mammalian embryonic development, and neural induction by RA in embryonal carcinoma cell lines and mouse embryonic stem cells has been extensively studied, (as previously discussed in chapter 1). To re-capitulate, RA was one of the first chemicals discovered to induce differentiation in embryonal carcinoma cell lines *in vitro*. When treated with RA, a subset of responsive embryonal carcinoma cells rapidly lost their malignant stem cell phenotype (Mummery *et al.* 1987, Rayner & Graham 1982). This applied to mouse embryonal carcinoma cells such as P19, F9 and the human embryonal carcinoma cell line NTERA2 (Alonso *et al.* 1991, Andrews 1998, McBurney 1993). Human EC cell lines NT2D1, TERA2.sp12 and the mouse EC line P19 have all been reported to respond to RA by differentiating along neural lineages (Andrews 1984, McBurney 1993, Przyborski 2001). Correspondingly, in mouse embryonic stem cells, the low levels of neural differentiation observed upon removal from feeder layers and culture to form embryoid bodies are markedly up-regulated if aggregated cells are treated with high concentrations of RA, and mesodermal gene expression is actively repressed (Bain *et al.* 1995, Bain *et al.* 1996, Fraichard 1995, Strubing *et al.* 1995).

As discussed in Chapter 1, TERA2.sp12 cells are presented a suitable model in which to study aspects of ectoderm development, as their differentiation behaviour suggests that unlike human ES cells, they more closely resemble cells of the ectoderm than cells of the

inner cell mass. Currently there are two alternative culture methods of differentiating TERA2.sp12 cells with RA, which generate markedly different responses.

3.1.2 Alternative methods of culturing retinoic acid treated TERA2.sp12 cells

TERA2.sp12 cells are routinely cultured on adherent electrostatically-coated plastic-ware, and upon differentiation with RA under these conditions, a small proportion of the population differentiates into maturing neurons. However, after 6 weeks of treatment with RA, aggregate-like formations are observed and these frequently detach from the culture surface and float in the media. When isolated and re-plated, cells growing out of these aggregate structures demonstrate marked morphological evidence of neural differentiation.

In an effort to promote neural differentiation, TERA2.sp12 cells were differentiated with RA while cultured in non-adherent conditions to form aggregates in suspension. Figure 3.2, published in (Horrocks *et al.* 2003) summarises the two alternative methods of TERA2.sp12 culture and differentiation with RA. These initial studies indicated that TERA2.sp12 cells cultured as aggregates and treated with RA prior to plating on an adherent surface generated markedly increased numbers of morphologically identifiable neurons compared to adherent cultures grown in parallel (Stewart *et al.* 2003). One possible explanation for the increased numbers of neurons observed in RA treated suspension cultures is that in these cultures an increased proportion of cells are differentiating into a neural fate. If this is indeed the case, comparison of these two culture systems would provide a simple basis from which to investigate mechanisms regulating neural induction in TERA2.sp12 cells, as a model of human ectoderm development. Accordingly, the central aim of the experiments in this chapter is to characterise and systematically compare the early stages of differentiation of TERA2.sp12 cells when induced to differentiate with RA under suspension conditions versus adherent conditions, in order to address the following questions:

1. Is neural differentiation indeed enhanced in RA treated TERA2.sp12 cells dissociated and cultured in suspension to form aggregates?
2. Is this up-regulation of neural differentiation associated with a reduction of differentiation into non-neural (putative epidermis) cell types?
3. What markers are expressed by the non-neural population of cells induced by RA treatment – can this population be definitively characterised as epithelial cells of the prospective epidermis?

A summary of the aims, objectives and experiments carried out is presented as a strategy flow chart in Figure 3.3.

Retinoic Acid Induced Neural Differentiation And The Formation Of Aggregates In TERA2.sp12 Cells Cultured Using Two Different Methods.

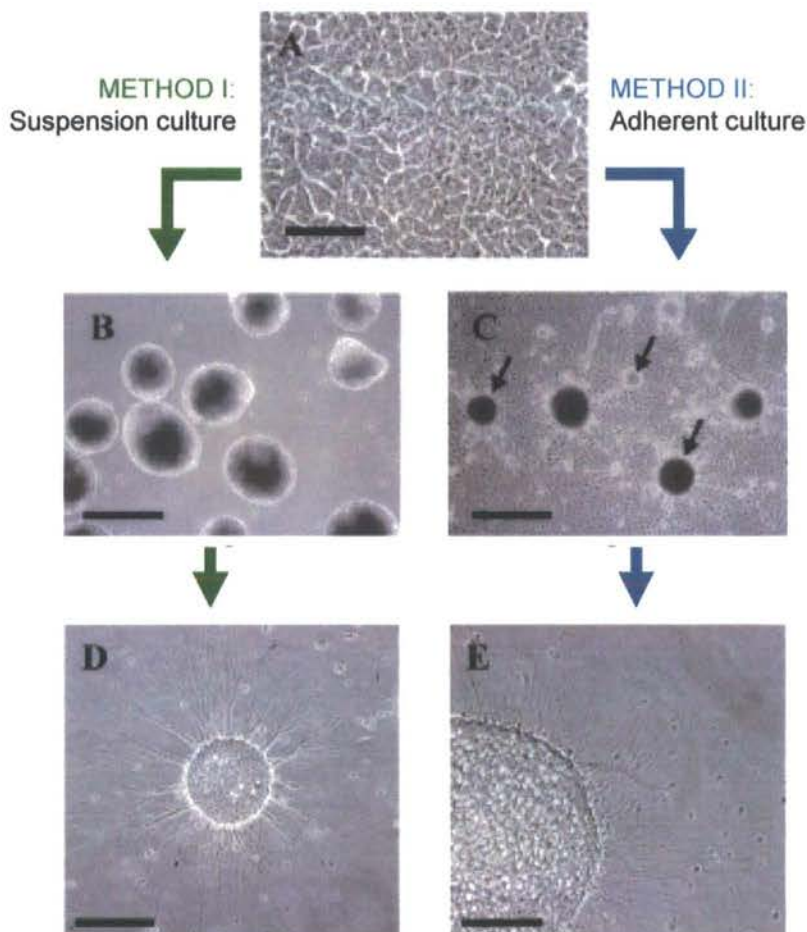
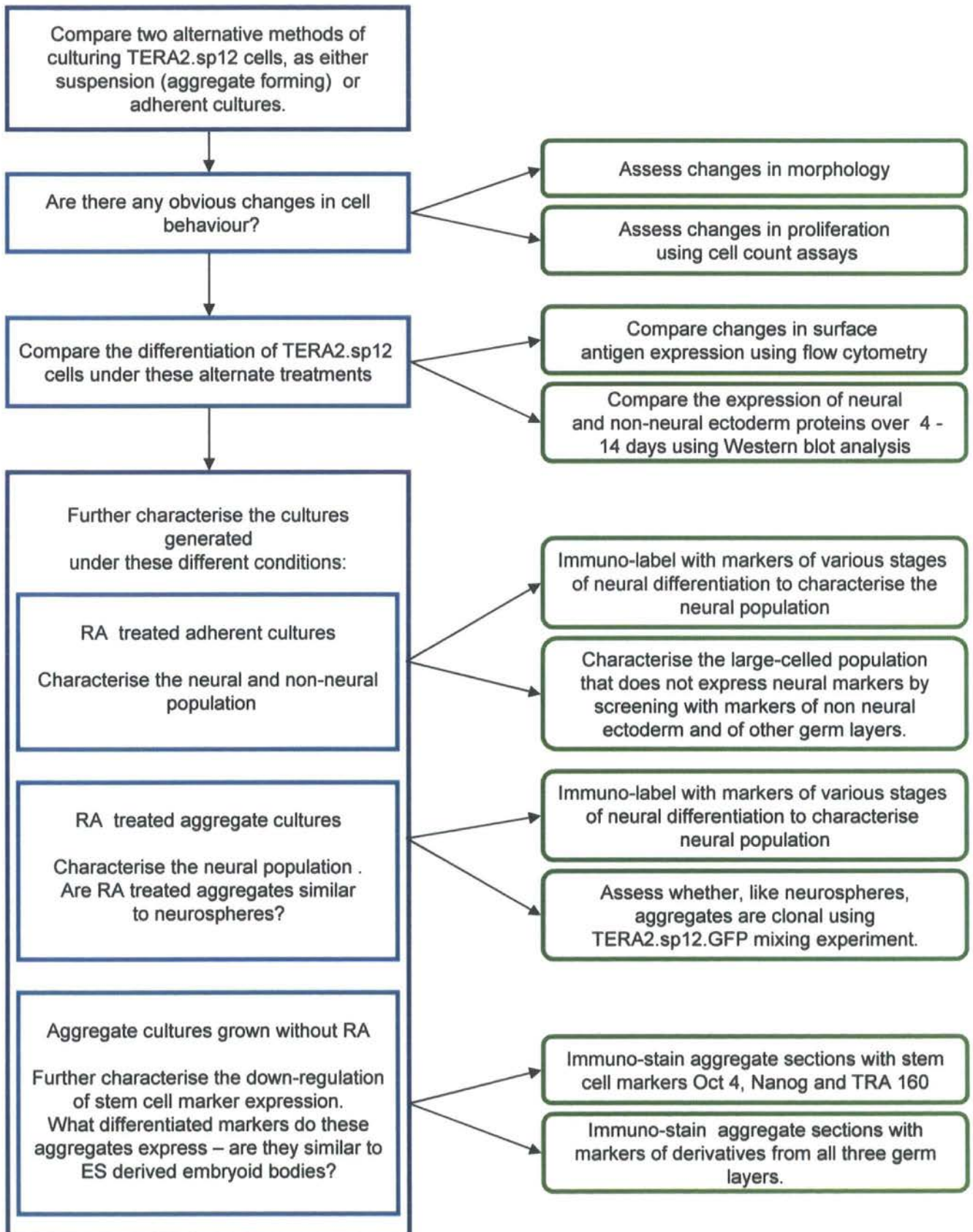


Figure 3.2. Formation of aggregate structures in retinoic acid differentiated TERA2.sp12 cells. Phase contrast images show live cultures taken at various stages of differentiation. TERA2.sp12 stem cells were grown and maintained as confluent monolayers (A). Cells exposed to 10 μ M retinoic acid were cultured either in suspension (B) and then plated onto an adherent surface (D) or cultured as adherent monolayers (C). Cultures of adherent cells produced large, dense accumulations of cells that form many well-developed neurites after 6 weeks of differentiation (C, arrows). A background of non-neuronal cell types surrounded these aggregates (C). Both methods ultimately result in the production of populations of aggregate structures that radiate large numbers of neurites (D,E). Scale bars: (A) 50:lm; (B) 750:lm; (C) 1500:lm; (D) 600:lm; and (E) 150:lm. Adapted from (Horrocks 2003) Figure 1.

Strategy Flow Chart For Investigating The Differentiation Of RA Treated TERA2.sp12 Cells Under Adherent Versus Non-adherent Conditions



3.2 RESULTS

3.2.1 General observations

TERA2.sp12 cells cultured under adherent conditions and treated with 10 μ M RA lost their characteristic stem cell morphology of a high nucleus to cytoplasm ratio with pale nuclei and prominent nucleoli, and developed into two morphologically distinct populations, depicted in Figure 3.4. The majority of the culture consisted of densely packed small cells which typically formed rosette-like structures (arrow) after 14 days of culture. A distinctive sub-population of cells was observed, consisting of large diameter cells with sizeable nuclei and prominent nucleoli, which formed small sheets of cells that grew in distinct patches and were easily distinguishable after 7 days of culture. These were given the term 'large cell plaques' (panels F and H). These 'large cell plaques' were observed at a consistent density over multiple experiments and appeared to increase in size over 7-14 days of culture. This phenotype was not observed in TERA2.sp12 cells grown as adherent cultures at differentiation density or in cultures vehicle treated with DMSO, indicating that the observed changes in RA treated cultures were indeed a result of RA exposure.

Cultures differentiated under non-adherent conditions formed small clusters of cells in suspension after 24 hours, and these clusters grew to form large floating aggregates of cells (Figure 3.5). These aggregates were usually variable in size, with smaller aggregates present even in cultures over 2 weeks old. However, the majority of aggregates reached a substantial size (1-2 mm) by 14 days in culture (panels D and G). Aggregates formed from cells cultured under non-adherent conditions and exposed to 10 μ M RA were initially more ragged in appearance than cultures grown without RA (panels B and E). However, by 7-14 days RA treated cells typically formed aggregates that were more regular and smaller than those grown without RA. TERA2.sp12 cells cultured in suspension without RA formed large and irregular aggregates after 14 days, and bud-like formations were frequently observed on aggregates between 7-14 days of culture.

3.2.2 Cell count assay

To compare the proliferation of TERA2.sp12 cells cultured under adherent versus non-adherent conditions, cultures were differentiated under either adherent or suspension conditions as described in Section 2.1.1 with three replicates set up for each time-point of 5, 7 and 14 days. At each time-point the selected cultures were individualised using Trypsin and counted using a haemocytometer as described in Methods Section 2.2.

Three different replicates of each sample were assayed using this method, and if any unusual cell counts were collected the counts were repeated. Cell counts were then used to estimate the

Changes In Morphology Of Adherent Cultures Of TERA2.sp12 Cells Treated With Retinoic Acid.

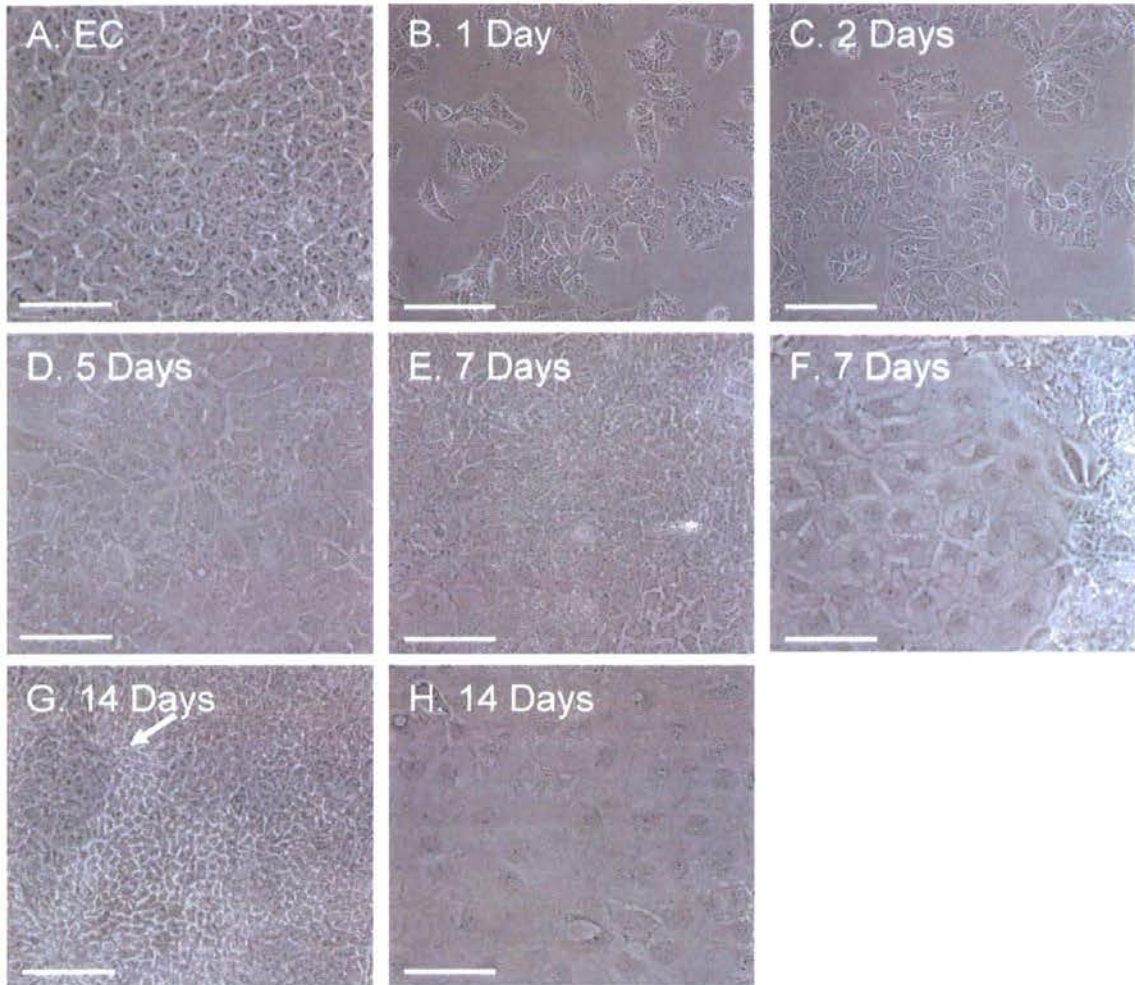


Figure 3.4. Phase contrast images of TERA2.sp12 cells grown as (A) a confluent adherent stem cell culture or seeded at a density of 20,000 cells/cm² and treated after 24 hours (B) with 10 μ M RA for 14 days. Representative images at 2 days (C), 5 days (D), 7 days (E, F) and 14 days (G,H) of culture demonstrate several changes in morphology. Undifferentiated TERA2.sp12 cells displayed a characteristic stem cell morphology of a high nucleus to cytoplasmic ratio, with prominent nucleoli, (A). Over time RA treated TERA2.sp12 cultures changed in morphology, forming more densely packed small cells after 7 to 14 days (E, G) with small rosette-like structures visible after 14 days of culture, as shown by the arrow in (G). By 7 days of treatment with RA, clusters of large-diameter cells with prominent nuclei and distinct boundaries (F) were visible, and these clusters had increased in size after 14 days (H). Scale bars = 50 μ m

Aggregate Formation Of TERA2.sp12 Cells Cultured Under Suspension Conditions
And Treated With Or Without Retinoic Acid

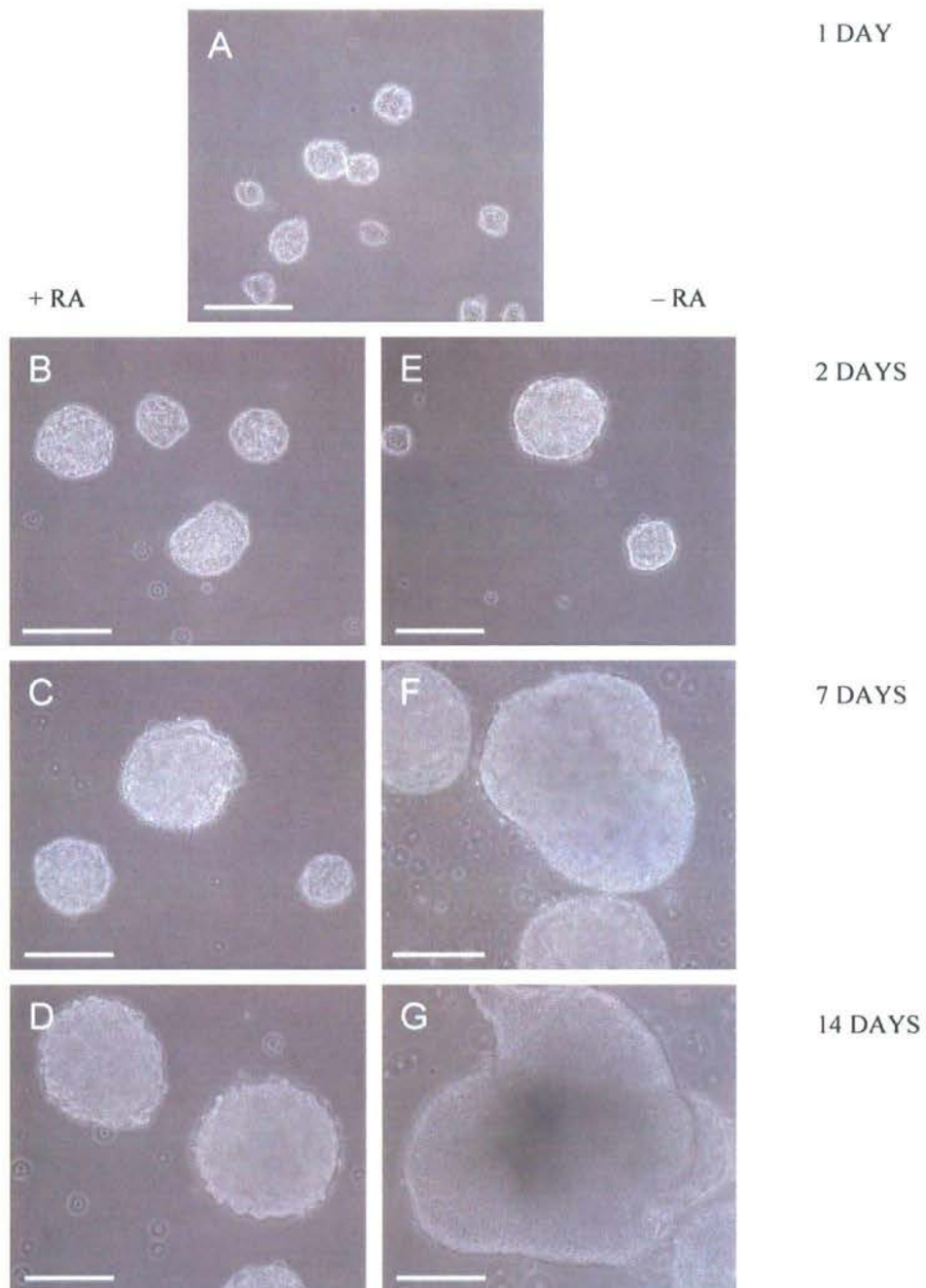


Figure 3.5 Representative phase contrast images of TERA2.sp12 cells cultured under suspension conditions to form aggregates. TERA2.sp12 cells were seeded at a density of 20,000 cells/cm² into non-adherent petri dishes and treated after 24 hours (A) with 10 μ M RA (B-D) or cultured without RA (E-G) for 14 days. Representative images at 2 days, 7 days and 14 days of culture depict aggregate development. TERA2.sp12 cells had formed small aggregates of cells after 24 hours of culture under non-adherent conditions (A). Cells cultured without RA rapidly formed larger, regular spheres (E), which then increased in size to form large irregular aggregates at 14 days (G). Initially, RA treated cultures formed small more irregular aggregates (B). These became more regular over time to form tightly packed spheres (D). Scale bars = 100 μ m

concentration of cells in each sample (count multiplied by 10,000) and the total cell number estimated by multiplying this value by the total volume of solution (10mls). These counts were also compared to the size of the pellets when centrifuged at 129g, to check that they were representative. The results are summarised in Table 3.2. Trypan blue exclusion was never more than 5% in all treatments, indicating that cell number was not markedly affected by cell death.

Table 3.2 Mean number of cells (per million) in adherent or suspension cultures of TERA2.sp12 cells treated with (+RA) or without RA over 14 days.

Days	Adherent	Adherent +RA	Suspension	Suspension +RA
0	1.50	1.50	1.20	1.20
5	8.85	10.93	3.23	3.83
7	24.87	28.33	8.13	5.57
10	33.10	22.40	5.27	3.50
14	-	35.80	11.47	8.83

As indicated in the graph in Figure 3.6, the increase in cell number over time was reduced by RA treatment under both suspension and adherent conditions. However, the most marked difference in rate of increase in cell number was between adherent and suspension cultures. Cells grown under suspension conditions and treated with RA ceased to increase in number by day 5, whereas cells grown under adherent conditions and treated with RA continued to increase in cell number, albeit at a slower rate by day 14. This suggests that proliferation in RA treated TERA2.sp12 cells is markedly inhibited under suspension conditions compared to adherent conditions, which may correspond to more a rapid departure from the stem cell phenotype. Despite a generally fast rate of increase, a distinct intermediate phase of slowed growth was observed between 7-10 days in adherent cultures treated with RA. This 'lag phase' was consistently observed over repeated experiments. This could be a result of growth inhibition as a result of increased cell-cell contacts as the population grows. This would prevent further growth in cultures of genetically normal cells but as observed here, contact-induced growth inhibition is overcome - a hallmark of tumourigenic cells.

Adherent cultures of TERA2.sp12 cells grown without RA initially demonstrated a similar rate of increase to adherent cultures treated with RA. However after 7 days the cell number escalated, such that cultures became so overgrown that they were no longer viable. After 7 days of culture, adherent cultures without RA were split and reseeded at 1:3, and the total count of three re-seeded flasks used to estimate the total population at 10 days. This rapid increase in cell proliferation after an initial lag-phase was observed over multiple repeated experiments. This was in contrast to cells grown in suspension culture conditions without RA – the rate of increase in cell number remained relatively steady, indicating that suspension conditions alone encouraged reduced proliferation and possibly differentiation. One source of

Cell Count Assay Of Adherent Or Suspension Cultures Of TERA2.sp12 Cells Treated Over 14 Days With Or Without Retinoic Acid.

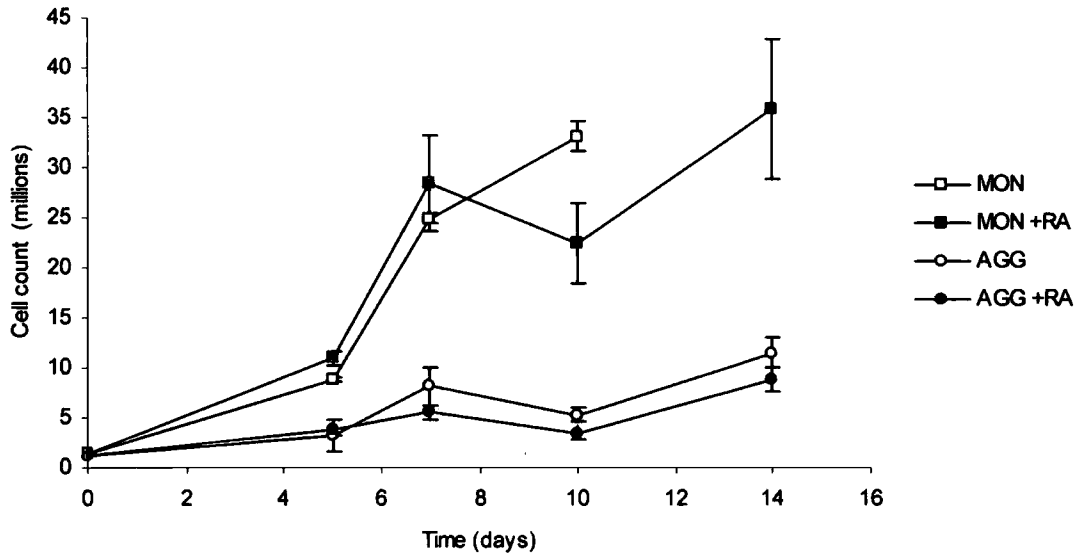


Figure 3.6. Graph presenting changes in mean cell count over 14 days for TERA2.sp12 cells cultured in adherent (MON) or non-adherent conditions (AGG) and treated with $10\mu\text{M}$ RA (+RA) or without RA. TERA2.sp12 cells were seeded at a density of 20,000 cells/cm² into either T75 culture flasks or bacteriological petri dishes and after 24 hours treated with either $10\mu\text{M}$ RA or untreated. At 5, 7 and 14 days 3 replicate cultures of each treatment were harvested and individualised using Trypsin, and samples counted with a haemocytometer. Adherent cultures demonstrated a markedly higher rate of increase in cell number compared to suspension cultures, with RA treated suspension cultures exhibiting the lowest rate of increase. N=3 for each data point and the error bars represent the standard deviation. There is no 14 day count for adherent cultures grown without RA (MON), because the cultures became too dense and were no longer viable.

experimental variation encountered was that cells forming aggregates under suspension conditions were physically more difficult to individualise with Trypsin treatment, with repeated aspiration necessary during this step. However, as a general comparison, the size of the pellet formed after centrifuging individualised cells from each culture matched the expected size estimated from cell counts.

3.2.3 Changes in cell surface marker expression

To compare the levels of expression of surface antigens associated with the stem cell phenotype (TRA 160, SSEA 3) or with the neural phenotype (VINIS 53, A2B5) in these treatments, at 7 and 14 days the remaining cells from the cell count assay were analysed using live flow cytometry following the procedure described in Section 2.3. Three replicates of cells from the same passage number were used for each treatment, and processed in parallel.

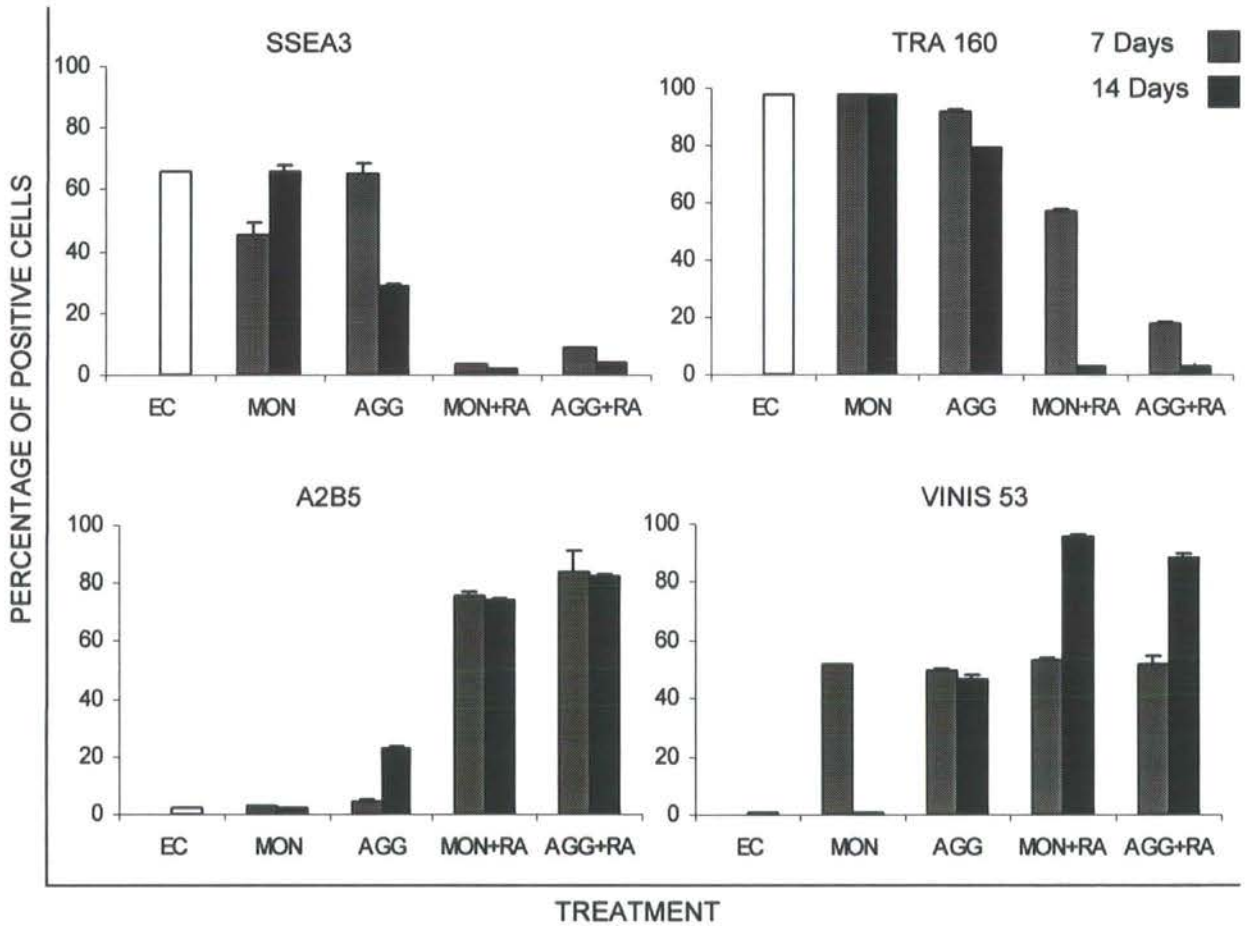
Comparisons of cell surface marker regulation in TERA2.sp12 cells treated with RA under adherent and suspension conditions demonstrated a similar pattern of regulation in both conditions, except under suspension conditions the down-regulation of stem cell marker expression was accelerated: Cells grown under both adherent and suspension conditions ceased to express stem cell markers TRA 160 and SSEA3, and up-regulated VINIS 53 and A2B5 expression after 2 weeks of treatment with RA (Figure 3.7). This corresponds with the results of the cell count assay in demonstrating that RA treated TERA2.sp12 cells cultured under suspension conditions more rapidly depart from the stem cell phenotype than those cultured under adherent conditions. Stem cell markers SSEA3 and TRA 160 also demonstrated variable levels of expression and sensitivity to treatment. In this experiment SSEA 3 was expressed in 64% of cells grown under maintenance conditions and down-regulated rapidly after RA treatment under both culture conditions, with less than 10% of cells positive after 7 days. In contrast, TRA 160 was expressed at a high intensity in 100% of cells grown under maintenance conditions, and after 7 days of RA treatment was still expressed in 60% of cells in adherent cultures compared to only 20% of cells suspension cultures.

The neural associated markers A2B5 and VINIS 53 were markedly up-regulated by RA treatment, following a similar trend in both culture conditions. A2B5 expression was up-regulated very rapidly, from less than 5% positive in EC cultures to 80-90% of cells expressing A2B5 after 7 days of treatment. VINIS 53 expression was up-regulated more gradually, with approximately 50% of cells positive after 7 days and 80-90% positive after 14 days. Together these data indicate that under both culture conditions, RA treated TERA2.sp12 cells are differentiating along a neural lineage.

Cells grown under suspension conditions for 14 days without RA treatment also

Comparing The Regulation of Surface Markers During RA Treatment Of TERA2.sp12 Cells Cultured Under Adherent Or Suspension Conditions.

i. Histograms Depicting Percentage Of Cells Positive For Stem Cell Surface Markers SSEA3, TRA 160 Or Neural Associated Markers A2B5 And VINIS 53 After 7 Or 14 Days Of Treatment, (Patterned Or Black Bars Respectively).



ii. Comparison Of Representative Flow Cytometer Traces For TRA 160 Detection After 14 Days In Adherent Cultures Treated With (+RA) or Without RA And Suspension Cultures Treated Without RA.

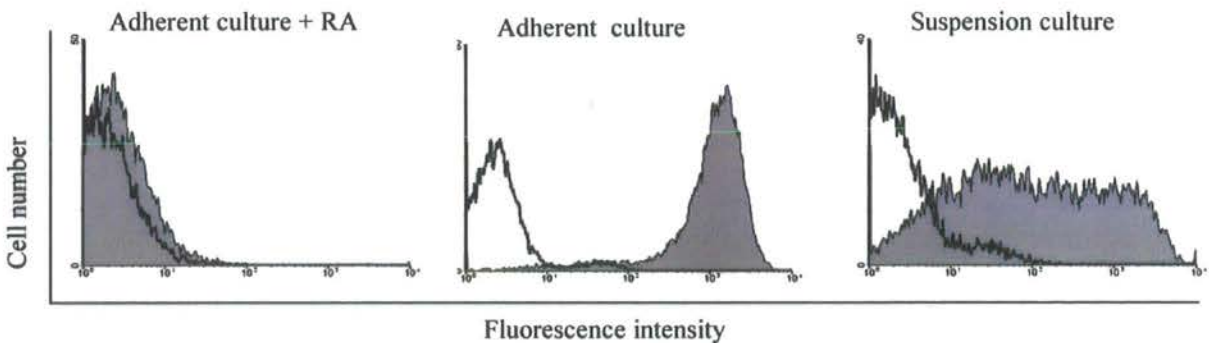


Figure 3.7. Detection of surface markers by flow cytometry. TERA2.sp12 cells were seeded at a density of 20,000 cells/cm² into either T75 culture flasks or bacteriological petri dishes and after 24 hours either treated with 10 μ M RA or untreated. At 7 and 14 days 3 replicate cultures of each treatment were harvested and individualised using Trypsin and immuno-labelled with antibodies to surface antigens SSEA3, TRA 160, A2B5, VINIS 53 or with the control antibody P3X. The percentage of immuno-positive cells in each sample was detected using a flow cytometer, with the mean percentage for each treatment generated from 3 replicates. Histograms in plate i depict the mean percentage of positive cells at day 7 (patterned bars) and day 14 (black bars) after live immuno-staining with either SSEA3, TRA 160, A2B5 or VINIS 53. A confluent flask of TERA2.sp12 cells cultured under maintenance conditions was used to monitor expression in EC cells (white bars). The percentage of immuno-positive cells in each sample was detected using a flow cytometer, with the mean percentage for each treatment generated from 3 replicates. The error bars represent the standard deviation of the mean. Mock treated cultures were split and re-seeded at a ratio of 1:3 at 7 and 10 days of culture (only one flask from each original culture was maintained, the remaining cells were discarded).

(ii) Representative flow cytometry traces of adherent cultures treated with or without RA and suspension cultures treated without RA and either immuno-stained with TRA 160 (grey trace) or with the control antibody P3X (clear trace). As depicted by the histograms in (i), both adherent and suspension cultures rapidly down-regulated the expression of stem cell markers SSEA3 and TRA 160, although in suspension cultures TRA 160 was more rapidly down-regulated such that after 7 days less than 25% of the population were immuno-positive, compared to over 50% in adherent cultures. Correspondingly, neural differentiation associated cell surface markers A2B5 and VINIS 53 were markedly up-regulated by RA treatment in both culture conditions, such that after 14 days over 80% and 90% of cells were positive for these markers respectively. Stem cell markers were down-regulated to some extent in cultures treated with-out RA, with a more permanent and marked down-regulation detected in suspension cultures. Although the percentage of cells positive for TRA 160 was still relatively high in suspension cultures treated without RA, comparisons of fluorescence intensity in (ii) demonstrate that the level of TRA 160 expression was markedly down-regulated in suspension cultures compared to adherent cultures treated without RA.

down-regulated the expression of stem cell markers TRA 160 and SSEA3 to some extent. This is clearly visible in representative traces of TRA 160 expression (plate ii, Figure 3.7). At 14 days the number of TRA 160 positive cells is still high, but compared to the pattern of expression in EC cells, there is a marked spread in fluorescence intensity, with a large proportion of cells down-regulating the expression levels of TRA 160. Correspondingly, in suspension cultures without RA treatment the number of cells positive for neural associated markers A2B5 and VINIS 53 was up-regulated to a certain extent (Figure 3.7), although in flow cytometry traces of A2B5 expression the staining intensity in positive cells was still very low compared to RA treated cultures. Taken together this suggests that a population of cells in aggregates cultured under suspension conditions in the absence of RA are embarking on differentiation (including neural differentiation).

Cells grown at the same density under adherent conditions without exposure to RA also showed some variation in cell surface marker expression. The proportion of cells positive for SSEA3 decreased after 7 days by approximately 20%, but after 14 days SSEA3 expression was once again similar to the expression in cells cultured under maintenance conditions. Correspondingly, VINIS 53 expression was up-regulated in these cultures at 7 days, although after 14 days the number of positive cells was reduced to levels seen in cells cultured under maintenance conditions. This implies that the regulation of expression of these markers is very sensitive in TERA2.sp12 cells, and that changes in the expression of these markers may be reversible to some extent.

3.2.4 Comparison of neural and non-neural ectoderm protein expression

To compare the expression of proteins associated with neural and non-neural ectoderm differentiation in TERA2.sp12 cells cultured with RA under either adherent or suspension conditions, the total level of expression of these proteins was monitored over 14 days using Western blot analyses. TERA2.sp12 cells were cultured under adherent or suspension conditions with or without RA as described in Section 2.1.1, with three replicate cultures for each treatment harvested at 4, 7 and 14 day time-points. At each time-point, cell pellets from sample cultures for each condition were collected as described in Methods Section 2.4, and cell pellets from IMR32 and FaDU cell lines were collected to use as positive controls for neural and non-neural ectoderm differentiation respectively. Protein was extracted from pelleted cell samples, electrophoresed in a 10% gel and transferred to PVDF membrane following the Western blot analysis protocol in Section 2.4. Blots were probed with antibodies to the neural progenitor protein Nestin, the neuronal proteins β III tubulin and NSE and epithelial keratins 8 and 18, following the protocol in Section 2.4. Equal loading was assessed by both Ponceau staining and by probing with an antibody to β -actin, and immuno-blots were

repeated three times with samples from different passage numbers.

Comparisons of the expression of neural and non-neural ectoderm proteins in RA treated cultures by Western blot analysis revealed differing trends in cells grown under adherent or suspension conditions, as depicted by Figure 3.8. Neural proteins were consistently up-regulated by RA treatment in both cultures (panels i and ii), but the total levels of these proteins were up-regulated to a greater extent over the first 14 days in cultures grown under suspension conditions. This marked difference was observed in every Western blot comparison of these cultures undertaken, and was most marked at 7-14 days of treatment. The neural progenitor protein Nestin also demonstrated a similar pattern of regulation, with protein expression detected after 4 days of treatment and increasing over 14 days in both RA treated cultures, although to a greater extent in cells cultured under non-adherent conditions. In adherent conditions, Nestin expression was subsequently steadily down-regulated between 14 and 28 days. No down-regulation in Nestin expression was observed in cultures grown under non-adherent conditions as they were analysed only for the first 14 days of treatment.

In parallel to the differences between levels of neural protein up-regulation in RA treated cultures grown under adherent or suspension conditions, the level of Keratin 8 protein as detected by Western blot analysis decreased rapidly in cells cultured to form aggregates, reaching almost undetectable levels by 7 days, whereas in cultures grown under adherent conditions, Keratin 8 levels increased over time, particularly between 7 and 14 days. These observations were made with data from more than 3 repeated experiments using cells grown from different passages. Keratin 8 levels in cells cultured under suspension conditions without RA also decreased, to a small extent. Neuronal β III tubulin levels were not markedly up-regulated, although the expression of Neuronal β III tubulin was variable over repeated experiments. Nestin expression also demonstrated variable levels of expression in repeated analyses, with the blot in panel iii in Figure 3.8 showing strong Nestin expression after 14 days of culture. Taken together the Western blot data indicate that under both suspension and adherent conditions, RA treated TERA2.sp12 cells are expressing developmentally regulated neural proteins as they undergo neuronal differentiation. However, compared to adherent cultures the rate of differentiation appears to be faster in suspension cultures and the proportion of cells differentiating along a neural lineage appears to be greater. This corresponds with evidence of minimal differentiation into non-neural ectoderm (as indicated by the expression of Keratin 8) under suspension conditions, compared to considerable levels of differentiation in adherent cultures.

For a more direct comparison between RA treated cultures grown under adherent and suspension conditions, protein expression was also compared in samples loaded onto the same gel, as depicted in the Western blot in Figure 3.9. Protein from RA treated cells cultured under

Western Blot Detection Of Neural And Non-neural Ectoderm Proteins In RA Treated Adherent
And Suspension Cultures Of TERA2.sp12 Cells

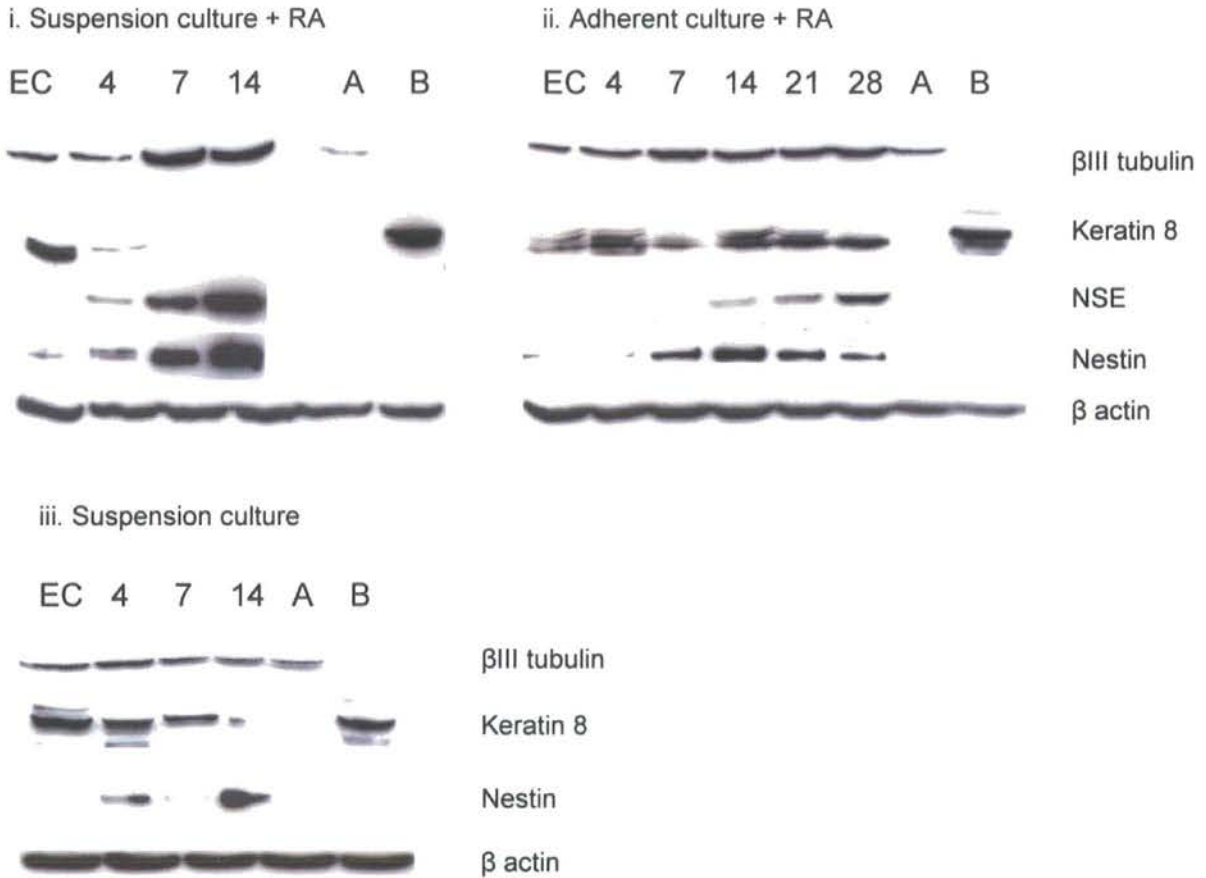


Figure 3.8 Western blot detection of neural and non-neural ectoderm markers from protein extracted at 4, 7 and 14 days from cells cultured under suspension culture conditions and either treated on day 1 with $10\mu\text{M}$ RA (i) or without RA (iii), and detection of the same markers from protein extracted at 4, 7, 14, 21 and 28 day time-points from TERA2.sp12 cells cultured under adherent culture conditions and treated on day 1 with $10\mu\text{M}$ RA (ii). Positive controls for neural protein (A) and non-neural ectoderm protein (B) were extracted from IMR32 and FaDU cells respectively, and protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions for EC samples. TERA2.sp12 cells grown as suspension cultures with RA demonstrated a marked increase in neural proteins β III tubulin and NSE, and the neural progenitor protein Nestin. Keratin 8 was down-regulated by 4 days of RA treatment. Note no controls were loaded for NSE and Nestin Western blots in panel (i).

Cells grown with RA as adherent cultures over 4-28 days (ii) demonstrated a gain in protein expression of neural markers β III tubulin and NSE, and a transient increase in expression of the neural progenitor protein Nestin. The levels of the simple epithelial protein Keratin 8 were also up-regulated with RA treatment. Cells grown as suspension cultures without RA treatment (iii) demonstrated some transient up-regulation of β III tubulin expression, and variable Nestin expression, with maximal levels detected at 14 days. Keratin 8 protein was down-regulated to some extent, reaching low levels after 14 days. Note that Keratin 8 protein is detected as two bands, at 54KDa and 45KDa (the band at 45KDa represents a degradation product of Keratin 8). β actin detection demonstrated equal loading in these samples.

Western Blot Comparison Of Keratin 8 and β III Tubulin Expression In RA Treated Adherent and Suspension Cultures Of TERA2.sp12 Cells

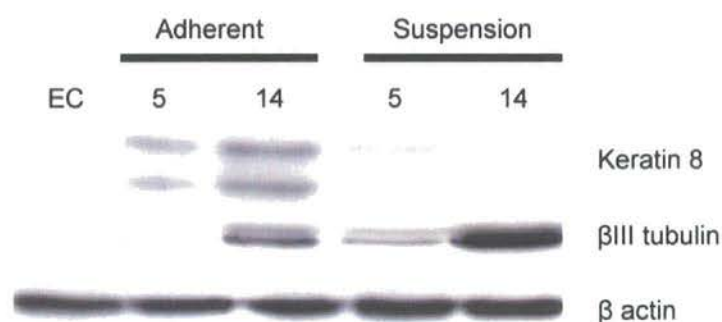


Figure 3.9. Western Blot detection of Keratin 8, β III tubulin and β actin from protein extracted at 5 and 14 days from TERA2.sp12 cells seeded either as adherent cultures or suspension cultures and treated after 24 hours with $10\mu\text{M}$ of RA. Protein was also extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions (EC sample). Keratin 8 expression was detected to increase over time in cultures grown under adherent conditions, whereas under suspension conditions Keratin 8 expression was detected to remain low and decrease to almost undetectable levels by 14 days of culture. Note that Keratin 8 protein is detected as two bands, at 54KDa and 45KDa (the band at 45KDa represents a degradation product of Keratin 8). Neuronal β III tubulin protein levels increased in both culture conditions after RA treatment, but to a much greater scale in cells cultured under suspension conditions. β actin detection demonstrated equal loading in these samples.

either suspension or adherent conditions for 7 and 14 days was electrophoresed on the same gel and transferred to PVDF membrane as previously described. Membranes were then immuno-blotted with antibodies to keratin 8 or neuronal β III tubulin. Equal loading was assessed by both Ponceau staining and by probing with an antibody to β -actin. The magnified up-regulation of neuronal β III tubulin in cultures treated with RA under aggregate-forming conditions compared to adherent conditions is marked, and correspondingly there is a marked down-regulation in Keratin 8 protein expression compared to an up-regulation in cells cultured under adherent and non-adherent conditions respectively. This confirmed that marked differences in the expression of markers for neural and non-neural ectoderm were generated in TERA2.sp12 cells treated with RA under either adherent or suspension conditions, suggesting that under adherent conditions differentiation into both neural and non-neural ectoderm is promoted, whereas under suspension conditions solely neural differentiation occurs.

3.2.5 Characterising the EC phenotype

To assess the undifferentiated TERA2.sp12 cell phenotype, cells were seeded at maintenance density into 12 well plates, and grown in maintenance media for 2-3 days until approximately 90% confluent. Cultures were then fixed in 4% PFA for 20 minutes at room temperature, and washed 3 times in PBS for 10 minutes. These cultures were then immuno-labelled following the protocol in Section 2.5 with antibodies to stem cell antigens TRA 160, SSEA4, Oct4, Nanog and with antibodies to the neural progenitor marker Nestin, neuronal markers β III tubulin, NF200, NF160, the epithelial keratins 8 and 18, and pan-epithelial keratin. Cultures from four different passages were immuno-stained in four repeated experiments.

Cultures grown under maintenance conditions immuno-stained positive for stem cell markers Nanog, Oct4 and TRA 160 in all cells observed (representative images for Oct4 and TRA 160 immuno-staining are depicted in Figure 3.10). However, these cells also faintly expressed Nestin and neuronal β III tubulin (Figure 3.10 plates D-F). Note that Nestin was undetectable in EC samples using Western blot analysis, whereas neuronal β III tubulin was present at low levels in all protein from EC samples in Western analyses. The expression pattern of both Nestin and neuronal β III tubulin was diffuse and not typical of neural cells, and β III tubulin expression was also detected in cells that appeared to be actively dividing (arrow). Thus under maintenance conditions TERA2.sp12 cells express the normal panel of stem cell markers indicative of a stem cell phenotype, although they also express low levels of some neural markers (in a pattern of expression that is not typical of neural cells).

3.2.6 RA induced differentiation as adherent cultures

To characterise the neural and putative epidermal differentiation of RA treated adherent

Immuno-staining Profile Of TERA2.sp12 EC Stem Cells

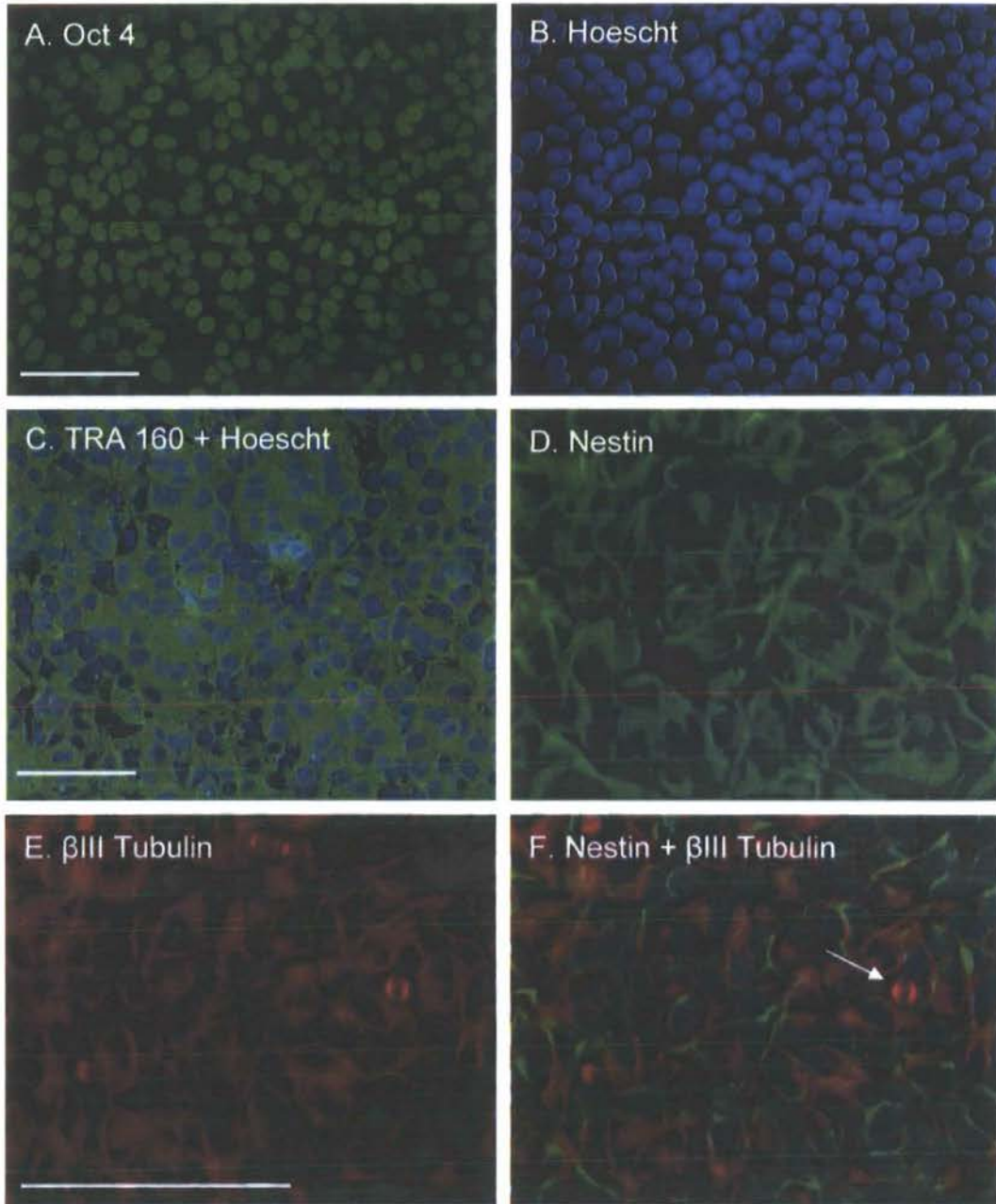


Figure 3.10. Confluent adherent cultures of TERA2.sp12 cells grown at maintenance conditions were fixed with PFA and immuno-stained with antibodies to Oct4 (A), TRA 160 (C), Nestin (D), β III tubulin (E) and the nuclei counterstained with hoescht (blue). TERA2.sp12 EC cells expressed stem cell transcription factor Oct 4 (A, with corresponding hoescht stain in B) and TRA 160 (overlaid with hoescht stain in C). However, TERA2.sp12 EC cells also expressed faint but significant levels of Nestin (D) and demonstrated weak staining for neuronal specific β III tubulin (E with overlay in F). Neuronal β III tubulin reactivity was also detected in actively dividing cells (arrow). Scale bars = 100 μ m

cultures, cells were seeded at 20,000cells/cm² into NUNC coated 12 well plates and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1 μ M pen/strep. After 24 hours cells the media was removed and replaced with the same media supplemented with 10 μ M RA. In experimental controls the media was replaced with the same media with no supplement or vehicle treated with media supplemented with 1 μ l/ml of DMSO. At 7 and 14 day time-points selected well plates for each treatment were fixed in 4% PFA for 20 minutes at room temperature, or fixed on ice in cold methanol for 10 minutes, washed three times in PBS for 10 minutes, and immuno-labelled following the procedure in Section 2.5 with the following antibodies: Neural progenitor markers Musashi and Nestin, neural markers MAP2, β III tubulin, NF160, NF200, and the glial marker GFAP (for GFAP immuno-staining cultures were fixed in methanol). To assay for epidermal differentiation cultures were immuno-stained with pan-epithelial keratin, keratin 8, keratin 18 and Tp63 antibodies. This immuno-labelling experiment was repeated twice for 7 day time-points and three times for 14 day time-points using TERA2.sp12 cells at different passages. As positive controls for neural and glial differentiation, cultured human IMR 32 cells and PFA fixed cryo-sectioned rat brain were immuno-stained in parallel. As a positive control for differentiation into prospective epidermis cultured FaDU cells were immuno-stained in parallel. As a negative control for non-specific binding of primary antibodies, FaDU cells were immuno-stained with neural antibodies, and IMR 32 cells immuno-stained with epithelial keratin and Tp63 antibodies.

In order to further characterise the expression profile of the neural and non-neural populations identified in RA treated adherent cultures, TERA2.sp12 cells were seeded into 12 well plates and cultured for 14 days with 10 μ M RA as described, fixed in 4% PFA, washed 3 times in PBS and double immuno-labelled following the protocol in Section 2.6.3 using the combinations of antibodies listed in Table 3.3

Table 3.3 Combinations of antibodies used to further characterise neural and non-neural ectoderm populations of RA treated TERA2.sp12 cells.

ANTIBODY COMBINATION	ANTIBODY TYPE	AIM OF IMMUNO-LABELLING
Nestin + β III tubulin (Covance)	Mouse + rabbit	To discriminate between neural progenitors and maturing neurons.
MAP2 + β III tubulin (Covance)	Mouse + rabbit	Characterise maturing neural cells.
NF160 + NF200	Mouse + rabbit	Characterise mature neurons
NF160 + β III tubulin (Covance)	Mouse + rabbit	"
Keratin 8 (ABCAM) + β III tubulin (Sigma)	Rabbit + mouse	Are any neural cells expressing Keratin 8?
Keratin 8 (ABCAM) + Nestin	Rabbit + mouse	Are any neural progenitor/ Nestin positive cells expressing Keratin 8?
Pan epithelial keratin + β III tubulin (Covance)	Mouse + rabbit	Discriminate between cells positive for neural and non-neural ectoderm antigens
Keratin 8 + 18	Rabbit + mouse	Characterise epithelial marker positive population
Pan epithelial keratin + TP63	Mouse + goat	Characterise prospective epidermis -like population.

As negative controls for non-specific binding of the secondary antibody, some wells were

incubated with no primary antibody, and then incubated with either a combination of mouse and rabbit secondary antibodies or mouse and goat secondary antibodies in antibody diluent. This immuno-labelling experiment was repeated four times with cultures at different passage numbers.

Neural differentiation

In accordance with the Western blot analyses, immuno-staining assays detected marked evidence of neural differentiation in RA treated adherent cultures: After 14 days a heterogeneous population of neural cells was displayed, with the majority of cells staining immuno-positive for neural progenitor markers Musashi and Nestin, and substantial populations expressing various neural markers including neuronal β III tubulin, MAP2 and neurofilaments. No cells stained GFAP positive by 14 days, although some cells expressing the astrocytic protein S100 were detected. After 14 days, the majority of cells stained brightly positive for Nestin or for neuronal β III tubulin, and distinctive rosette or ridge formations were observed at high frequencies within all cultures examined, as represented in Figure 3.11. These rosette formations were also visible under phase microscopy. Maturing neurons with well developed axons and expressing neurofilaments such as NF200 or NF68 were also detected (Figure 3.11), usually growing in small clusters on top of the other cells. By 21 days of culture, Nestin expression was down-regulated such that most cells demonstrated no or very low staining intensity for Nestin, and neuronal β III tubulin staining was dominant. However, in an exception to this trend, after 21 days some large aggregations of cells had formed within these cultures, and these stained positive for Nestin (Figure 3.12) and incorporated BrDU. Aggregate-like structures also showed strong immuno-reactivity to MAP2 and NF200 and demonstrated distinct regional staining (Figure 3.13). Some structures observed appeared almost tubular, as depicted in Figure 3.13 plate D, where MAP2 immuno-positive cells surround the outer edge and NF200 positive cells form a ring around the centre of the structure.

Non-neural differentiation

The large plaques of cells observed under phase contrast were negative for all neural and neuronal markers tested, including Nestin, Musashi, neuronal β III tubulin, MAP2, neurofilaments, GFAP and S100. An example of positive β III tubulin immuno-staining in surrounding cells is presented in Figure 3.14. However, these large cells showed strong immuno-reactivity to pan epithelial keratin, and in larger plaques a proportion of cells were faintly positive for the epidermis stem cell marker TP63 (Figure 3.14). These 'large-cell plaques' were also negative for all stem cell markers tested, although there were a few exceptions: In cultures treated for 7 days with RA, a small number of plaques, which were

Neural Differentiation In RA Treated Adherent Cultures Of TERA2.sp12 Cells

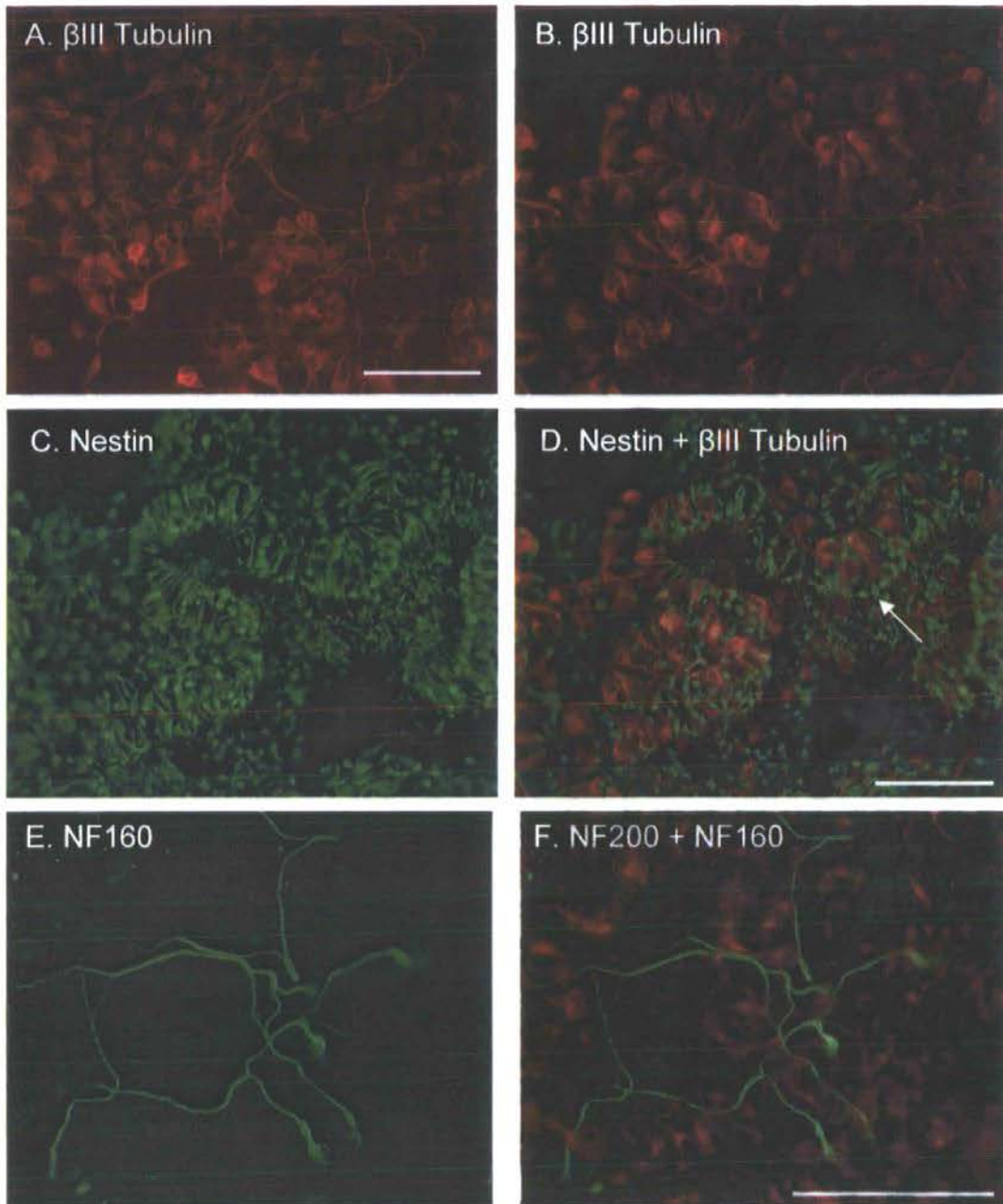


Figure 3.11. TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 10 μ M RA for 14 days prior to fixation in 4% PFA and immuno-labelling with either Nestin (green) and neuronal β III tubulin (red), (plates A-D) or with neurofilaments NF200 (red) and NF160 (green), (plates E-F). Note that the majority of cells expressed high levels of β III tubulin or Nestin. Plates A and B depict positive β III tubulin staining in two different regions within the same culture, (with corresponding Nestin staining and overlay for plate B in C and D). Note that Nestin and β III tubulin positive regions surrounding weakly Nestin positive and β III tubulin negative cells were frequently visible in these cultures. As depicted in B,C (overlay D), some complex structures were also observed. The arrow denotes the edge of a thick ridge of very strong Nestin positive immunostaining, bounded by clusters of strongly β III tubulin positive cells which were often orientated with nuclei at the top of the ridge with axonal like projections leading down or away from the ridge. As depicted in E, F, neurons immuno-positive for NF160 tended to grow on top of other cells and exhibited well defined axons. NF200 immuno-reactivity was also detected in cells displaying a less mature neuronal morphology, and some developing neurons did not show reactivity to both NF68 and NF200 (F). Scale bars = 100 μ m.

Zones Of Proliferation In 21 Day RA Treated Adherent Cultures Of TERA2.Sp12 Cells

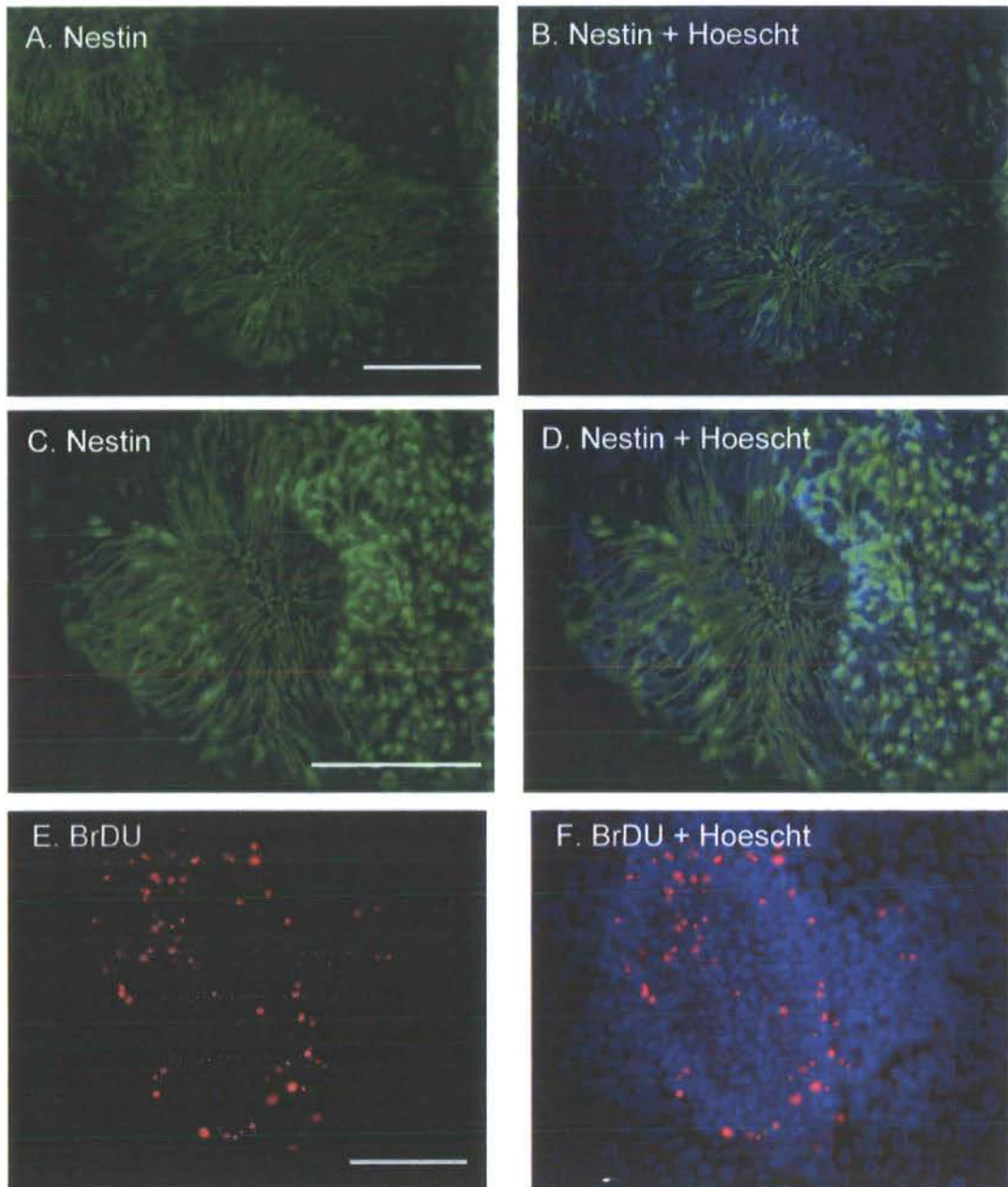


Figure 3.12. TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 10 μ M RA for 21 days. Twenty four hours prior to fixation in 4% PFA, 10 μ M of BrDU was added to the culture media. Fixed cultures were then immuno-labelled with either Nestin or BrDU and the nuclei counterstained with hoescht. Minimal Nestin immuno-staining was observed throughout the culture with the exception of aggregate-like structures. Two different examples of positive Nestin staining are depicted in A (with hoescht overlay in B) and C (with hoescht overlay in D). Note that the stained cells are aligned in a radial orientation within the aggregate-like structure (A, overlay with hoescht in B), and that in C, (overlay with hoescht in D) an invagination is apparent within the centre of the aggregate-like structure. As depicted by a representative image in E (with hoescht overlay in F), BrDU uptake and cell proliferation was rare except within these aggregate-like structures. Note that clusters of BrDU positive cells were situated predominantly around the edge of these structures. Scale bars = 100 μ M

NF200 And MAP2 Immuno-positive Aggregate-like Structures In 21 Day RA Treated Adherent Cultures Of TERA2.sp12

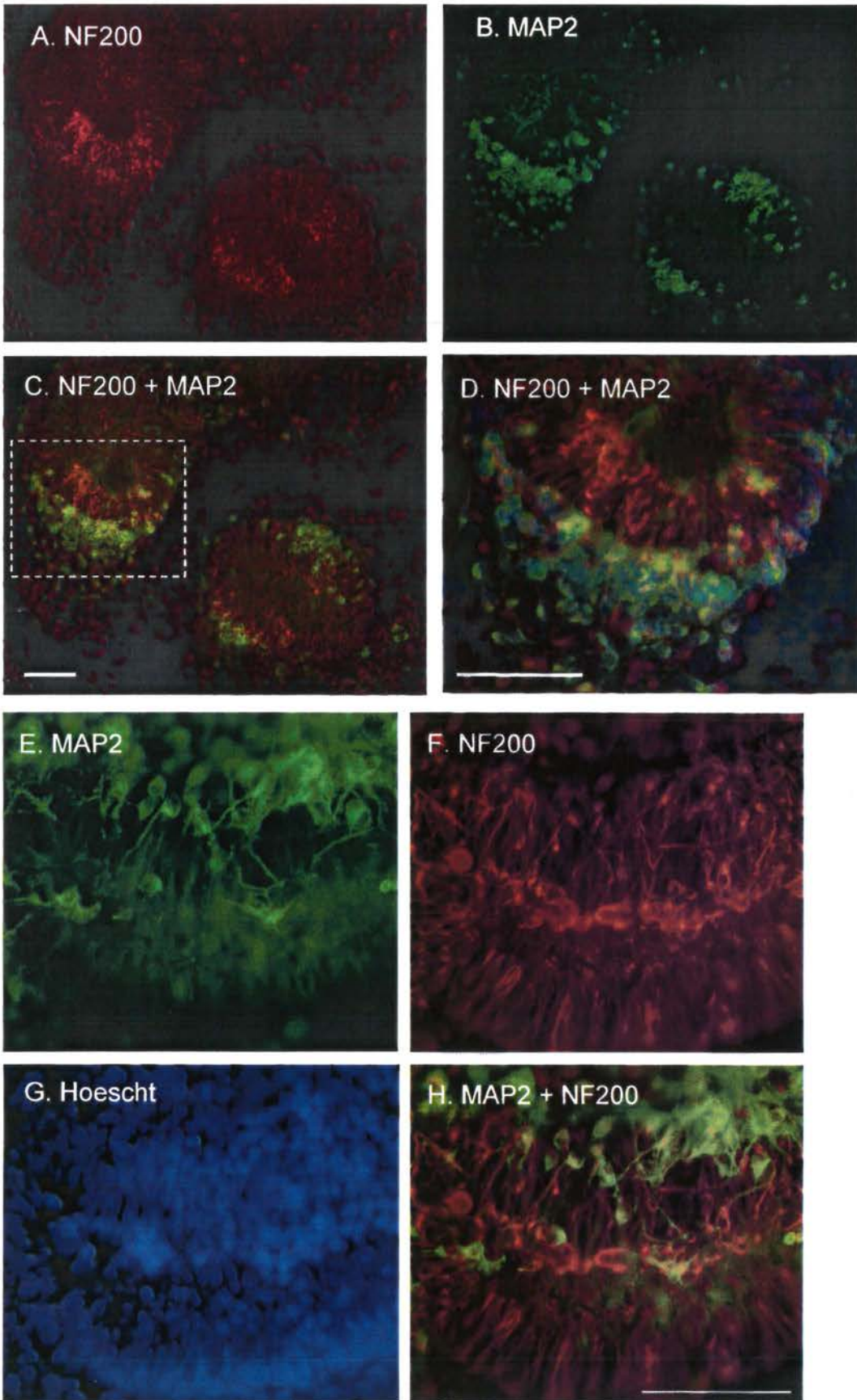


Figure 3.13 NF200 And MAP2 immuno-positive aggregate-like structures in 21 day RA treated adherent cultures. TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 10 μ M RA for 21 days. Cultures were then fixed in 4% PFA and double immuno-labelled with MAP2 (green), and NF200 (red) and the nuclei counterstained with hoescht. Aggregate-like structures demonstrated strong reactivity to MAP2 and NF200 with distinct regional staining. Some of these structures appeared almost tubular, as depicted in (A-C with magnified image in D). Note that MAP2 immuno-positive cells surround the outer edge and NF200 positive cells form a ring around the centre of the structure. As presented in E-F (with corresponding hoescht stain in G and overlay in H) distinct regional staining was visible within the structure and cells were aligned in a radial orientation. Scale bars = 100 μ m

A Distinctive Sub-population Of Retinoic Acid Treated Adherent TERA2.sp12 Cells Express Markers Of Non-neural Ectoderm.

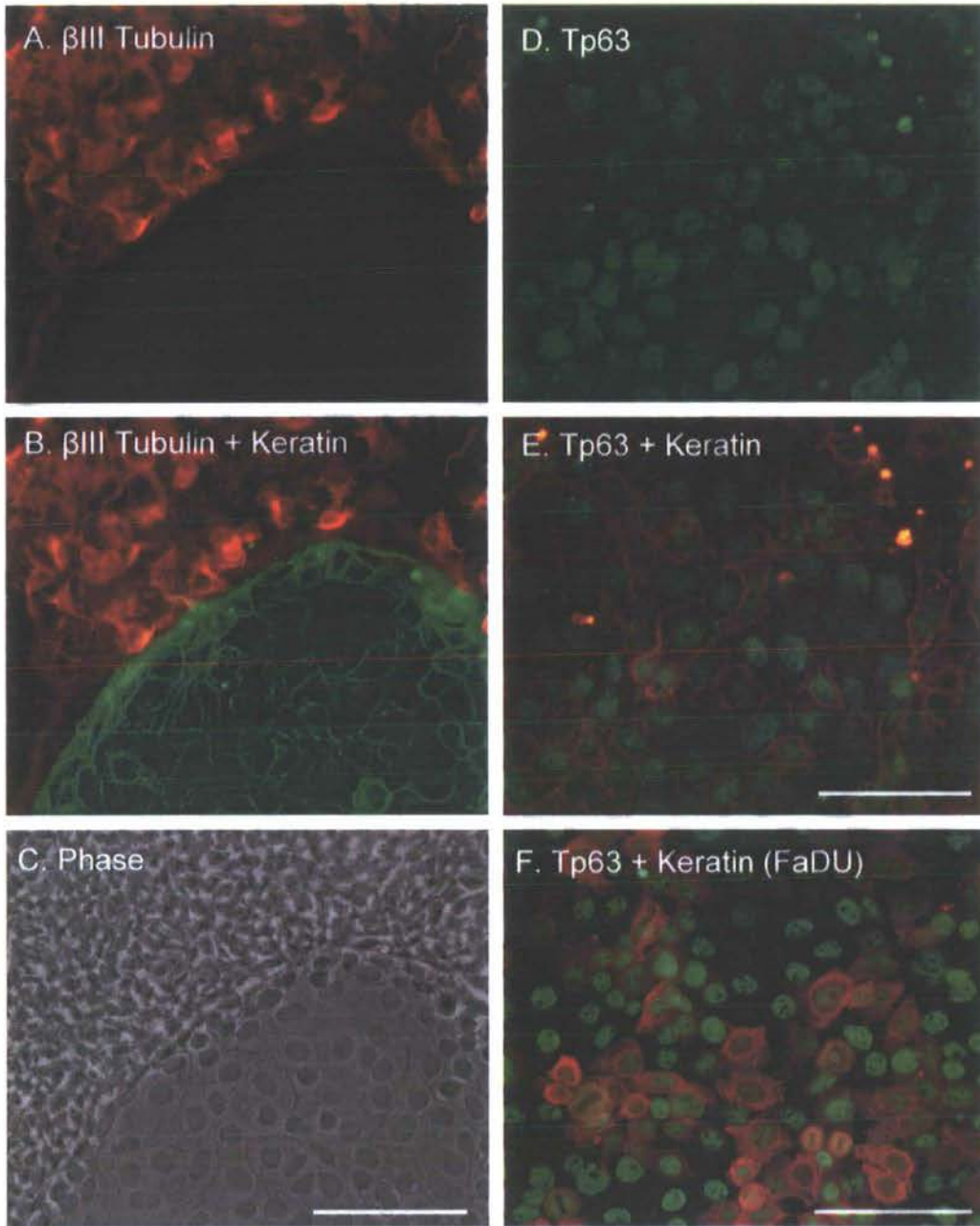


Figure 3.14. A distinctive sub-population of retinoic acid treated adherent cells express epithelial markers. TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 10 μ M RA for 14 days prior to fixation in 4% PFA and double immuno-labelled with neuronal β III tubulin (red) and pan-epithelial keratin (green), (A-C) or with pan-epithelial keratin (red) and the stem cell antigen Tp63 (green), (D-E). Distinctive populations of large diameter cells formed plaques negative for neuronal β III tubulin (A) and positive for pan-epithelial keratin (in overlay B), creating a marked boundary of differential staining between the edge of the area of large cells and densely packed neural cells. Note that this partition is reflected in the contrasting, non overlapping morphologies between the two populations in the corresponding phase contrast image (C). Cells within these large plaques were detected to co-express nuclear localised Tp63 (D) and pan-epithelial keratin (overlay in E). The expression pattern of TP63 and pan-epithelial keratin was similar to that of the positive control population of cultured FaDU cells (F) although the staining intensity for both pan-epithelial keratin and Tp63 was much brighter in the positive control population. Scale bars = 100 μ m

smaller in size, displayed some positive Nanog and TRA 160 immuno-staining, and the staining intensity was low in both cases.

In order to further characterise the ‘large-celled plaques’ adherent cultures of TERA2.sp12 treated for 14 days with RA were screened with a panel of differentiation markers of other germ layers. Immuno-staining was negative in the large celled population within adherent cultures for all of these markers. However the endoderm marker HNF3 β was detected in less than 0.5% of the remaining cells, with positive nuclear localised staining typically occurring in a group of 2-3 cells. The previously identified marker of early mesoderm differentiation, Brachyury, was also detected at low levels in RA treated adherent cells. Expression was still very low, detected in less than 1% of the population, with positive nuclear localisation typically occurring in small groups of 4-8 cells. In both cases the cells were morphologically indistinguishable from the surrounding neural population under phase contrast microscopy, and no expression was detected in cells cultured under maintenance conditions. To summarise, the immuno-staining experiments identified both a neural and non-neural population of cells in RA treated adherent cultures. The neural population consisted of both neural progenitors and a range of progressively more mature neuronal cells, whereas the morphologically distinct ‘large cell plaques’ expressed markers supporting their characterisation as non-neural ectoderm. Screening with markers for non-ectoderm derivatives also confirmed that the predominant differentiation in RA treated TERA2.sp12 cells is ectoderm derived.

3.2.7 RA induced differentiation as suspension cultures

Assay of aggregate formation – similar to neurospheres?

To determine whether RA treated aggregates shared characteristics typical of neurospheres, such as their formation from a single cell, a simple mixing experiment where aggregates were formed from a mixture of normal TERA2.sp12 cells and GFP transfected TERA2.sp12 single cells was performed, and aggregate sections analysed for GFP expression. The GFP transfected TERA2.sp12 cell line (with GFP expression promoted by the CAG promoter); termed TERA2.sp12.GFP was used in addition to TERA2.sp12 cells. Confluent T75 flasks of TERA2.sp12 or TERA2.sp12.GFP were individualised using Trypsin as previously described to form a single cell suspension. Then different mixtures of TERA2.sp12.GFP and TERA2.sp12 cells were seeded at a density of 20,000cells/cm² into 90mm bacteriological dishes as summarised in Table 3.4. Cultures were treated with RA and cultured following the protocol in Section 2.11. To assess the effect of cell density on aggregation formation, single cells were also seeded following the same ratios as in Table 3.4

at half the normal differentiation density, (10,000cells/cm²).

After 5 days, aggregate cultures were then fixed in 4% PFA, embedded in OCT embedding medium and cryo-sectioned following the procedure in Section 2.5.2. Sections were collected on histobond slides and mounted under glass coverslips in vectashield mounting medium with 0.1% Hoescht, before being examined under a Nikon diaphot 30 fluorescence microscope with filters suitable for the detection of GFP fluorescence and Hoescht fluorescence.

Table 3.4 Summary of combinations of individualised TERA2.sp12.GFP and TERA2.sp12 cells seeded into bacteriological dishes.

Treatment	Percentage of TERA2.sp12.GFP cells	Percentage of TERA2.sp12 cells	AIM
1	100	0	Are all cells in aggregates derived from TERA2.sp12.GFP cells detected to express GFP?
2	0	100	Check that no aggregates derived from TERA2.sp12 cells are detected to express GFP
3	50	50	Do aggregates derived from a seeding mixture of both TERA2.sp12 and TERA2.sp12.GFP cells contain either GFP expressing or non-GFP expressing cells (supports clonal Ho) or a mixture?

Cultures seeded entirely with the TERA2.sp12.GFP cell line formed aggregates in which all the cells observed were GFP positive, as shown in a representative aggregate section in panels A-C of Figure 3.15, although the level of GFP fluorescence detected was highly variable from cell to cell. Control cultures seeded entirely with non-GFP expressing TERA2.sp12 cells formed aggregates in which no GFP fluorescence was detected. Cultures seeded from a 50:50 mixture of normal and GFP expressing cells formed aggregates that all contained both GFP positive and GFP negative regions (representative section in panels D-F of Figure 3.15). No aggregates containing solely GFP positive or GFP negative cells were observed, even in aggregates formed from cells seeded at a lower density – in this case the aggregates formed were smaller in size. This experiment indicates that under these conditions aggregates of TERA2.sp12 cells are not formed from individual cells, rather from a combination of cells aggregating together and proliferating. Therefore they do not exhibit one of the defining features of neurospheres, and should not be described as such.

Histology of aggregates

To investigate the internal structure of aggregates formed with RA treatment under suspension conditions, sections of aggregates formed after RA treated culture under suspension conditions were stained with haematoxylin and eosin. To summarise, TERA2.sp12 cells were cultured with 10µM RA under suspension conditions for 7 or 14 days as described in Section 2.1.1, fixed in 4% PFA and either embedded in OCT embedding medium and cryo-sectioned following or dehydrated and embedded in paraffin wax and sectioned using a microtome

TERA2.sp12 Aggregate Formation Assay

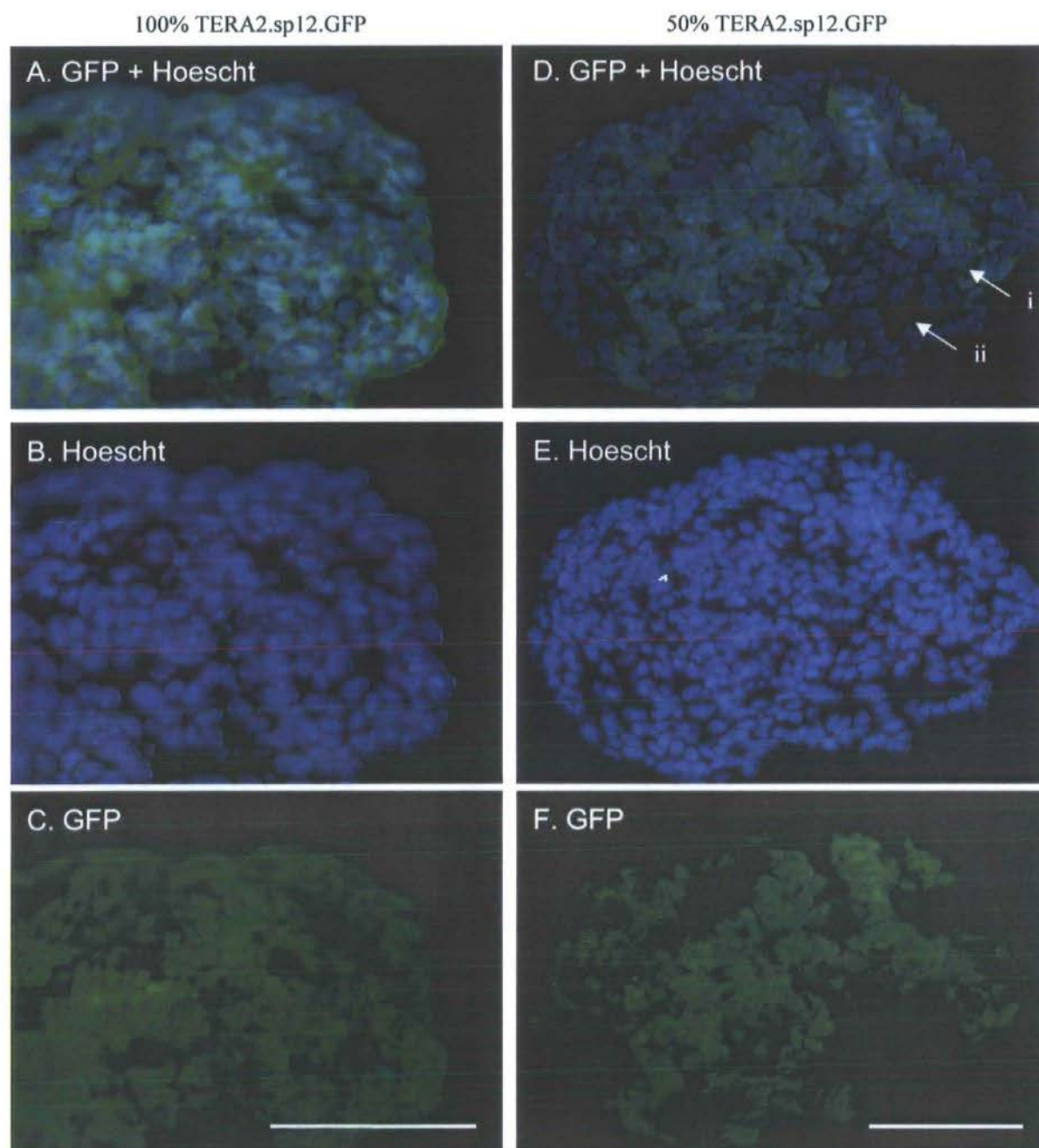


Figure 3.15. Cryostat sections of aggregates formed from individualised cells seeded at 20,000cells/cm² into bacteriological dishes and treated with 10 μ M RA for 4 days prior to fixation in 4% PFA. Cultures were either seeded entirely with TERA2.sp12.GFP cells, with a 50:50 mixture of TERA2.sp12 : TERA2.sp12.GFP cells, or entirely with TERA2.sp12 cells. Fixed aggregates were embedded in OCT embedding medium, cryostat sections collected on slides counterstained with hoescht (blue), and GFP fluorescence (green) detected using a filter suitable for GFP fluorescence under a fluorescence microscope. As depicted in a representative aggregate section (overlay in A, hoescht stain in B, GFP fluorescence in C), GFP fluorescence was detected in all cells within aggregates generated from cultures seeded with 100% TERA2.sp12.GFP cells, although levels of GFP expression were variable from cell to cell. However, as depicted by a representative aggregate section (overlay in D, hoescht stain in E, GFP fluorescence in C), aggregates grown from a seeding mixture of 50% TERA2.sp12.GFP and 50% TERA2.sp12 cultures contained distinct regions displaying either the presence (arrow i) or complete absence of GFP (arrow ii). This indicates that under these conditions aggregates form from multiple cells and are not clonal in origin. Scale bars = 100 μ m

Haematoxylin And Eosin Stained Sections Of RA Treated Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

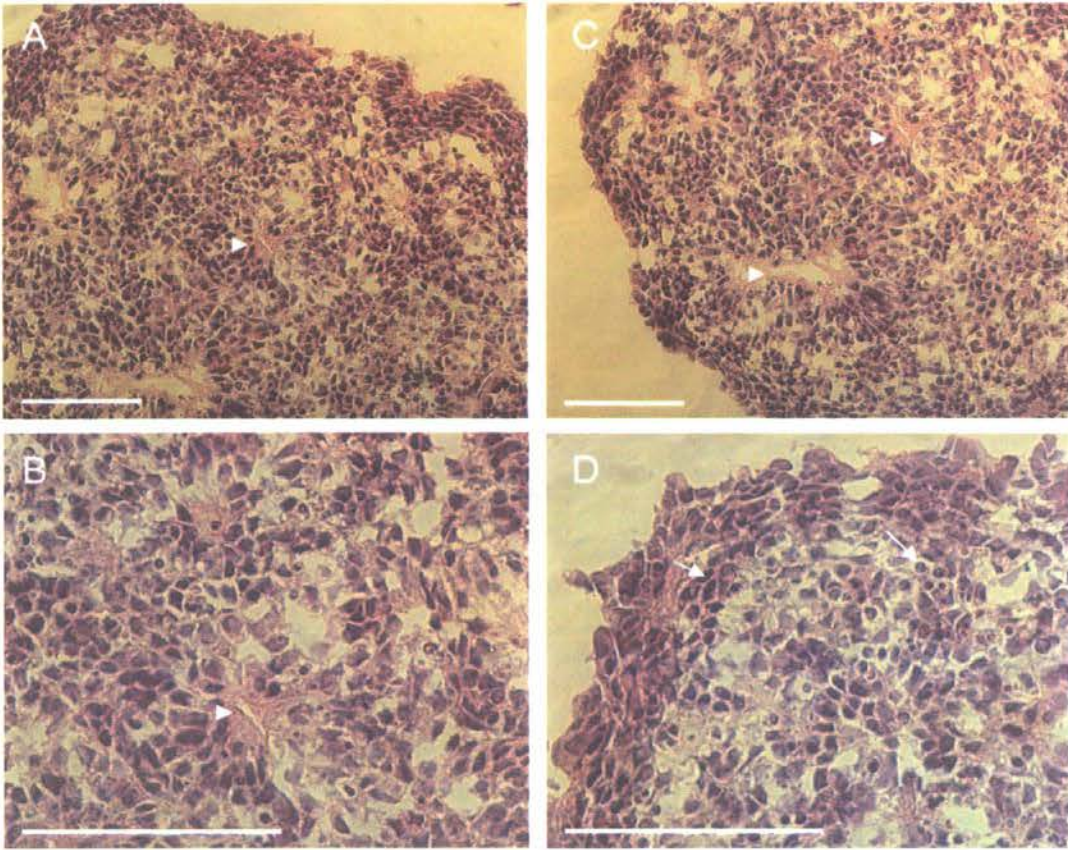


Figure 3.16. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and treated with 10 μ M RA for 14 days to form suspension aggregates. These were fixed in 4% PFA, embedded in OCT embedding medium and 10 μ m cryostat sections collected on slides and stained with haematoxylin, eosin, and alkaline alcohol to blue the nuclei. Phase contrast images of sections of the same aggregate (A,B) and two different aggregates (C, D) under 10x and 20x magnification demonstrate that the internal structure is not homogeneous. Cells were often arranged around distinct tubular formations, (arrow heads) and at least two different cell morphologies were visible, with some cells displaying prominent round nuclei and others displaying a more angular morphology with a low cytoplasm to nucleus ratio (arrows). Scale bars= 100 μ m

following the protocol in Section 2.6. Sections were then collected on Histobond slides and stained with haematoxylin and eosin as described in Section 2.6.

Sections demonstrated that aggregates were typically solid in structure, and in sections of 14 day aggregates some small cavities or tubular formations were observed (depicted by arrowheads in Figure 3.16). Staining also revealed that after 14 days cells within these aggregates were not homogeneous, with at least two different cell morphologies visible (arrows in Figure 3.16).

Location of dividing cells within aggregates

To locate the population of dividing cells within RA treated aggregates and identify any patterns of localisation, suspension cultures of RA treated aggregates were pulsed with BrDU prior to processing and sectioning at 5, 7 and 14 day time-points. For each time-point, 24 hours prior to fixation, 3.26 $\mu\text{l/ml}$ of a 10 mg/ml stock solution of 5-bromo-2'-deoxyuridine, BrDU (Sigma) in PBS was added to the cell culture media to make a final concentration of 10 μM . Collected aggregates were then fixed in 4% PFA, processed and immuno-stained with the G3G4 antibody to BrDU (hybridoma) following the corresponding immuno-labelling protocols in Section 2.5, except an additional step was required. Prior to the blocking stage, cells were treated for 10-15 minutes with 4NHCL to denature the double stranded DNA and allow penetration of the BrDU antibody, and then washed in PBS for 5 minutes. In addition, the diluted primary antibody was pre-warmed to 37°C prior to application. As a negative control, cultures which had not been pulsed with BrDU prior to fixation were also immuno-labelled with the antibody to BrDU in parallel.

As demonstrated by representative sections in Figure 3.17, positive immuno-staining for incorporated BrDU was very frequent in 5 day aggregates, with BrDU positive cells distributed evenly through-out the aggregate. The frequency of BrDU positive cells decreased over time such that 14 day aggregates contained lower numbers of immuno-positive cells, although different aggregates demonstrated different levels of positive staining. No distinctive regional localisation of dividing cells was observed within the aggregates, although in the older and larger aggregates BrDU staining was typically observed less frequently in the centre. In general, larger aggregates also appeared to contain fewer cells that had incorporated BrDU than smaller aggregates. These data suggest that over time there are fewer dividing cells within RA treated aggregates. Although there is no distinct localisation of dividing cells within these aggregates after 5 days of culture, after 14 days there may be a trend whereby dividing cells are localized to the centre of the aggregates, although this needs to be further analysed.

Down Regulation Of Stem Cell Marker Expression And Proliferation In RA Treated Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

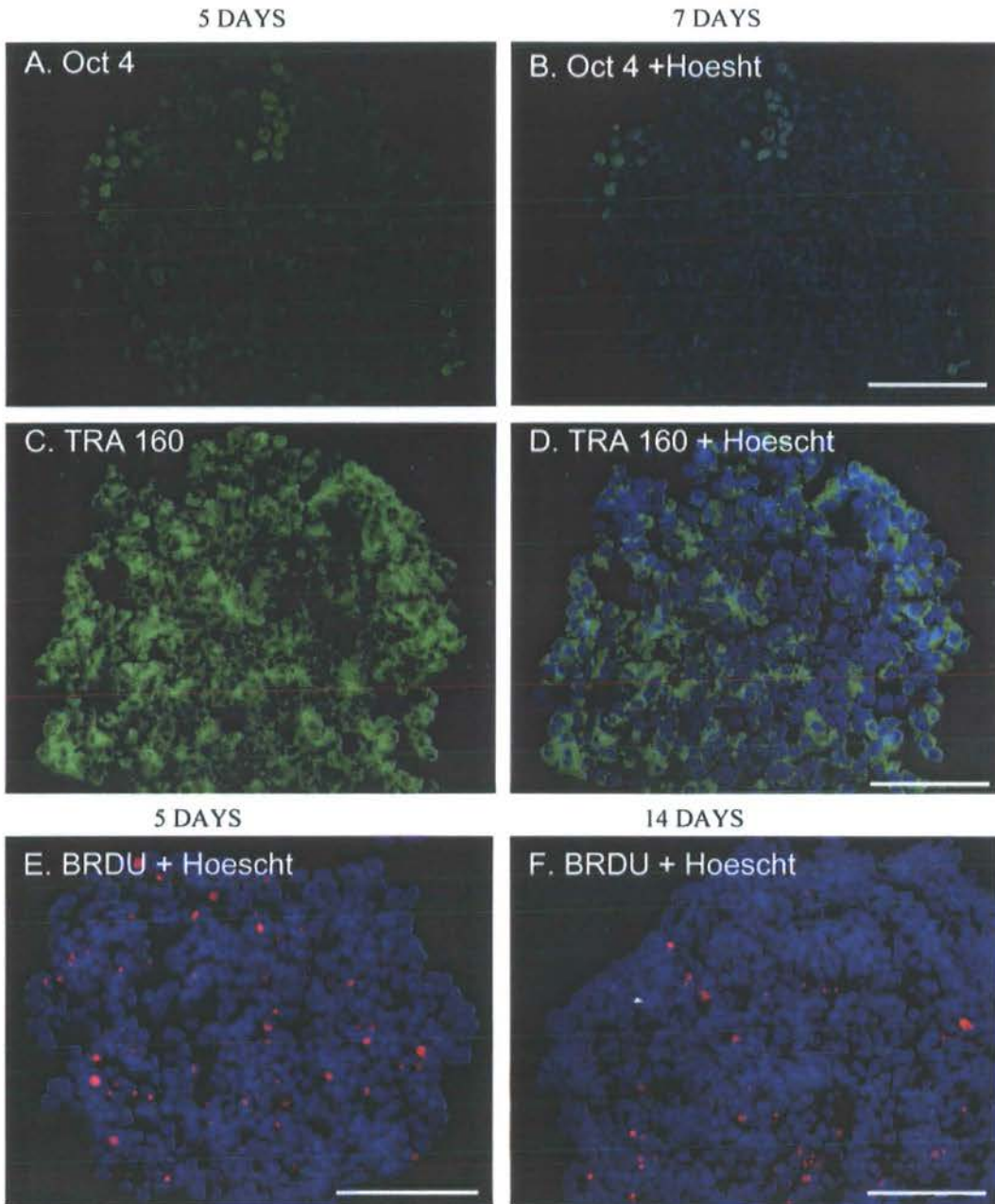


Figure 3.17. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and treated with 10 μ M RA to form suspension aggregates. After 5 or 7 days aggregates were fixed in 4% PFA, embedded in OCT embedding medium and 10 μ m cryostat sections collected on slides. Sections of day 5 aggregates were immuno-labelled with an antibody to Oct4 (A) and the nuclei counterstained with hoescht (overlay in B). Sections of 7 day aggregates were immuno-labelled with an antibody to TRA 160 (C) and the nuclei counterstained with hoescht (overlay in D). Nuclear expression of the stem cell marker Oct4 was down-regulated in the majority of cells within 5 day aggregate sections, with a minority of cells situated towards the edge of the aggregate demonstrating positive nuclear expression (B). Note that a substantial proportion of cells within 7 day aggregates immuno-stained positive for TRA 160. To detect proliferating cells in aggregates suspension cultures were grown as above and fixed at 5 or 14 days, except 24 hours prior to fixation, 10 μ M of BrdU was added to the culture media. After fixation aggregates were embedded and sectioned as above and immuno-labelled with an antibody to BrdU (red) and the nuclei counterstained with hoescht (blue). After 5 days of culture the frequency of BrdU labelled cells was relatively high in a typical aggregate (E), with positive cells distributed fairly evenly throughout the structure. In 14 day aggregates, the frequency of BrdU labelled cells was lower. An aggregate section demonstrating relatively high positive staining is depicted in F. Scale bars = 50 μ m

Pattern of stem cell marker down-regulation within aggregates

The flow cytometry analyses demonstrated a rapid down-regulation of stem cell markers SSEA3 and TRA 160 in RA treated aggregate cultures, although 20% of the population was still immuno-positive for TRA 160 after 7 days of treatment. To confirm flow cytometry data and investigate the pattern of expression of other key stem cell markers in RA treated aggregates, TERA2.sp12 cells were differentiated with RA under suspension conditions as described in Section 2.1.1 for 5, 7 and 14 days, embedded in OCT mounting medium and 10 μ m cryo-sections immuno-labelled with the stem cell markers TRA 160, Oct4 and Nanog following the protocol in Section 2.5.

Immuno-staining for stem cell markers TRA 160 and Oct4 in sectioned aggregates revealed markedly different patterns of expression and down-regulation (Figure 3.17). TRA 160 was expressed in distinct sub-populations through-out the aggregates after 7 days of culture, and down-regulated such that no positive cells were detected in aggregates cultured for 14 days. In contrast, nuclear localised Oct4 expression was rare in aggregates after only 5 days of culture, with small pockets of immuno-positive cells typically occurring close to the surface of the aggregates, and no positive staining for nuclear localised Oct4 was observed in aggregates cultured for 7 days. This indicates that Oct4 and TRA 160 are not always co-expressed, and supports the hypothesis that cells that have already down-regulated the stem cell transcription factor Oct4 may still be expressing TRA 160.

Characterising aggregate differentiation

To characterise the neural differentiation within RA treated aggregates and assay for evidence of non-neural ectoderm differentiation, TERA2.sp12 cells were differentiated with RA under suspension conditions as described in Section 2.1.1 for 5, 7 and 14 days, embedded in OCT mounting medium and 10 μ m cryo-sections immuno-labelled using the protocol described in Section 2.5.2 with antibodies to the following: Neural progenitor markers Musashi and Nestin, neuronal markers MAP2, β III tubulin, NF160, NF200 and glial marker GFAP (note that for GFAP immuno-labelling aggregates were fixed in methanol instead of PFA). To screen for evidence of non-neural ectoderm differentiation sections were immuno-stained with pan-epithelial keratin, keratin 8 and keratin 18. To further characterise the neural populations in RA treated aggregates, slides were also double immuno-labelled as described in Section 2.5.4 with the combinations of antibodies listed in rows 1-4 of Table 3.3.

As detected by Western blot analyses, immuno-staining demonstrated strong up-regulation of neural differentiation markers in RA treated suspension cultures. Sections of aggregates generated from 7 days of suspension culture stained brightly immuno-positive for Nestin and neuronal β III tubulin, and immuno-positive cells for these markers very closely

associated. However the staining intensity was greater in sections of aggregates cultured for 14 days. In a proportion of 14 day aggregates, tubular or rosette-like staining of radially arranged Nestin positive and β III tubulin negative cells around a small cavity was observed (Figure 3.18). In some cases an aggregate section contained up to three of these rosette-like structures, and multiple sections through the same aggregate revealed that some of these cavities extended through the aggregate and were tubular in structure.

Cells expressing neuronal filaments NF200 and NF160 and demonstrating a more mature morphology were frequently detected in immuno-labelled sections of 14 day RA treated aggregates, usually situated close to the surface of the aggregate (Figure 3.19, F). These neurofilaments were not expressed in cells within or close to rosette-like structures (arrow head). Immuno-staining with MAP2 identified immuno-positive cells localised within a region close to the surface of the aggregate, such that sections through the middle of the aggregate frequently contained a ring of immuno-stained positive cells (Figure 3.19). In summary, immuno-staining analyses demonstrated that within RA treated aggregates formed from suspension culture, extensive neuronal differentiation was occurring in the absence of differentiation into non-neural ectoderm. Complex structures resembling neural tube formations were present and aggregates contained a marked level of organisation with neurons at different developmental stages regionally localised.

No markers of other germ layer derivatives were detected by immuno-staining in RA treated aggregate sections (in contrast to positive controls), and immuno-staining for epithelial keratins was negative in most aggregates. However within one batch of aggregates in rare cases a ball of cells close to the centre of the aggregate was detected to express keratin 8 and this exhibited corresponding negative staining for neural markers. This indicates that on rare occasions some RA treated cells cultured under suspension conditions are able to differentiate into aggregates containing non-neural ectoderm, which may be associated with variations in the culture conditions.

3.2.8 Aggregates formed from suspension culture without RA

Histology of aggregates

To study the internal structure of aggregates formed from culture under suspension conditions in the absence of RA, TERA2.sp12 cells were cultured under suspension conditions for 7 or 14 days as described in Section 2.1.1, fixed in 4% PFA and either embedded in OCT embedding medium and cryo-sectioned, or dehydrated, embedded in paraffin wax and sectioned using a microtome following the protocols in Section 2.6. Aggregate sections were collected on Histobond slides and subsequently stained with haematoxylin and eosin

Formation Of Neural Tube-like Structures Within RA Treated Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

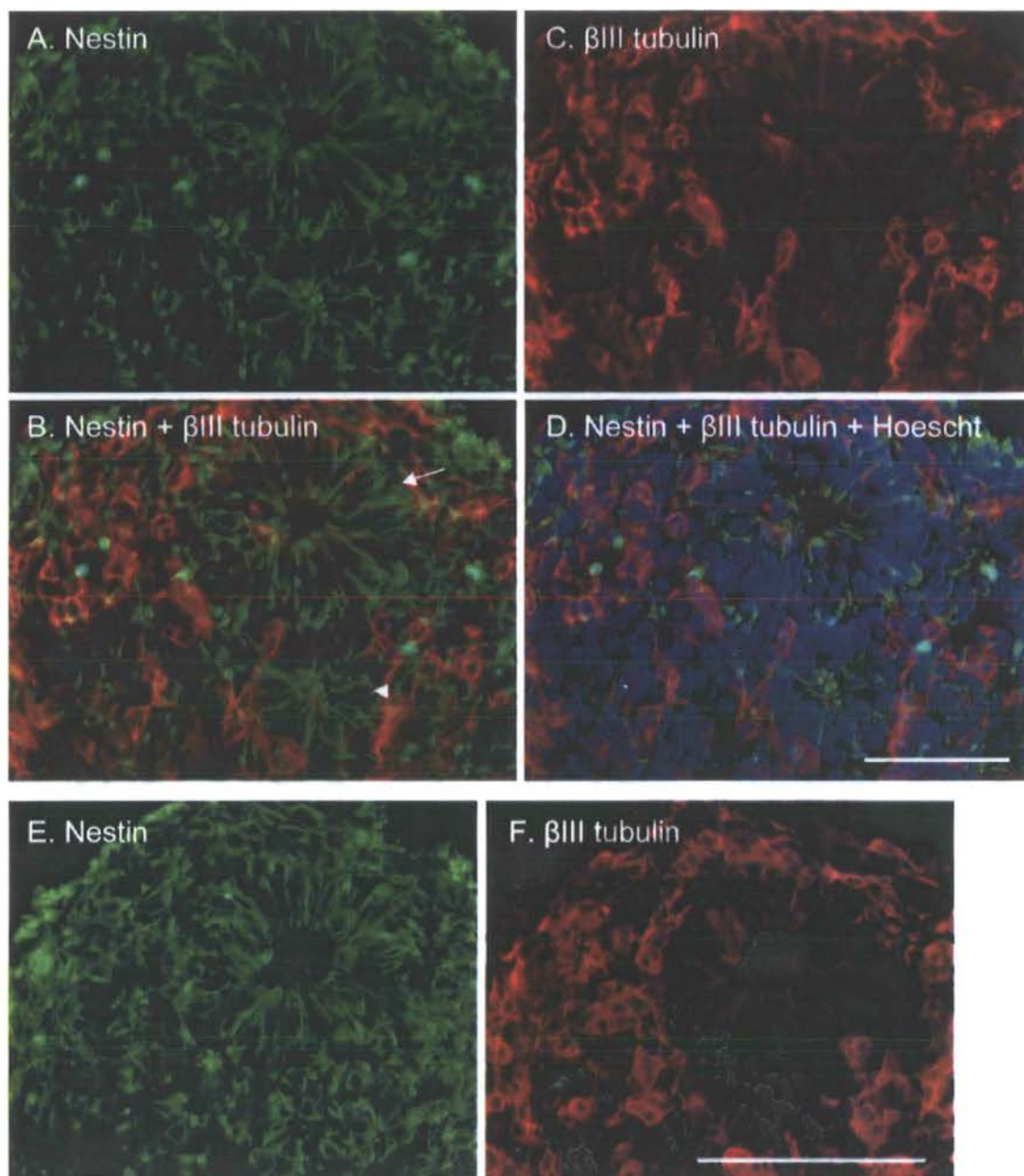


Figure 3.18. TERA2.sp12 cells were seeded at 20 000 cells/cm² into bacteriological petri dishes and treated with 10 μ M RA to form suspension aggregates. After 14 days aggregates were fixed in 4% PFA, embedded in OCT embedding medium, 10 μ m cryostat sections collected on slides and double immunolabelled with antibodies to neuronal β III tubulin (red) and Nestin (green) and the nuclei counterstained with hoescht (blue). Two different sections (A-D and E-F) through the same aggregate present distinct rosette (arrow head) and tubular (arrow) formations that appear to form a tube through the aggregate. These structures were composed of Nestin immuno-positive cells that orientated radially from the centre and were negative for neuronal β III tubulin. Overlay images (C and with hoescht in D) show that many cells within the aggregate stained positive for Nestin or for neuronal β III tubulin (although no cellular co-localisation was detected). Scale bars = 50 μ m

Regional Staining For Neurofilaments And MAP2 Within RA Treated Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

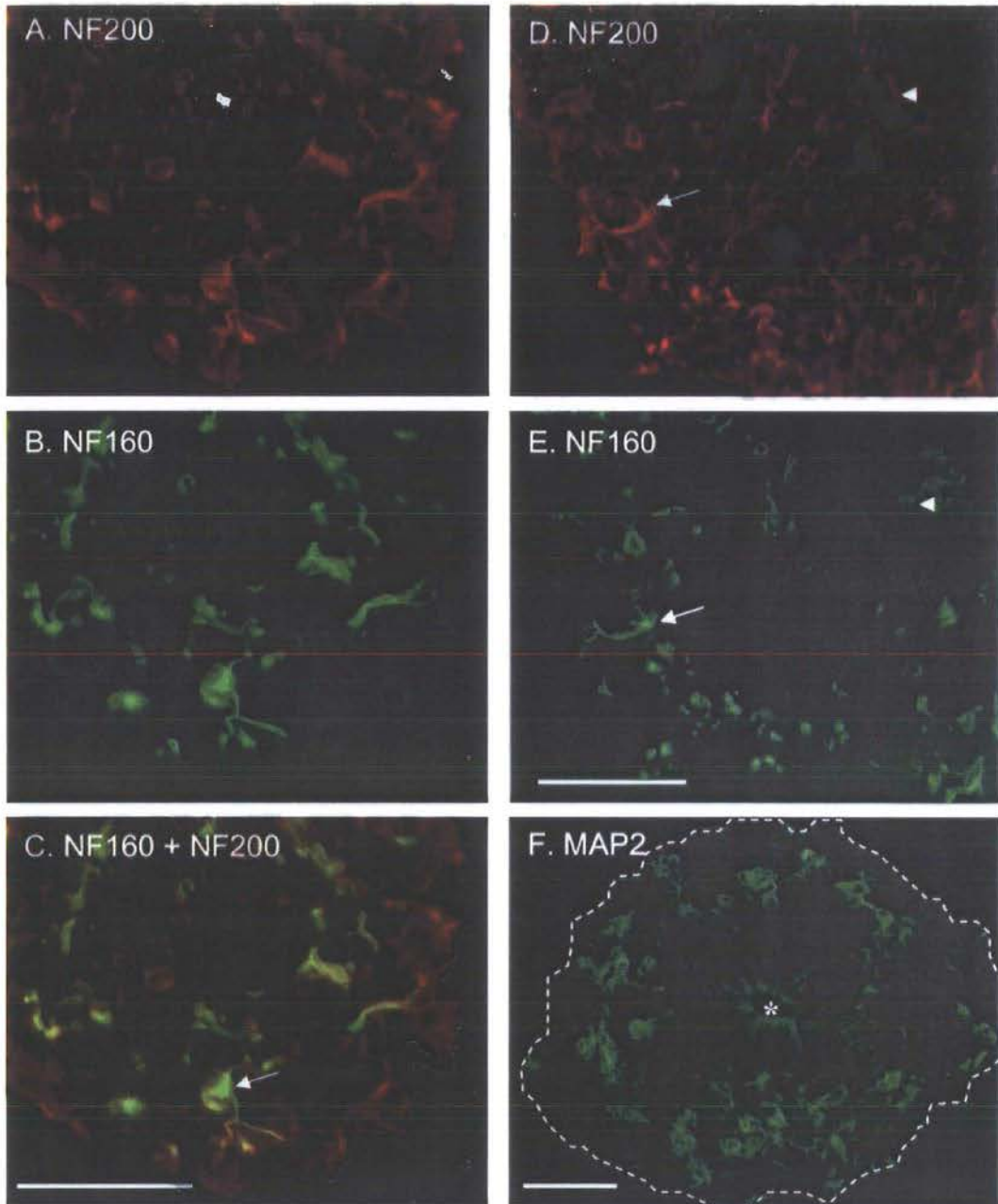


Figure 3.19. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and treated with 10 μ M RA to form suspension aggregates. After 14 days aggregates were fixed in 4% PFA, embedded in OCT embedding medium, 10 μ m cryostat sections collected on slides and double immuno-labelled with antibodies to NF200 (red) and NF160 (green) in plates A-C and plates D-E or with an antibody to MAP2 (green, plate F). Sections depicted by examples of two different aggregates (A,B with corresponding overlay D) and (E,F) contained a population of cells with a neuronal morphology which co-expressed the neurofilaments NF160 and NF200. Cells expressing high levels of these neurofilaments were typically situated close to the surface of the aggregates (arrows), and neither neurofilament was expressed in cells within or close to putative tubular structures (arrow heads). A representative section of an aggregate stained with MAP2 as shown in F, contains MAP2 bright cells situated in a ring close to the surface of the aggregate (outlined with white dashes) with very strong staining in the cytoplasm around the nucleus. As depicted by an asterix, cells within putative tubular structures contained cells that were faintly positive for MAP2. Scale bars = 50 μ m

following the protocol Section 2.6.2.

Aggregates formed from culture under non-adherent conditions and without RA were usually larger than those formed with RA treatment, and frequently contained cavities as indicated by arrow heads in Figure 3.20. Cells usually lined these cavities in an organised manner, such that the surface of the cavity was relatively smooth, and immuno-staining of these aggregates with the stem cell surface marker TRA 160 stained continuous membranes of the cells surrounding these cavities, an indication that these cavities were not formed from mechanical stresses during processing. Haematoxylin and eosin staining identified at least 3 morphologically different cell phenotypes within aggregate sections (arrows), indicating that these aggregates were heterogeneous in nature.

Down-regulation of stem cell marker expression

To characterise the population of cells within aggregates that had down-regulated surface stem cell antigens SSEA3 and TRA 160 as identified by flow cytometry, TERA2.sp12 cells were cultured as aggregates in suspension as described in Section 2.1.1 (without the addition of RA) for 5, 7 and 14 days. The following procedures were carried out as detailed in Section 2.5.2. At the selected time-points the aggregates were collected, fixed in 4% PFA, embedded in OCT embedding medium and cryo-sectioned. Ten micrometer sections from 5, 7 and 14 day aggregates were then immuno-labelled with the stem cell antibodies TRA 160 and Oct4.

Immuno-staining of sections of these aggregates with stem cell markers confirmed the flow cytometry results; regions of cells within the aggregates were slowly losing stem cell marker expression. After 7 days of culture under non-adherent conditions, small regions of adjacent cells within an aggregate (usually close to the surface) were negative for stem cell marker TRA 160, whereas marked down-regulation of Oct4 was already present in aggregates after 5 days (Figure 3.21). After 7 days almost no positive staining for nuclear Oct4 was detected, whereas the down-regulation of TRA 160 was more prolonged. After 14 days larger areas within the aggregates (typically closer to the centre) had down-regulated TRA 160 expression, and a corresponding down-regulation in nuclear localised Oct4 staining was also observed, with roughly 50% of cells within an aggregate exhibiting negative staining for nuclear localised Oct4. These data support the hypothesis whereby an increasing number of cells within these aggregates are down-regulating stem cell antigens and differentiating. An alternative explanation for this pattern of expression could be that a sub-population of stem cell antigen negative cells within the culture has expanded in number and merged with aggregates consisting of stem cell antigen positive cells. However, the stem cell antigen positive population decreases over time, and no increase in propidium iodide exclusion was detected, indicating that this decrease is not due to cell death.

Haematoxylin And Eosin Stained Sections Of Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

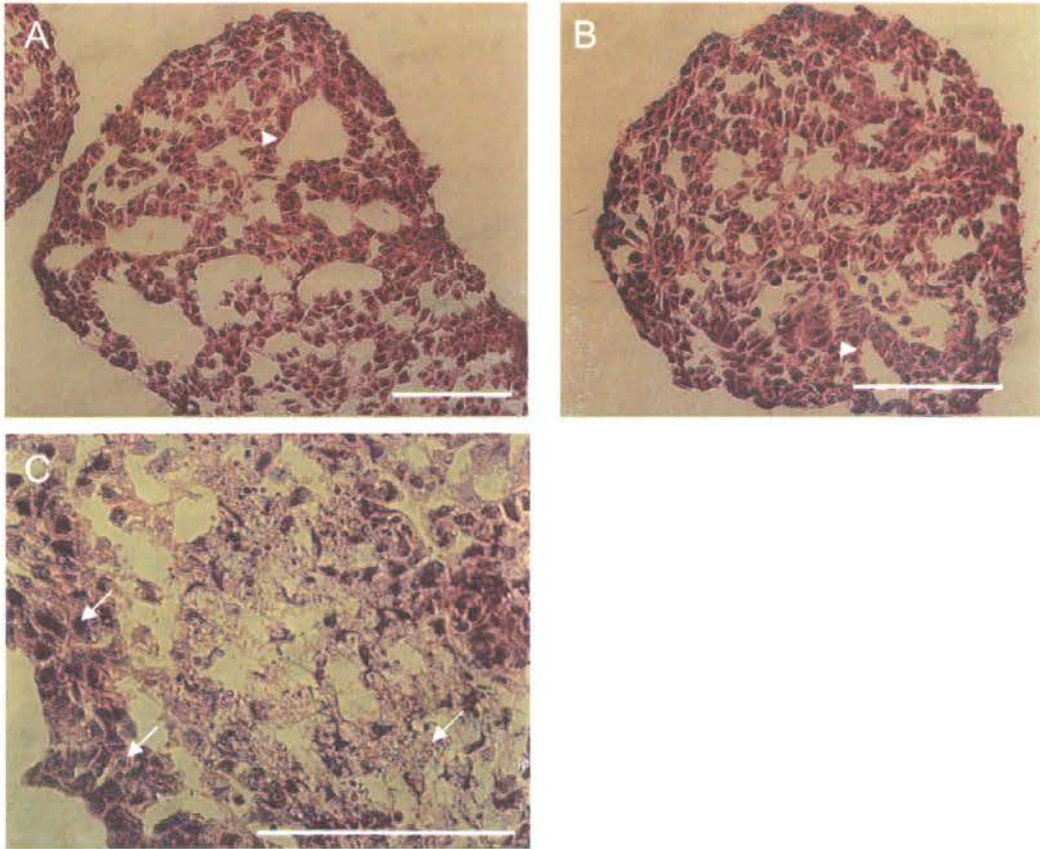


Figure 3.20. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and cultured for 14 days to form suspension aggregates. These were fixed in 4% PFA, embedded in OCT embedding medium and 10 μ m cryostat sections collected on slides and stained with haematoxylin, eosin and alkaline alcohol to blue the nuclei. Phase contrast images of sections of three different aggregates viewed at 10X magnification (A,B) and 20X magnification (C) reveal that the aggregates were heterogeneous in structure, with some cavities present (arrow head), that are typically lined with an ordered arrangement of cells. At least 3 different cell morphologies were observed, as depicted by arrows in C. Scale bars = 100 μ m

Down-regulation Of Stem Cell Markers Within Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.

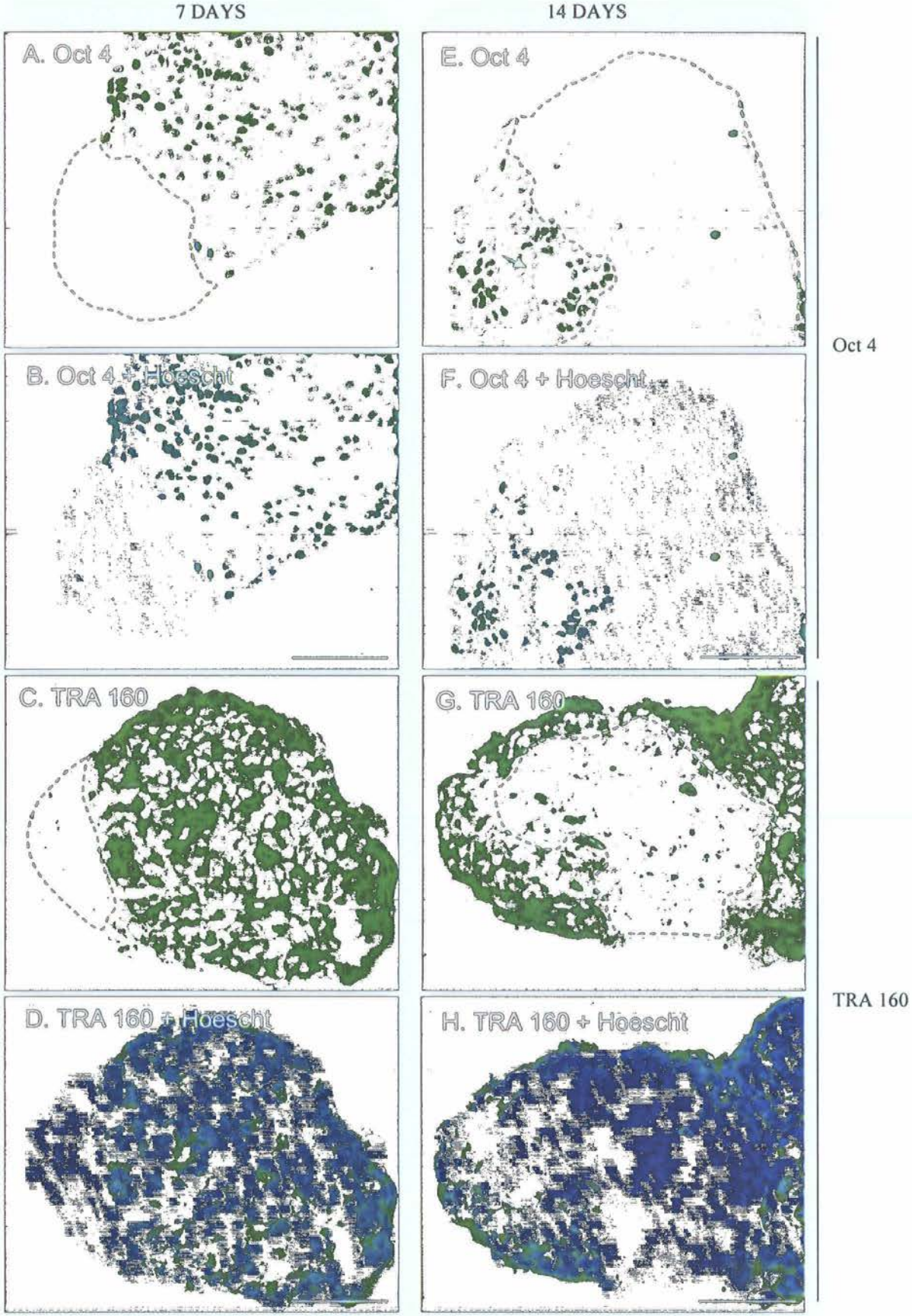


Figure 3.21. Down-regulation of stem cell markers within aggregates formed from suspension culture. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and cultured for 7 or 14 days to form suspension aggregates. Aggregates were fixed in 4% PFA, embedded in OCT embedding medium, 10µm cryostat sections collected on slides and immunolabelled with an antibody to either the stem cell transcription factor Oct4 (A-B, E-F) or the stem cell surface antigen TRA 160 (C-D, G-H) and the nuclei counterstained with hoescht (blue). Regions negative for TRA 160 or nuclear localised Oct4 staining are outlined in white dashes. A representative section through an aggregate cultured for 7 days contains a small distinct region towards the edge of the aggregate exhibiting down-regulation of nuclear localised Oct4 (A with hoescht overlay in B). A similar pattern of down-regulation is also observed in a representative 7 day aggregate section stained with TRA 160 (C with overlay in D). After 14 days of culture, a representative aggregate section (G with overlay H) demonstrates more substantial down-regulation of this stem cell marker, with a large region exhibiting negative staining for TRA 160 within the centre of the aggregate. Correspondingly at 14 days an aggregate section stained with an antibody to Oct4 (E with overlay F) demonstrates that a substantial proportion of cells within aggregates had down-regulated the expression of nuclear localised Oct4. Note that cells surrounding the cavity (arrow) in this section remained positive for nuclear localised Oct4. Scale bars = 100µm

Characterising differentiation

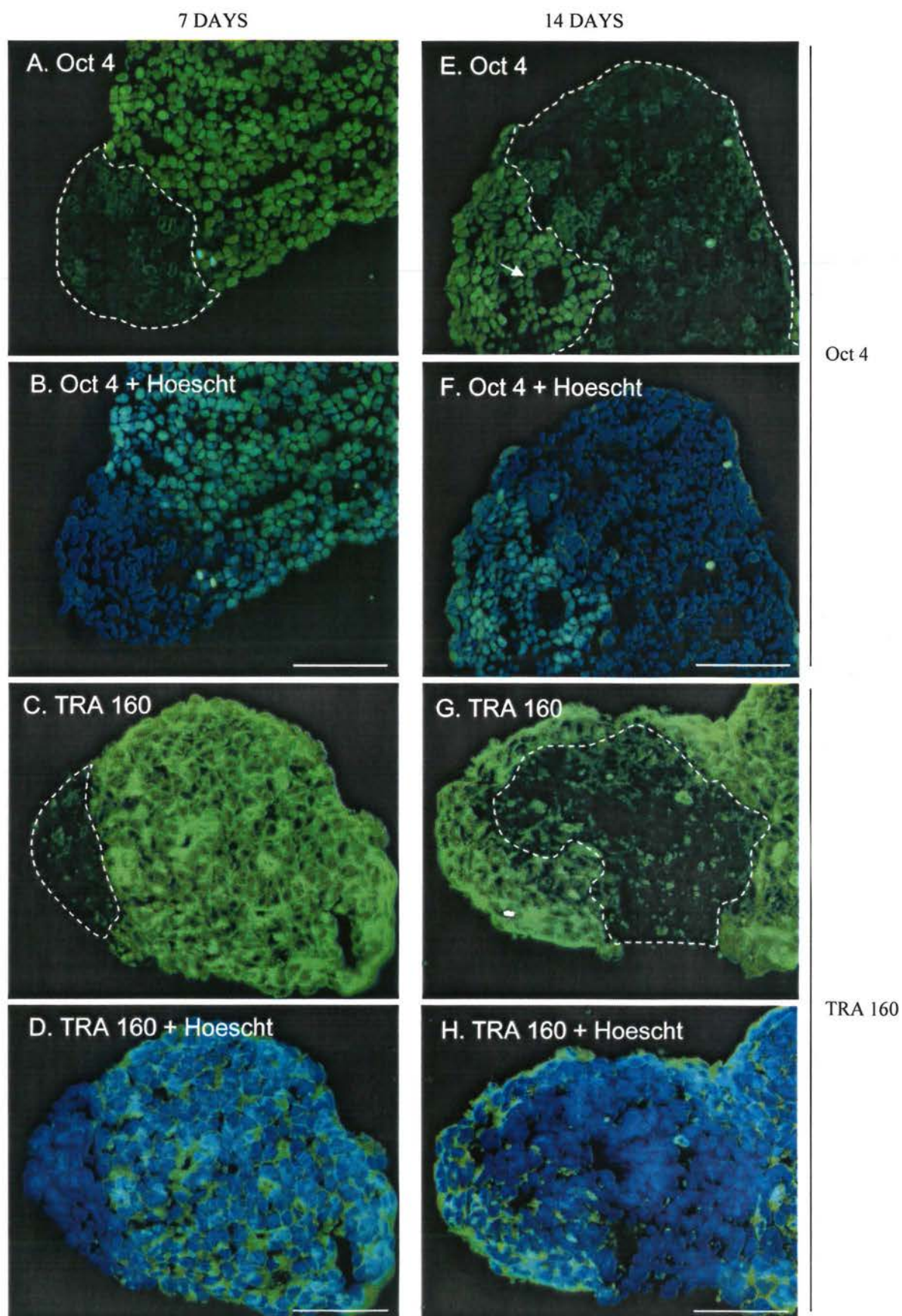
To identify the phenotype of the cells that had down-regulated stem cell marker expression and determine whether, like ES cell derived embryoid bodies, these aggregates contained differentiated derivatives from all three germ layers, cryostat sections of day 7 and 14 aggregates cultured without RA were immuno-stained with the following panel of markers for different germ layer derivatives and with antibodies to extraembryonic tissues.

Table 3.5 Details of antibodies used in immuno-labelling screen for markers of differentiation

ANTIGEN	ANTIBODY	SOURCE	DILUTION	FIXATIVE	CATEGORY
Nestin	MouseIgG	Chemicon	1:400	PFA	Ectoderm- neural progenitor
β III Tubulin	Rabbit IgG	Covance	1:400	PFA	Ectoderm –neuronal
NF160 or NFM	MouseIgG	Chemicon	1:100	PFA	"
NF200 or NFH	Rabbit IgG	Chemicon	1:400	PFA	"
MAP2	MouseIgG	Sigma	1:200	PFA	"
TP63 / TP73L	GoatIgG	R&D systems	1:50	PFA	Ectoderm – epidermis
Keratin 8	Rabbit IgG	Abcam	1:100	PFA	"
Keratin epithelial	MouseIgG	Chemicon	1:400	PFA	"
Gata 4	Goat IgG	R&D systems	1:50	PFA	Endoderm
AFP	MouseIgG	Sigma	1:100	PFA then 1 min with ethanol	Endoderm and primitive endoderm
HNF3 β	Goat IgG	R&D systems	1:50	PFA	Endoderm
Brachyury	Goat IgG	R&D systems	1:50	PFA	Mesoderm
α smooth muscle actin	mouse IgG	Sigma	1:100	PFA	smooth muscle
HCG	mouse IgG	Abcam	1:50	PFA	Trophoblast - trophoectoderm
Keratin 8	Rabbit IgG	Abcam	1:100	PFA	Nuclear localised marker of trophoblast cells

No mature neurons were observed at 14 days in the sections sampled, although most aggregates contained Nestin and β III tubulin positive cells. However, the pattern of staining for both Nestin and β III tubulin was diffuse and very similar to that observed in undifferentiated TERA2.sp12 cells. In contrast, large regions within the aggregates frequently stained immuno-stained positive for pan epithelial keratin (Figure 3.22), and cells within these regions were morphologically different under phase microscopy. Many cells within aggregate sections also exhibited positive nuclear staining for the early endoderm transcription factor Gata4 and mesoderm transcription factor Brachyury, as depicted by representative sections in Figure 3.22, whereas no positive immuno-staining for the endoderm transcription factor HNF3 β , the smooth muscle protein α -smooth muscle actin or the trophoblast hormone HCG was detected. In rare cases (less than 5% of aggregates screened), 2-3 cells within a sectioned aggregate stained positive for the primitive endoderm protein AFP. These immuno-staining analyses provide supporting evidence that TERA2.sp12 cells within aggregates formed from culture under suspension conditions in the absence of RA are differentiating into non-neural ectoderm, mesoderm and endoderm progenitors. This draws comparisons to the formation of

Down-regulation Of Stem Cell Markers Within Aggregates Formed From Suspension Culture Of TERA2.sp12 Cells.



Expression Of Mesoderm, Endoderm And Ectoderm Markers Within Aggregates
Formed From Suspension Culture Of TERA2.sp12 Cells.

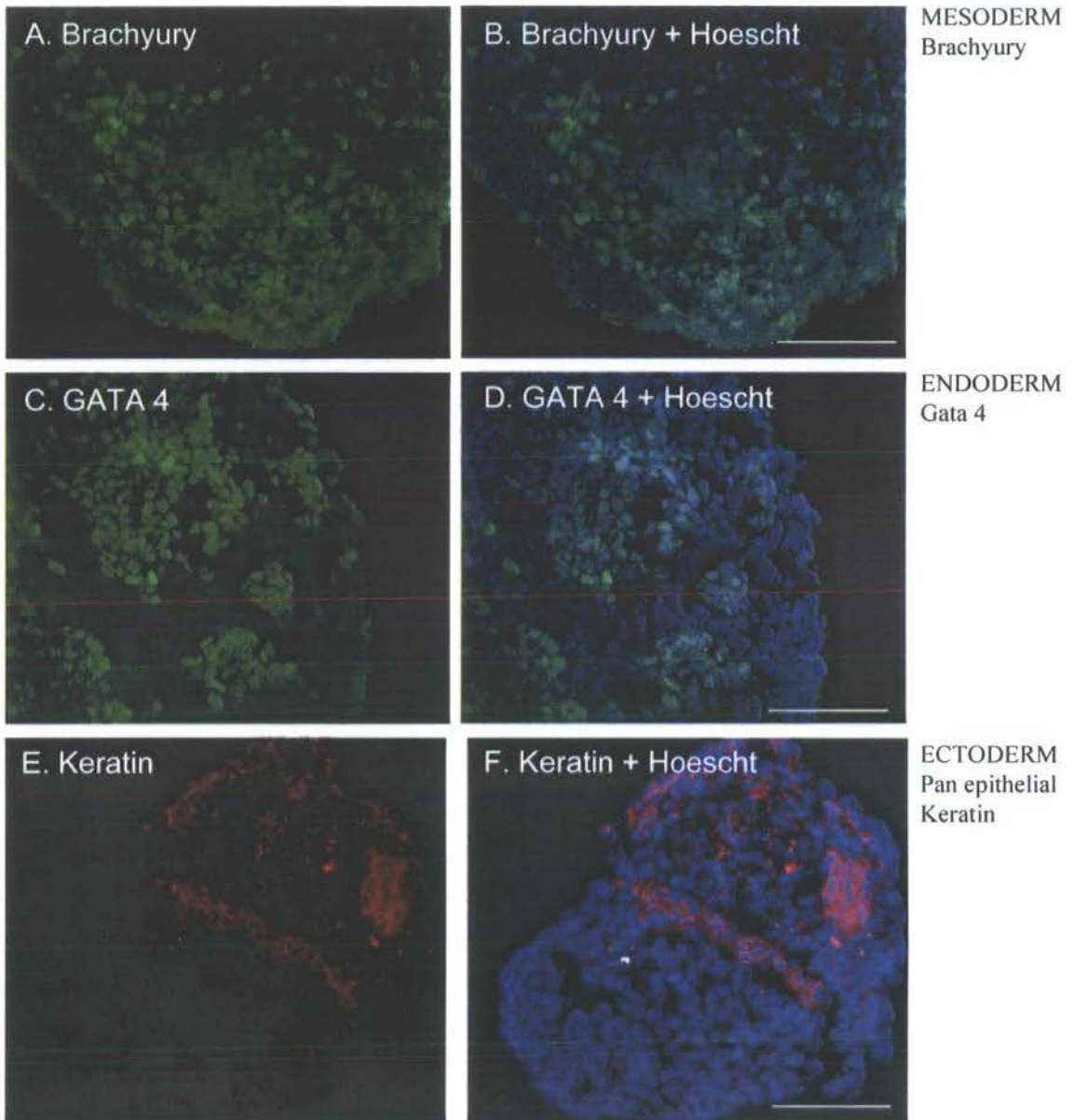


Figure 3.22. TERA2.sp12 cells were seeded at 20,000 cells/cm² into bacteriological petri dishes and cultured for 14 days to form suspension aggregates. Aggregates were fixed in 4% PFA, embedded in OCT embedding medium, 10 μ m cryostat sections collected on slides and immuno-labelled with an antibody to the mesoderm transcription factor Brachyury (A), the endoderm transcription factor Gata4 (C), or the ectoderm expressed pan epithelial keratin (E) and the nuclei counterstained with hoescht (blue). A representative aggregate section in A (with hoescht overlay in B) shows variable staining intensities for nuclear localised Brachyury, although a substantial number of cells display bright nuclear staining. In representative aggregate section C (with hoescht overlay in D), Gata4 positive cells demonstrate bright nuclear staining and occur in distinct patches throughout the aggregate. The Keratin immuno-stained section in E (with hoescht overlay in F) contains distinct regions that are immunopositive for pan-epithelial keratins. Scale bars = 100 μ m

Embryoid bodies from ES cells, which contain derivatives of all three germ layers.

3.2.9 Summary of results

A summary of the results generated from comparisons between TERA2.sp12 cells treated with RA under adherent or suspension conditions is depicted in Table 3.6. TERA2.sp12 cells cultured under suspension conditions without RA treatment also down-regulated stem cell antigens (to a slower extent), and after 14 days of culture the aggregates formed were detected by immuno-staining to express early markers indicative of all three germ layers.

Table 3.6 Summary comparison of RA induced differentiation in TERA2.sp12 cells cultured under adherent versus non-adherent conditions.

FORM OF ANALYSIS	ADHERENT CONDITIONS	NON-ADHERENT CONDITIONS
Aggregate formation	Adherent aggregate- like structures formed after more than 21 days	Small clusters of cells formed after 24 hours, free floating aggregates formed after 2-3 days.
Estimated mean rate of proliferation over 0-14 days (increase in cell number per 24 hours).	2.45 cells/24 hours	0.545/24 hours
Loss of surface carbohydrate stem cell markers	Both SSEA3 and TRA 160 down-regulated to less than 4% by 14 days. 56.35% of cells immuno-positive for TRA 160 at 7 days.	Both SSEA3 and TRA 160 down-regulated to less than 4% by 14 days. 17.7% of cells immuno-positive for TRA 160 at 7 days.
Gain of surface glycolipid antigens VINIS 53 and A2B5	Both antigens up-regulated. 75% of cells immuno-positive for A2B5 and 96% immuno-positive for VINIS 53 after 14 days.	Both antigens up-regulated. 82% of cells immuno-positive for A2B5 and 89% immuno-positive for VINIS 53 after 14 days.
Western blot detection of neural markers	Up-regulation of neuronal β III tubulin after 7 days (+), levels maintained for 28 days. NSE detected after 14 days, reaching maximum levels after 28 days (++). Levels of Nestin protein increased from 7-14 days (maximum ++), then decreased gradually to low levels at 28 days.	Strong up-regulation of neuronal β III tubulin after 7 days (++), and NSE detected at low levels after 4 days, increasing to high levels after 14 days (+++). Nestin expression up-regulated after 4 days, reaching high levels after 14 days, (+++).
Western blot and immuno-staining detection of simple epithelial protein Keratin 8 and epidermal stem cell transcription factor Tp63	Keratin 8 levels up-regulated over 14 days, and decreased slightly after 28 days. TP63 was not detected in Western blot analyses, although it was detected by immuno-staining in plaques of large cells.	Keratin 8 rapidly down-regulated to very low levels after 7 days. TP63 was not detected in Western blot or immuno-staining analyses.
BrDU up-take	A large proportion of cells had taken up BrDU after 5 days of culture, but after 14 days only scattered cells had taken up BrDU, and after 21 days BrDU up-take was rare. An exception was observed within aggregate-like growths of cells in the culture, which contained a high frequency of BrDU incorporating cells.	At 5 days scattered cells within the aggregate had taken up BrDU, and at 14 days the frequency was even lower. BrDU immuno-staining tended not to be localised in the centre of the aggregate.

3.3 DISCUSSION

3.3.1 Summary

The aim of this investigation was to compare and characterise the RA induced differentiation of TERA2.sp12 cells cultured under either adherent conditions or suspension conditions (in which floating aggregates form), and determine whether neural differentiation was indeed up-regulated as a result of suspension culture. Stem cell marker expression and proliferation rate were both more rapidly down-regulated in RA treated cells cultured under suspension conditions compared to adherent conditions, indicating that suspension cultures more rapidly embarked on differentiation. Correspondingly, the expression of neuronal proteins was more rapidly up-regulated and to higher levels as detected by Western blot analysis, and the increase in epithelial Keratin 8 expression observed in adherent cultures was reversed, supporting the hypothesis that increased neural differentiation and decreased non-neural ectoderm-like differentiation was occurring in suspension cultures. However, Keratin 8 was subsequently demonstrated to be expressed in two different populations within adherent cultures; in a non-neural morphologically distinct population that also expressed the epidermal stem cell marker TP63, and also in a proliferative Nestin positive population.

Aggregates generated from RA treatment under suspension conditions were also further characterised and although solely neural sub-types were identified, they did not resemble neurospheres in their structure or in other key characteristics. However, it was of interest to observe that distinct zones of proliferation and neuronal differentiation were displayed within these aggregates (and also to some extent in adherent cultures), with organised structures consisting of aligned proliferative neural progenitors and maturing neurons present after 14 days of RA treatment. These structures frequently resembled neural tube structures and could potentially provide useful models in which to study neural tube formation and patterning.

Culture under suspension conditions in the absence of RA treatment also induced stem cell marker down-regulation and a decrease in proliferation in TERA2.sp12 cells, although the time course of stem cell marker down-regulation was much longer than in RA treated cultures. Early markers of all three germ layers were detected in immuno-stained sections of aggregates after 14 days of culture (neural differentiation was not observed in the sections sampled), implying that these aggregates are similar to simple embryoid bodies formed from human ES cells and suggesting that TERA2.sp12 cells are not limited in their differentiation potential to solely ectodermal derivatives.

3.3.2 The stem cell phenotype

TERA2.sp12 cells have previously been reported to express stem cell surface markers TRA160 and SSEA3 which are down-regulated upon RA treatment (Przyborski 2001) and in this study we demonstrated that TERA2.sp12 cells also show nuclear localised immunostaining for Oct4 and Nanog; two key homeodomain transcription factors reported to be essential to the pluripotent and self-renewing phenotypes of embryonic stem cells (Chambers *et al.* 2003, Mitsui *et al.* 2003, Pesce & Scholer 2001). Oct4 is described as a key gate keeper in the beginnings of mammalian development and synergises with Sox2 in pluripotent cells to promote the transcription of target genes (Ben-Shushan *et al.* 1998, Chew *et al.* 2005, Nishimoto *et al.* 2005) including the sox2 and Oct4 encoding genes and the pluripotent marker Nanog (Rodda *et al.* 2005). In an initial study, the stem cell markers TRA 160, SSEA3, Oct4 and Nanog were utilised to compare the effect of RA treatment on the loss of stem cell phenotype in TERA2.sp12 cells cultured in either suspension or adherent culture conditions.

The effect of suspension culture – accelerated departure from the stem cell phenotype

Initial comparisons of stem cell marker expression and changes in proliferation rate indicate that RA treated cells cultured under suspension conditions departed from the stem cell phenotype more rapidly than cells cultured in adherent conditions: Cells grown under suspension conditions down-regulated the expression of the cell surface antigen TRA 160 to low levels in approximately half the time of cells treated in adherent conditions, and nuclear expression of the transcription factor Oct4 was absent in cells after 5 days of RA treatment in cultures grown under non-adherent conditions.

However, this trend was not observed for all stem cell markers. SSEA3 was observed to be down-regulated at relatively the same rate at the time-points tested in both culture conditions. This could be due to differential regulation of these stem cell markers, (the pathways controlling the expression of cell surface markers such as SSEA3 and TRA 160 have not yet been elucidated). However, it is more likely to be due to the limitations of this experiment. SSEA3 appears to be lost very early in TERA2.sp12 differentiation and was already down-regulated to low levels after 7 days of RA treatments in both sets of cultures. Rapid down-regulation of SSEA3 upon differentiation has been reported in both human embryonal carcinoma and embryonal stem cells (Draper *et al.* 2002, Fenderson *et al.* 1987). Hence it is likely that SSEA3 was down-regulated during the first few days of culture, and any difference in the speed of down-regulation was not detected because the first time-point of detection was too late. Live surface staining analyses indicated that by 4 days of culture SSEA3 expression was down-regulated to minimal levels in aggregate cultures compared to

significant expression in monolayer cultures. However in this experiment whole aggregates were immuno-stained, and as a result SSEA3 expression was only monitored on the surface of aggregates.

Oct4 protein levels were down-regulated in both sets of RA treated cultures after 14 days of treatment. This corresponds with previous studies that report that Oct4 expression can be regulated through the binding of nuclear receptors (including the RA receptors RAR/RXR) to its proximal promoter (Barnea & Bergman 2000, Ben-Shushan *et al.* 1995), and reports that Oct4 expression is down-regulated by RA treatment (Okamoto *et al.* 1990). It was also interesting to note that in these immuno-staining experiments – where Oct4 staining was absent from the nucleus, there was still low level staining in the surrounding cytoplasm of these cells. This could simply represent non-specific background staining. However, controls using secondary antibodies alone did not yield a similar background effect, and although this staining could represent non-specific interactions between the primary antibody and the stained cells, there is a possibility that the staining represents Oct4 protein in the cytoplasm. In the latter case this could reflect a post-translational means of regulating Oct4 activity, through translocation from the nucleus. However, there are no reports to date of regulation of Oct4 through this mechanism.

Stained sections of aggregates cultured with RA indicate that Oct4 was rapidly down-regulated in these aggregates, whereas it is interesting to note after 7 days of culture, sectioned aggregates still exhibited substantial TRA 160 expression – when Oct4 expression is almost entirely absent. Again this reflects the differing sensitivities and kinetics of regulation for the range of stem cell markers tested. As Oct4 is considered to be the key ‘gate keeper’ for stem cell maintenance, this also implies that cells that have begun to depart from the stem cell phenotype may still express some stem cell antigens (such as TRA 160), an indication that caution should be used when identifying stem cells with only a limited number of antigens. It would be interesting to determine whether TRA 160 is directly regulated by Oct4. One approach would be to use transfection or RNAi to directly inhibit Oct4 activity, and determine whether TRA 160 is also down-regulated. It would also be interesting to determine whether any other stem cell markers are also expressed in cells which remain positive for TRA 160, and to generate an overview of the kinetics of down-regulation of a range of stem cell markers. This would lead to a better characterisation of the kinetics of stem cell marker expression and a better understanding of the process by which cells depart from the stem cell phenotype.

As discussed later, negative staining for nuclear localised Oct4 was also detected in a proportion of cells within aggregates cultured without RA, indicating that the more rapid loss

of stem cell markers in RA treated cultures grown under non-adherent versus adherent conditions may be due in part to signalling events resulting from aggregate formation alone.

Cell confluency and the maintenance of stem cell phenotype

TERA2.sp12 cells are routinely seeded at a lower density (20,000cells/cm²) than maintenance culture density prior to RA treatment, so that the cultures do not overgrow during the experiment. In both adherent and non-adherent conditions, cells were seeded at this density prior to treatment with RA. However, cell-confluence is known to inhibit differentiation in embryonal carcinoma cells (for review Andrews 2002). Thus there is the possibility that some of the differentiation observed could be due to the initial low seeding density. To control for this possibility, cells were cultured in parallel at the same density and cultured without RA. In adherent cultures, cell growth initially slowed, and the percentage of cells expressing SSEA3 had decreased by roughly 15% after 7 days, although TRA 160 expression remained high. However, cells began to rapidly proliferate after 5 days such that the culture became over-confluent and the cells expired within 24 hours if they were not split and reseeded at maintenance density. After 14 days the proportion of cells expressing SSEA3 was equivalent to that of maintenance cells at 60 %. This raises an important point. Either SSEA3 expression had been up-regulated to original levels in these cells, or in a more likely scenario, the remaining SSEA3 positive cells had proliferated rapidly such that the increased number of SSEA3 negative cells had become insignificant after 14 days. It is interesting to note that TRA 160 was not down-regulated by seeding at low-density, in contrast to the sensitivity of SSEA3 expression. This may support the theory that stem cells can occupy various stages of 'stemness' where they have not yet committed to differentiate but have down-regulated some stem cell markers. Variable SSEA3 expression has been reported within populations of human embryonic stem cell cultures, for example approximately 65% of the culture of the H7 cell line express SSEA3 (Draper 2002) and indeed SSEA3 expression is not ubiquitous in maintenance cultures of TERA2.sp12. This raises the question of whether SSEA3 loss of expression in culture is reversible, and also whether down-regulated SSEA3 expression actually confers a restricted differentiation capability and reduced pluripotency. Fluorescence activated cell sorting of SSEA3 positive and negative cells is one technique that could be used to separate these populations and compare their differentiation profiles. It would also be useful to determine the expression of a wider range of stem cell markers (including Oct4 and Nanog) in cells seeded at this lower density. However, due to time constraints this was not carried out in the course of this study.

It is important to note that cells cultured without RA and seeded at the same density under adherent or suspension conditions behaved markedly differently. In adherent conditions,

as discussed previously, a transient down-regulation in cell proliferation and the proportion of SSEA3 positive cells were observed. In suspension conditions, levels of SSEA3 expression and the proportion of cells expressing SSEA3 were steadily down-regulated and this was permanent. In addition, other stem cell markers such as TRA 160 and nuclear localised Oct4 were also down-regulated.

Undifferentiated TERA2.sp12 cells express neuronal β III tubulin and Nestin

Both Western blot and immuno-staining analyses demonstrated positive expression of neuronal β III tubulin in undifferentiated cultures of TERA2.sp12 cells that were uniformly positive for Oct4, Nanog and TRA 160. This was the case with two different anti- β III tubulin antibodies, raised in different hosts, and control cultures of the human squamous carcinoma FaDU cell line were negative. Hoescht co-staining of cell nuclei indicated that β III tubulin expression was also present in cells that were actively dividing. Cells in undifferentiated cultures also immuno-stained positive for Nestin. Importantly, the expression of these markers was not only unusual in context, but also in phenotype; in both cases the immuno-staining was more diffuse and the cells did not display a neural morphology.

This aberrant expression could be a result of the abnormal genetic phenotype of TERA2.sp12. These embryonal carcinoma cells exhibit marked trisomy, and one could argue that these cells are inherently genetically instable. Another possibility is that this aberrant expression is an artefact from prolonged cell culture. Although this phenotype was observed in many different passages of TERA2.sp12, including those cultured from very early passage numbers, TERA2.sp12 cells are derived from passage 15 of TERA2 cells, and therefore even the earliest passages have undergone substantial cell culture.

The intermediate filament protein Nestin was originally categorised as a neural progenitor marker (Fuchs & Weber 1994), but recently this has been questioned in several reports documenting the expression in various non-neural lineages, including mesenchymal stem cells, angiogenic endothelial cells and pancreatic islets of langerhans (Hunziker & Stein 2000, Lardon *et al.* 2002, Woodbury *et al.* 2002). Nestin is also expressed in at least some human ES cell lines (Linda Lako, personal communication). Neuronal β III tubulin expression has also been reported in non-neuronal lineages including mesenchymal stem cells (Tondreau *et al.* 2004), and there have been some reports of neuronal β III tubulin expression in undifferentiated cultures of both mouse and human ES cell lines (Ginis *et al.* 2004). In one study where undifferentiated H1 cell cultures were reported to stain positive for β III tubulin, this staining displayed filamentous localisation in cells that co-stained positive for the stem cell marker TRA 181 (Carpenter *et al.* 2001). However, Neuronal β III tubulin is also expressed in non-neuronal tumours and is associated with de-differentiation and an ascending

histological grade of malignancy in gliomas and lung cancer (Katsetos *et al.* 2003). As non-neural β III tubulin expression is associated with 'de-differentiation' in cancer, it may be expressed in TERA.sp12 cells as a consequence of selection for an undifferentiated phenotype. In addition, recent investigations have highlighted the genetic instability of human stem cell lines cultured for prolonged periods (Draper *et al.* 2004). Thus low levels of β III tubulin expression in undifferentiated cell cultures of human stem cell and embryonic carcinoma cells could be an aberrant phenotype acquired during prolonged culture, in association with strong selection for the stem cell phenotype. It would be of interest to test very early passages of human embryonic stem cells, and discern whether β III tubulin expression in undifferentiated cells is acquired after subsequent passaging.

The aberrant expression of Nestin and neuronal β III tubulin underscores the importance of using multiple markers to characterise a phenotype, and in determining whether marker localisation is typical. To distinguish between undifferentiated neuronal β III tubulin and Nestin expression in this study, in all cases immuno-staining was carried out in addition to the Western blot analyses.

3.3.3 Neural differentiation in RA treated TERA2.sp12 cells

RA treatment induced neural differentiation in both suspension and adherent culture conditions: TERA2.sp12 cells gained neural marker expression in a regulated series of molecular events that appeared to mimic neuronal differentiation during embryogenesis. Similar to previously published studies with NTERA2 and TERA2.sp12 (Przyborski 2001, Przyborski *et al.* 2000), cells initially increased expression of the neural progenitor marker Nestin, followed by an increase in NeuroD1, expressed when cells exit the cell cycle and begin to differentiate, and the up-regulation of β III tubulin, NSE and neurofilament expression typical of maturing neurons. However, in aggregate cultures, the up-regulation of Nestin, NeuroD1, and maturing neuronal marker proteins started 2-3 days earlier, and peak expression levels (especially for β III tubulin) were generally higher. This indicates that induction of neuronal differentiation is occurring earlier, and neural differentiation may be occurring to a greater extent in aggregate cultures compared to monolayer cultures.

Immuno-staining analyses in adherent cultures demonstrated that by 14 days the majority of the cell culture consisted of proliferating Nestin positive cells, closely associated with β III tubulin and MAP2 positive cells that were distinctly neuronal in morphology. The expression of neuronal filaments was detected in a small proportion of neuronal cells exhibiting long axons that were detected in clusters on the top of the remaining cells, and increased numbers of these cells were observed with longer culture periods. Distinct rosette like structures of Nestin positive cells were observed between 7-14 days of RA treatment,

consisting of columnar, Nestin negative cells within the centre, and occasionally distinct ridges of orientated Nestin positive progenitor cells closely associated with β III tubulin positive neural cells were detected. Nestin positive cells invaginating to form a tubular structure were also observed, and after 21 days larger tubular structures had developed, consisting of distinct zones of neural cells expressing MAP2 and more mature neurofilaments. These structures demonstrated a marked similarity to various stages of neural tube formation, and similar observations have recently been reported in human embryonic stem cell cultures that have been differentiated towards the dopaminergic lineage with FGF treatment (Li *et al.* 2005, Yan *et al.* 2005). In parallel, neural tube like structures consisting of radially aligned Nestin positive cells surrounded by β III tubulin positive cells were also generated within aggregates formed from RA treated TERA2.sp12 cells cultured in suspension. These tube-like structures extended through several consecutive sections of aggregates, and had formed after 14 days of culture rather than 21 days in adherent conditions.

Further investigation to characterise these neural tube-like structures would be valuable, as these could be potentially useful models in which to study human neural tube formation and differentiation *in vitro*. In particular the neural tube-like structures formed within aggregates may re-capitulate the *in vivo* situation more closely as these cells are within a three-dimensional context. Immuno-staining for markers of various stages of neural tube formation, known patterning markers such as Sonic hedgehog (Shh) and Fibroblast growth factors (FGFs), and markers for positional identity in the neural tube would help to establish whether these structures could be useful models in which to study aspects of neural tube development.

As previously discussed, neural markers such as NSE, β III tubulin and Nestin were up-regulated more rapidly in cells cultured under non-adherent conditions, and this could be closely linked to the rapid loss of stem cell marker expression. However, another possibility is that neural differentiation was up-regulated in TERA2.sp12 cells cultured as aggregates in suspension as a result of neural differentiation being promoted over other forms of differentiation, such as non-neural ectoderm differentiation. This is supported by early observations of divergent Keratin 8 expression detected by Western blot analysis in differentiating adherent versus suspension cultures. Keratin 8 expression increased over 14 days in cells cultured under adherent conditions, and decreased in cells cultured in suspension. Keratin 8 is a type I acidic intermediate filament and together with Keratin 18 forms the major components of simple epithelia (Moll *et al.* 1982). Taken together with the observation that large-diameter cells similar in morphology to simple epithelia were observed in RA treated adherent cultures and not in suspension cultures of TERA2.sp12, this supports the hypothesis that neural and non-neural ectoderm differentiation is occurring in RA treated adherent

cultures, whereas neural differentiation alone occurs in suspension cultures, which could account for the enhanced levels of neural proteins detected by Western blot analysis in non-adherent cultures.

3.3.4 Non-neural differentiation in RA treated TERA2.sp12 cells

Keratin expression – indicative of differentiation into non-neural ectoderm?

Keratin 8, 18 and pan-epithelial keratin were detected by immuno-staining in the plaques of large-diameter cells observed in adherent cultures, supporting evidence that these cells were non-neural ectoderm in character, and immuno-staining analyses demonstrated that pan-epithelial Keratin and Keratin 8 expression was almost non-existent in aggregate cultures compared to adherent cultures.

However, the monoclonal anti Keratin 8 antibody (Sigma) also immuno-stained cells which were not within these 'large-celled plaques', in this case co-staining Nestin positive cells. This was in contrast to pan-epithelial keratin and Keratin 18 antibodies. A polyclonal anti-Keratin 8 antibody raised in a different host (ABCAM) solely immuno-stained large diameter-cells. One explanation for the different staining specificity is that the ABCAM sourced antibody recognises more restricted isoforms of Keratin 8 compared to the Sigma antibody. Westerns repeated with the ABCAM sourced antibody to Keratin 8 demonstrated no difference in expression to the antibody sourced from Sigma. This suggests that preparation of the samples for Western blot analyses enabled all forms of Keratin 8 to be detected by the ABCAM antibody. As a consequence, Keratin 8 expression detected in Western blot analyses is likely to represent at least two different populations of Keratin 8 expressing cells. Indeed, Western blot analyses indicate that undifferentiated TERA2.sp12 cells express Keratin 8.

Although typical of simple epithelia, Keratin 8 expression is not confined to cells of the non-neural ectoderm, and demonstrates a varied expression pattern during development. Keratin 8 and 18 are the first intermediate filaments to be expressed in embryogenesis, at the 8 cell stage of mouse blastomeres (Jackson *et al.* 1980). Keratin 8 expression has also been identified in the foetal heart (Markl 1991), and in astrocytic progenitor cells in the developing or repairing CNS of vertebrates (Groff *et al.* 1997, Heatley 1996). This correlates to some extent with the observed expression in undifferentiated cells, and the population of Nestin and Keratin 8 positive cells in differentiating RA treated cultures could represent neural stem cells or astrocytic progenitors. Therefore the up-regulation of total Keratin 8 protein detected by Western analysis in adherent cultures may reflect both an up-regulation in the proportion of neural progenitors and the presence of non-neural cell plaques in adherent cultures. Correspondingly, the observed down-regulation of Keratin 8 protein in TERA2.sp12 cultures

is likely to reflect the rapid departure of these cells from the stem cell phenotype and earlier neural differentiation.

Non-ectodermal differentiation of RA treated TERA2.sp12 cells?

In order to characterise these keratin positive 'large-celled plaques', and determine whether these cells were non-neural ectoderm precursors or another germ layer derivative, adherent cultures were immuno-stained with further markers of ectoderm derivatives and also markers of endoderm, mesoderm and tropho-ectoderm derivatives. These cells were negative for all markers tested, with one exception: Some cells within the larger cell plaques immuno-stained positive for TP63, a marker of epidermis progenitor cells (Koster & Roop 2004, McKeon 2004, Pellegrini *et al.* 2001), which has recently been reported to be temporally expressed in human embryonic stem cells differentiating into keratinocytes (Green *et al.* 2003).

During this screen, the endoderm transcription factor Gata4, mesoderm transcription factor Brachyury, and in very rare cases endoderm transcription factor HNF3 β was detected in small populations of cells within the culture that were morphologically indistinct under phase microscopy. This questions the supposition that TERA2.sp12 cells are limited to ectodermal differentiation. However, these markers are early markers of differentiation, and markers of later stage differentiation, (such as α smooth muscle actin and AFP) were not detected. In addition, Brachyury expression is reported to occur with no other evidence of mesoderm differentiation in NTERA2 cells (Gokhale *et al.* 2000) implying that the Brachyury expression detected may not be indicative of true mesoderm differentiation.

3.3.5 Characterisation of aggregates formed from suspension culture

Aggregation has been used in many cell systems to derive differentiated cells: Embryoid bodies are generated from mouse and human embryonic stem cells to form differentiated derivatives of all three germ layers, and neurospheres are routinely cultured from dissociated embryonic or adult CNS tissue exposed to growth factors (Campos 2004, Reynolds & Weiss 1992, Reynolds & Weiss 1996). The aggregates formed from culture of TERA2.sp12 cells under suspension conditions either with or without RA treatment displayed distinctive characteristics in both structure and protein expression.

Aggregates formed from RA treated suspension cultures

As previously discussed, TERA2.sp12 cells cultured as aggregates in suspension and treated with RA demonstrated accelerated down-regulation of stem cell marker expression and enhanced neural marker expression, with immuno-staining of aggregate sections suggesting that these aggregates were entirely neural in characteristics.

The neural characteristics of these aggregates raised the question of whether RA induced aggregates were comparable to neurospheres. Neurospheres are formed from dissociated embryonic or adult CNS tissue exposed to growth factors and consist of both neural progenitors and more differentiated progeny. One key feature used to classify neurospheres is that they are clonal in origin, meaning each neurosphere is derived from a single cell (Campos 2004, Reynolds & Weiss 1996).

To investigate whether RA treated aggregates formed from TERA2.sp212 cells were clonal in origin, aggregates were cultured from single cells of either TERA2.sp12 or GFP expressing TERA2.sp12.GFP cells at various cell densities. These aggregates consistently contained populations of both GFP positive and negative cells, indicating that under these conditions aggregate formation was literally due to 'aggregation' of single cells and proliferation. In addition, the pattern of neural marker expression within differentiating cells differed markedly from that of neurospheres, which are described to have a core of differentiating β III tubulin and GFAP positive cells surrounded by Nestin positive progenitor cells (Reynolds & Weiss 1996). In contrast, aggregate sections showed no immuno-reactivity to GFAP. This indicated that although the aggregates shared some properties with neurospheres, they did not satisfy all of the key characteristics for neurosphere formation. Another essential difference between neurospheres and TERA2.sp12 aggregates is their proliferative capacity. Neurosphere formation is a routine method to isolate and propagate neural stem cells, whereas TERA2.sp12 cells are being treated with a differentiation agent, and ultimately demonstrate very low proliferation rates. It would be interesting to try to promote neural differentiation and then proliferation with FGF and EGF to more closely mimic neurosphere generation from TERA2.sp12 cells.

Aggregates formed from RA treatment under non-adherent conditions frequently displayed radial zones of neural marker expression, with more immature markers (such as MAP2) coinciding with Nestin in the centre of the aggregate, and then maturing neurons expressing neurofilaments situated closer to the outer edge of the aggregate. This indicates some organisation in neural development was occurring in these aggregates. It is possible that cells within the aggregates were exerting differential effects through paracrine signals in order to achieve this level of organisation, and further characterisation of these signals could prove useful.

Aggregates formed from suspension culture without RA

Aggregates cultured without retinoic acid formed large irregular aggregates which were often more tubular in shape and frequently contained small cavities. Cells within these aggregates slowly down-regulated stem cell marker expression, such that the proportion of cells immuno-

positive to SSEA3 and TRA 160 had dropped to 30% and 80% respectively, and TRA 160 expression was also markedly lower in intensity in the remaining positive cells after 14 days of culture. Immuno-stained sections of aggregate cultures identified regions within the aggregate down-regulating TRA 160 expression, and also distinct regions were evident where nuclear staining of Oct4 was negative, which had expanded to dominate aggregate sections after 14 days. Negative staining for nuclear localised Oct4 was always detected in a patch of adjacent cells – not in scattered cells throughout the aggregate, suggestive that stem cell marker down-regulation was occurring through a localised community effect.

Immuno-staining identified frequent keratin positive regions within these aggregates, and also positive regions of expression for the endoderm marker Gata4 and for the mesoderm marker Brachyury, suggestive that epithelial, endodermal and mesodermal differentiation was occurring. As previously discussed, a report based on a NTERA2 EC cells indicates that Brachyury expression can occur in the absence of mesoderm differentiation (Gokhale 2000). However, the up-regulation of Brachyury under these conditions was marked, and much higher than in other treatments. Although markers of more mature derivatives such as smooth muscle were not detected, strong up-regulation of Brachyury does suggest the presence of an early mesoderm derivative.

The use of suspension culture and the detection of early markers from all three germ layers in these aggregates closely resemble the method of generation and key characteristics of embryoid bodies. Embryoid bodies (EBs) were first grown to form differentiated derivatives of all three germ layers *in vitro* from mouse embryonic stem cells in 1985 (Doetschman *et al.* 1985). There are two main methods of creating embryoid bodies with mouse ES cells *in vitro*, in both cases cells are either removed from their feeder layers or cultured without LIF to remove stem cell maintenance factors, and cultured under conditions which prevent the cells from adhering to a surface: Cells can be cultured as hanging drops in suspension in an inverted Petri dish and then transferred to an un-coated Petri dish for further differentiation in suspension, or cultured as single cells in an uncoated non-adherent Petri dish. In these structures, the developmental program of ICM/epiblast cells is thought to be re-activated in the ES cells (Smith 2001). Cellular differentiation is described to proceed on a schedule similar to that in the embryo but in the absence of proper axial organization or elaboration of a body plan. A basic description of the formation of EBS from mouse ES cells (extracted from Doetschman 1985) is as follows: Within the first few days of differentiation, EBs generate populations of cells that express genes indicative of primitive endoderm and mesoderm. While first simply appearing as a ball of cells, the embryoid body takes on an increasingly more complex appearance, becoming more cavitated, then forming a hollow or cystic embryoid body which can eventually form internal structures such as a yolk sac, and actively beating

cardiomyocytes.

As with murine ES cells, when human ES cells are cultured in suspension they can spontaneously create EBs, including cystic EBs. However, embryoid bodies are reportedly more difficult to derive from human ES cells partly due to the difficulties associated with culturing them as single cells (Ng *et al.* 2005) and not all human ES cell lines appear to be capable of forming EBS (Reubinoff *et al.* 2000). Human EBs are typically generated by roughly chopping large clumps of cells and culturing these in petri dishes or culturing cells as hanging drops at high densities. The H9 human ES cell line was reported to successfully form embryoid bodies containing derivatives of all three embryonic germ layers when cultured in non-adherent Petri dishes at high densities, although they were less organised than in mouse ES cells, as no pattern formation or organogenesis was observed (Itskovitz-Eldor *et al.* 2000).

As a basis from which to determine whether human embryoid bodies are similar to the aggregates formed from TERA2.sp12 cells, the reported characteristics of EBs derived from the human ES cell line H9 are directly compared with aggregates derived from TERA2.sp12 cells in Table 3.7.

Table 3.7 List of typical characteristics of embryoid bodies derived from the human ES cell line H9 and TERA2.sp12 aggregates formed under similar suspension conditions.

CHARACTERISTIC	EMBRYOID BODIES FORMED FROM H9 ES CELLS	AGGREGATES FORMED FROM TERA2.sp12 CELLS.
Culture conditions for formation	Cultured in non-adherent Petri dishes in the absence of feeder layers	Culture in non-adherent Petri dishes.
General morphology in culture under phase contrast	Initially form compact simple embryoid bodies, cavitated and cystic EBs observed under phase microscopy between 7 and 14 days.	Cavities observed at 14 days, highly cystic structures not observed under phase microscopy.
Morphology of haematoxylin and eosin stained sections	Can contain extra-embryonic tissues such as blood islands, highly cystic, and multiple cell morphologies observed. Cavities observed that are lined with two layers of epithelial and endoderm-like cells.	At least 3 different cell morphologies visible, cavities are less distinctly defined, and no distinct layers of cells surrounding cavities observed.
Markers of different germ layers detected	ECTODERM: NF68	ECTODERM: No neurofilament expression, pan-epithelial keratin,
	ENDODERM: AFP	ENDODERM: Gata4, in very rare cases AFP. Negative for gut endoderm marker HNF3 β
	MESODERM: α globin, α cardiac actin.	MESODERM: Brachyury Negative for smooth muscle marker α -smooth muscle actin.
Other features	Some embryoid bodies reported to pulse synchronously and express the myocardium marker α cardiac actin.	

Comparisons in Table 3.7 suggest that TERA2.sp12 aggregates formed from culture under suspension conditions do share several similarities with embryoid bodies derived from human ES cells. However, they appear to contain fewer organised structures than HES derived

embryoid bodies and were not observed to gain a highly cystic morphology. In addition, taken together with reports linking expression of the mesoderm marker Brachyury with an absence of mesoderm differentiation, this study does not yet provide convincing evidence that aggregates derived from TERA2.sp12 can form derivatives of all three germ layers.

A key difference between ES derived embryoid bodies and TERA2.sp12 derived aggregates is that embryoid bodies are formed in culture conditions where stem cell maintenance factors have been removed, such as LIF in mouse ES cultures or fibroblast feeder layers in human ES cultures. TERA2.p12 cells do not require feeder layers or additives to retain their stem cell phenotype, and thus the differentiation in TERA2.sp12 cells is entirely due to culture in non-adherent conditions and aggregate formation. This demonstrates that aggregation alone is enough to induce differentiation, and as discussed, patterns in stem cell marker down-regulation present strong evidence of a community-effect occurring within aggregates. The capacity for aggregation alone to induce differentiation is addressed somewhat in mouse ES cells, as when aggregates of mouse ES cells are cultured in suspension in the presence of LIF, the outer layer of cells down-regulate Nanog expression and express markers of visceral endoderm (Hamazaki *et al.* 2004). However, differentiation is restricted to the outer layer of cells, and shows no comparable trend in the regional down-regulation of Nanog expression in TERA2.sp12 aggregates. An investigation into the effect of aggregation on human ES cells that are cultured in stem cell maintenance conditions has only recently been possible, with the development of feeder free culture systems (Xu *et al.* 2001), and would be of interest here.

In summary, these results demonstrate the powerful effect of aggregation on differentiation of TERA2.sp12 cells, which reflects the increasing complexity of cell-cell interactions gained from cells cultured as aggregates. Evidence suggestive of a community effect within these aggregates, with regions of cells adjacent to each other embarking on proliferation, could be due to local changes in cell signalling that polarise neighbouring cells towards differentiation and may be mediated through cell-contact derived signalling or through local paracrine signalling events. A future investigation to elucidate the signalling events which are inducing differentiation in these aggregates could prove highly relevant to the signalling events that induce differentiation in the developing epiblast, as the 3-dimensional cell-cell contacts and close interactions generated in an aggregate are more similar to the environment experienced by embryonic cells *in vivo*. The mechanisms for down-regulation of Oct4 in the compacted morula and trophoectoderm differentiation have not yet been determined, although it has been proposed that Oct4 may be repressed as a consequence of specific cell-cell adhesion (Pesce & Scholer 2001), which would correspond with the observations in this study.

3.3.6 Synopsis

- RA induced neural differentiation of TERA2.sp12 cells reflected many aspects of neural differentiation in early development, with structures similar to the neural tube observed in both adherent and non-adherent cultures.
- RA treated cells grown under adherent conditions
 - Display many neural characteristics, with regionally organised expression of neural markers of different stages.
 - Also differentiate into large-diameter cells which were solely detected to express markers of non-neural ectoderm.
- RA treated cells grown under non-adherent conditions to form aggregates
 - Demonstrate accelerated loss of stem cell markers and both accelerated and up-regulated gain of neural markers compared to adherent cultures.
 - Share some characteristics with neurospheres, although they are unique in many aspects and should not be described as neurospheres.
- Cells cultured under non-adherent conditions to form aggregates (without RA)
 - Contain marked regions of stem cell marker down-regulation and differentiation in neighbouring cells that is suggestive of a community effect.
 - Share similarities with embryoid bodies derived from HES cells, although key differences exist – the reduced complexity, absence of evidence of mature derivatives from all three germ layers and the method whereby in ES cells maintenance factors are removed from the culture.

3.3.7 Conclusions

As well as more comprehensively characterising the RA induced differentiation of TERA2.sp12 cells under aggregate conditions, and demonstrating that aggregation alone can induce differentiation in TERA2.sp12 cells, this study demonstrates that RA treatment of TERA2.sp12 cells under non-adherent conditions does indeed up-regulate neural differentiation compared to adherent cultures, and two possible explanations for this difference are apparent:

One possible explanation is based on the accelerated loss of stem cell markers and reduction in proliferation in TERA2.sp12 cells cultured with RA under suspension conditions as opposed to adherent conditions. The accelerated neural differentiation observed could be due to a more rapid departure from the stem cell phenotype and differentiation in aggregates. However, this does not explain the marked up-regulation in neural marker expression, as theoretically the maximum expression level of neural markers would eventually be the same, and this is not the case. Another possible explanation is that as adherent cultures exhibit

evidence of both neural and non-neural ectoderm differentiation (in contrast to solely neural differentiation in suspension cultures), the lower level of neural differentiation is a direct consequence of a proportion of TERA2.sp12 EC cells differentiating along a non-neural instead of neural fate.

It is likely that the accelerated and up-regulated neural differentiation observed in RA treated cells cultured as suspension as opposed to adherent cultures is generated by a combination of the above. This raises an important question: Why is an up-regulation of neural differentiation and inhibition of non-neural ectoderm differentiation manifested in suspension cultures – and do key differences in signalling levels underlie these differences?

As reviewed in the next chapter, in the vertebrate ectoderm BMP signalling plays a key role in determining whether cells are specified towards prospective neural or epidermal lineages, and early dissociation experiments with *Xenopus* ectoderm explants were central to generating the generally accepted model of ‘default’ neural induction and BMP mediated inhibition of this process. This draws several parallels to the work in this chapter, suggesting that BMP signalling may be playing a similar role in neural induction decisions in TERA2.sp12 cells. In the following chapter I attempt to determine whether underlying changes in BMP signalling induced by these alternate culture methods are indeed the cause of these marked differences in neural and non-neural ectoderm differentiation, as a means to investigate neural induction events in TERA2.sp12 cells as a model of human ectoderm.

CHAPTER 4
BMP SIGNALLING AND CELL FATE DETERMINATION IN
TERA2.SP12 CELLS

4.1 INTRODUCTION

The following introduction discusses neural versus non-neural ectoderm specification during vertebrate development and current theories for neural induction in vertebrates. BMP signalling is presented as a key regulator of neural versus non-neural fate determination in the ectoderm, and subsequently the BMP signalling pathway and its regulatory components are outlined. The roles of other signalling proteins such as fibroblast growth factor (FGF) and Wnt in neural induction are also discussed in relation to recent work in chick embryos, focussing on their potent regulatory effects on BMP pathway activity. Finally, the current work on BMP signalling and neural induction in human and mouse embryonal carcinoma cells is surveyed and TERA2.sp12 cells are presented as a suitable model in which to study this key developmental process.

4.1.1 Neural versus non-neural fate specification in the ectoderm

In vertebrates, the dorsal ectoderm will give rise to the entire nervous system, while the ventral side will generate the epidermis. The developmental signals controlling the specification of naive ectoderm along neural as opposed to non-neural fates (termed neural induction) has been a subject of intense interest since the first clues emerged in an experiment in the 1920s by Spemann and Mangold. Using differentially pigmented newts they observed that transplantation of the most dorsal lip of the blastopore (prospective mesoderm) to the ventral side of another embryo which would normally generate epidermis (prospective epidermis) at gastrula stage, generated a second axis with a central nervous system almost entirely derived from the host prospective epidermis (Spemann & Mangold 1924, 2001) (see reviews (Hemmati-Brivanlou & Melton 1997, Stern 2005, Weinstein & Hemmati-Brivanlou 1999). This region in the dorsal lip was termed the 'organiser' and similar regions were identified in the prospective mesoderm of other species such as *Xenopus* and chick that were able to induce neural tissue upon transplantation, (Spemann & Mangold 1923 (2001)) and see reviews (Hemmati-Brivanlou & Melton 1997, Stern 2005, Weinstein & Hemmati-Brivanlou 1999). This established the concept of neural induction as an instructive interaction between the dorsal lip of the blastopore, (the 'organiser') and the neighbouring ectoderm. Consistent with this idea, studies identified secreted neural inducers Follistatin, Noggin and Chordin expressed in the organizer region of *Xenopus* embryos that could induce neural markers in blastula stage animal cap explants (Hemmati-Brivanlou *et al.* 1994a, Lamb *et al.* 1993, Sasai *et al.* 1995).

However, subsequent studies generated an accumulating body of contradictory

evidence, including key observations from the culture of animal cap explants (naive ectoderm) from the pre-gastrula stage of *Xenopus* embryos. Although intact cultured explants of pre-gastrula ectoderm formed epidermis, temporary dissociation of these explants led to neural differentiation, in the absence of any interaction with the organiser (for review Stern 2005). One suggested explanation for this effect was that signalling between early ectodermal cells (possibly mediated by a secreted protein) normally suppressed neural differentiation, and dissociation disrupted this suppression through a dilution effect of the secreted protein.

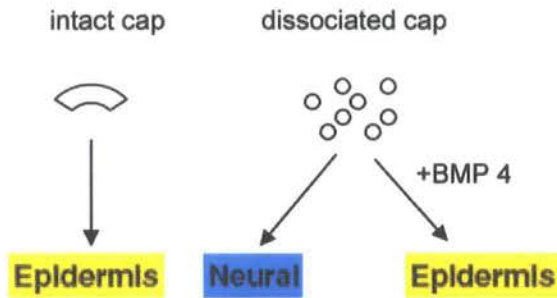
Furthermore, whilst studying the effect of Activin on mesoderm formation in *Xenopus*, Hemmati-Brivanlou and Melton (1992) observed that treatment of cultured animal cap explants with a broad range inhibitor of Activin receptors, that actually inhibits a range of Transforming growth factor β (TGF β) ligands, namely Activin, mature Vgl and Bone morphogenetic protein 4, inhibited mesoderm formation, but also generated neural tissue (Hemmati-Brivanlou & Melton 1994b). Taken together with the reports of neural differentiation in dissociated *Xenopus* explants, this led them to propose a default model of induction, namely that cells would become neural in the absence of other signals, and actually required instructive signals to form epidermis. They subsequently supported this model and identified Bone morphogenetic protein 4 (BMP4) as one such instructive signal using a series of classic dissociation experiments with *Xenopus* cultured animal caps, outlined in Figure 4.1.a. In summary, they demonstrated that BMP4 treatment could 'rescue' the neuralising effect of dissociation in cultured *Xenopus* gastrula ectoderm explants, preventing these cells from forming neural tissue and instead inducing differentiation into epidermis (Wilson & Hemmati-Brivanlou 1995).

Taken together with the finding that Noggin, Chordin, Follistatin and other neural fate inducing molecules such as Cerebrus inhibited BMP signalling in the extracellular space (Fainsod *et al.* 1997, Piccolo *et al.* 1996, 1999, Zimmerman *et al.* 1996), this led to more widespread acceptance of a 'default model' of neural induction in *Xenopus*. In essence, the default model proposes that neural induction occurs as a result of the inhibition of BMP signalling in the embryonic ectoderm, and that in the absence of external signalling cues, ectodermal cells will adopt a neural fate. BMP2 and BMP7 are also expressed in the ectoderm at this time and Suzuki *et al.* subsequently reported that BMP2 and BMP7 could also inhibit neuralisation and mediate epidermis formation in developing *Xenopus* (Suzuki *et al.* 1997a). In addition, transcription of BMP RNA is maintained by the activity of BMP protein (Biehs *et al.* 1996) which explains the disappearance of BMP4 and 7 expression from the vicinity of the organizer (which secretes BMP inhibitors) at the gastrula stage (Fainsod *et al.* 1994, Hawley *et al.* 1995).

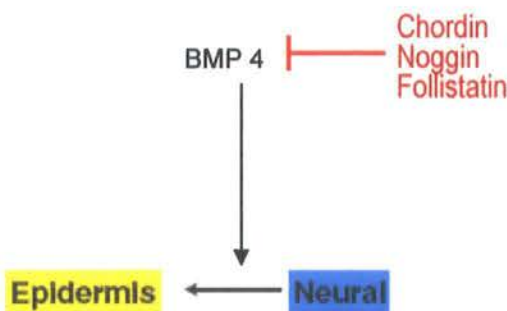
To summarise, signalling through BMP2, 4 and 7 is thought to inhibit neural

The Default Model Of Neural Induction In Developing *Xenopus laevis*

A Removal of explant from naïve ectoderm in pre-gastrula stage and culture of animal cap *in vitro*



B Inductive interactions in the default model



C A rough fate map of a blastula-stage embryo

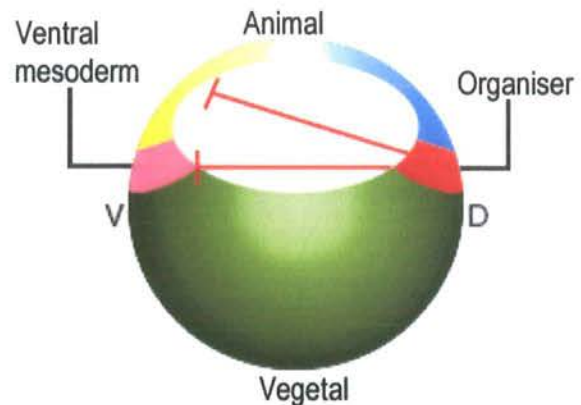


Figure 4.1 Key Dissociation experiments carried out by Hemmati-Brivanlou et al. that support the default model of neural induction (A), an outline of the inductive interactions in the default model of neural induction (B) and a rough fate map of the blastula-stage embryo (C).

(A) In key dissociation experiments by Hemmati-Brivanlou et al. (1995) on explanted pre-gastrula animal caps, they demonstrated that the addition of BMP4 could rescue the neuralising effect of dissociation. Excised animal caps matured *in vitro* differentiate into epidermal tissue. However, if the animal caps are dissociated for more than 5 hours, re-associated tissue will differentiate into neural cells. This effect is rescued if the dissociated cells are exposed to BMP4 protein, when neural differentiation is suppressed and epidermal tissue forms.

(B) A diagram of the inductive interactions proposed by the model: Ectoderm cells have an autonomous tendency to differentiate into neural tissue, but are prevented from doing this and are directed instead to epidermis by BMP4, which is expressed ubiquitously. Near the organizer, BMP antagonists block BMP4 signalling, allowing neighbouring ectoderm cells to develop according to their 'default' neural fate.

(C) A rough fate map of a blastula-stage embryo. Prospective territories are the organizer in red, ventral mesoderm in pink, neural tissue in blue, epidermis in yellow and yolk endoderm in green. The red lines represent BMP antagonist activity emanating from the organizer. V (ventral), D (Dorsal).

(Figures B and C adapted from Stern 2005 Figure 1)

induction and promote the generation of epidermis in the *Xenopus* developing ectoderm, and neural induction is proposed to occur as a result of suppression of the activity of these BMPs, mediated by BMP inhibitors secreted from the organizer region of the underlying mesoderm. This is outlined in sections B and C of Figure 4.1.

4.1.2 Bone morphogenetic proteins

BMPs form a sub-group of the TGF β family of secreted proteins that were originally isolated from their ability to induce bone and cartilage formation when implanted at ectopic sites in rats (Wozney *et al.* 1988). Subsequent studies have demonstrated that BMPs are multifunctional signalling molecules that regulate a vast array of cellular processes including differentiation, apoptosis and proliferation and play powerful roles in multiple aspects of development, such as mesoderm ventralisation, limb patterning, haematopoiesis and neural induction, see reviews (Dale & Jones 1999, Graff 1997, Hogan 1996a, Hogan 1996b, Mehler *et al.* 1997). It is also important to note that signalling mediated through the Nodal/Activin branch of the TGF β pathway induces mesodermal gene expression in ectodermal cells, reflecting the powerful roles of TGF β signalling in fate determination.

As a subgroup of the TGF β (transforming growth factor) family, these proteins are initially synthesised as large precursors that form homo- or hetero-dimers, and the active dimers are released by proteolytic cleavage. These active dimers bind to pairs of serine/threonine membrane receptor kinases, (types I and II), mediating the formation of a hetero-tetrameric receptor complex which initiates signalling transduction through Smad proteins. An outline of the TGF β signalling pathway is depicted in Figure 4.2.

In summary, the Type II receptors are constitutively active kinases, and trans-phosphorylate Type I receptors upon ligand binding and hetero-tetrameric receptor complex formation. The activated kinases of Type I receptors are subsequently able to phosphorylate and activate various intracellular Receptor smads (R smads), and thus determine the specificity of intracellular signals. Specific receptors for BMPs have been identified (BMPRIA, BMPRIB, and BMPRII), but some BMPs also interact with receptors with high affinity for Activins (ActRI, ActRII, ActRIIB). Phosphorylated Receptor smads are then able to complex with Common smad 4, (Co smad 4) and accumulate in the nucleus, where the hetero-complex binds to DNA and complexes with a range of DNA binding co-factors to induce transcriptional activation or repression of a host of genes. Subsequent Receptor smad de-phosphorylation then causes their return to the cytoplasm. For reviews see (Balemans & Van Hul 2002, Hill 2001, Itoh *et al.* 2000, Kawabata *et al.* 1998, Massague *et al.* 2005, Massague & Wotton 2000, Shi & Massague 2003).

Summary Of The TGF beta Signalling Pathway

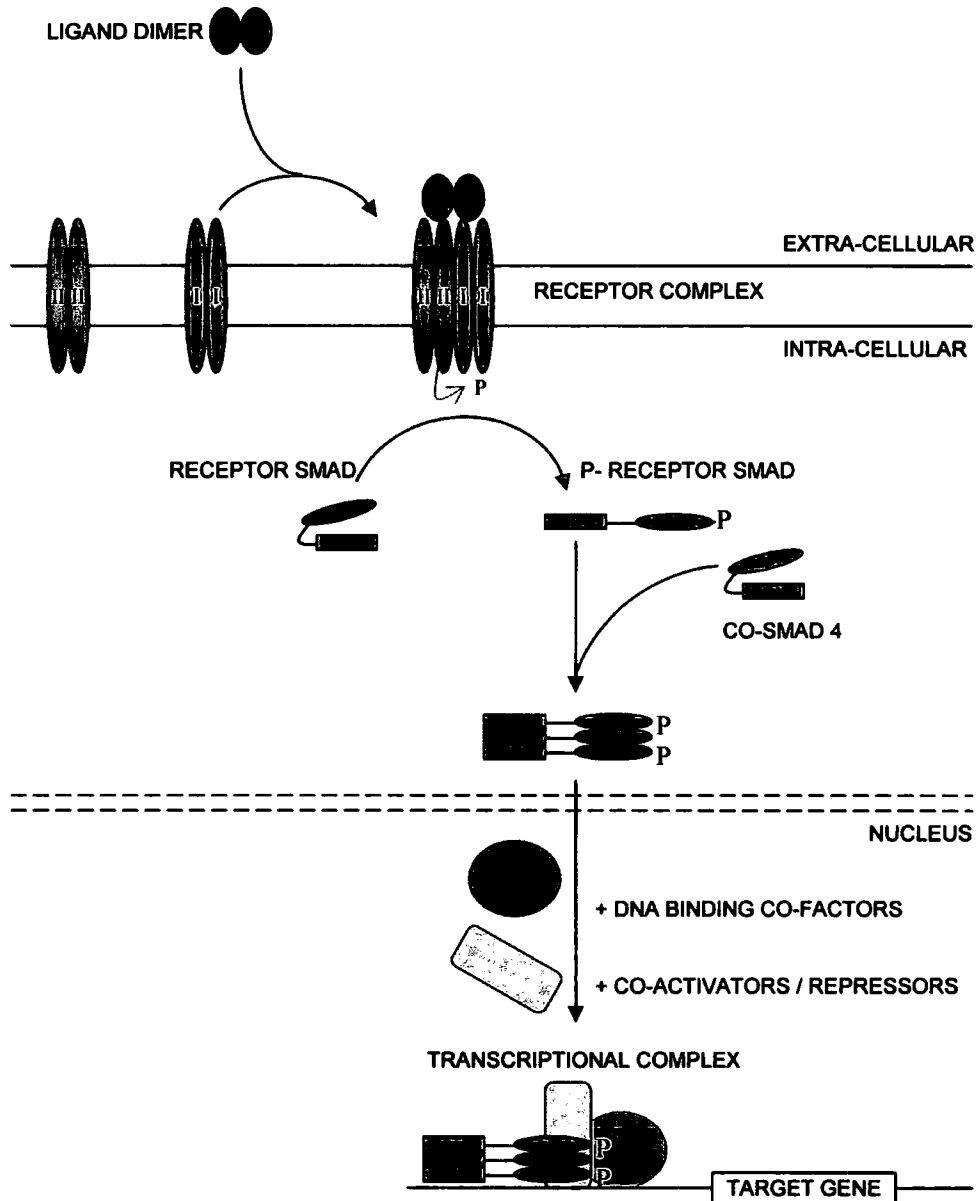


Figure 4.2. A basic outline of the TGF beta signalling pathway.

Upon ligand-induced heteromeric complex formation and activation of type I and type II receptors, R-Smads are phosphorylated and form heteromeric complexes with Co-Smads that translocate to the nucleus, where in cooperation with DNA binding co-factors and co-activators or repressors they control the expression of target genes in a cell type specific manner

Smads – the mediators of BMP signalling

The name smad is derived from a fusion of the terms *sma* and *mothers against dpp* (*Mad*), after the homologues identified in *C. elegans* and *Drosophila* respectively (Derynck *et al.* 1998, Savage *et al.* 1996), and as outlined above, there are three distinct subgroups of smads, the receptor smads (R smads), Common smad 4, and inhibitory smads. All of these smads have been identified in humans and these well characterised transduction proteins are extensively reviewed by Massague *et al.* (Massague 2005, Shi & Massague 2003). Smads 1, 5 and 8 are activated by the principle type I BMP receptors BMPR-IA or BMPR-IB, (whereas receptor Smads 2 and 3 mediate Activin, TGF β and Nodal receptor signalling), and Smad4 serves as a common partner for all R Smads. Smad6 and Smad7 are inhibitory Smads that interfere with Smad–receptor or Smad–Smad interactions.

All smad proteins consist of two globular domains, mad-homology 1 (MH1) and mad-homology 2 (MH2) coupled by a linker region: The N-terminal domain, or “Mad-homology 1” (MH1) domain, is highly conserved in all R Smads and Smad4 but not in the inhibitory Smads, and mediates DNA binding. The MH2 domain is conserved in all Smad proteins, and mediates interactions with cytoplasmic retention proteins, nucleoporins of nuclear pore complexes, and with DNA-binding cofactors. R-Smads also have a conserved C-terminal motif, Ser–X–Ser, that is phosphorylated by the activated receptor. The linker region is quite divergent between the various subgroups, and a region overlapping the linker and MH2 regions (the Smad4 activation domain) also mediates interactions with transcriptional activators and repressors.

To summarise, the structural analysis of smad proteins has revealed multiple sites for protein and DNA binding and for phosphorylation, which manifests the complexity of interactions mediated by these multi-functional signalling proteins and generates an insight into the level of sophistication of this apparently simple signalling pathway.

Regulation of BMP pathway activity

Finely tuned signals between cells are necessary to coordinate all aspects of development, and as a key signalling pathway BMP signalling is tightly controlled by a web of regulatory proteins acting at multiple levels, particularly at the stages of receptor activation and receptor smad activity. These participate in negative feedback loops and also as a mechanism of integration with other signalling pathways: Figure 4.3 outlines the key modulators of BMP pathway activity in the embryo.

In summary, extracellular proteins prevent receptor activation by binding to extracellular BMPs and preventing their interaction with receptors. Noggin (Zimmerman 1996) and Chordin (Piccolo 1996) both bind to BMP2 and BMP4 with high affinity (Noggin

Outline Of The Actions of Negative Regulatory Proteins On BMP Pathway Activity

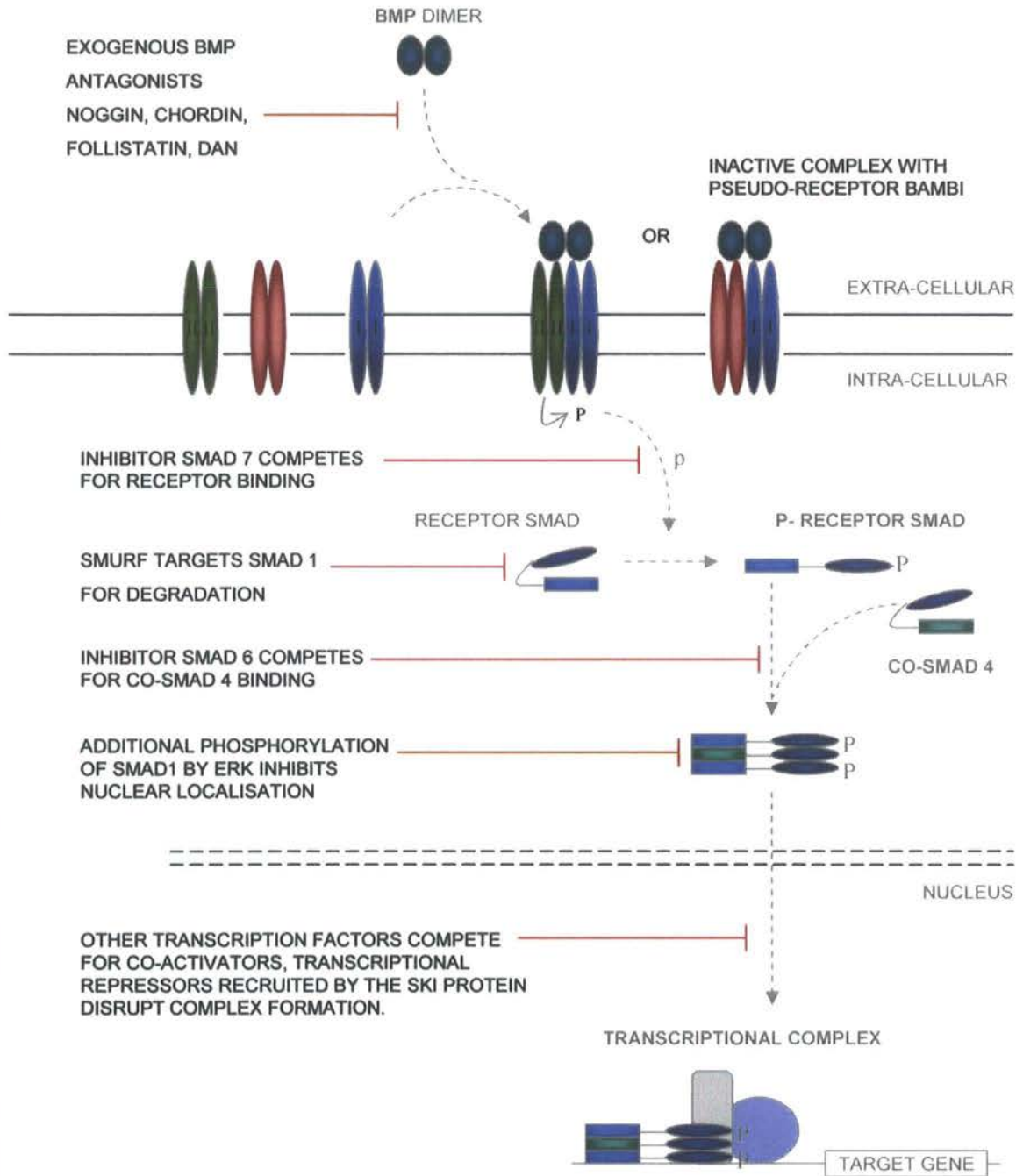


Figure 4.3. An outline of Modulation of BMP pathway activity in the embryo.

In the embryo, BMP pathway activity is modulated at several levels. Extracellularly, various secreted inhibitors such as Noggin, Chordin and Follistatin bind and inhibit BMP ligands. At the extracellular membrane, a pseudo-receptor (BAMBI) competes with Type II receptors for Type I receptors and forms an inactive complex. In the cytosol, the inhibitory Smad 6 competes with Smad 4 for binding to activated Smad 1, whereas Smad 7 is thought to exert part of its inhibitory activities by preventing the activation of Smad 1. In addition, Smurf1 and 2 target Smad 1 and the TGF receptors for degradation by the proteasome. Additional phosphorylation of Smad 1 mediated by ERK1/2 in the MAPK pathway leads to inhibition of nuclear accumulation, and finally, within the nucleus transcriptional repressors recruited by the Ski protein can disrupt smad mediated transcriptional activation of a variety of target genes.

also binds with lower affinity to BMP7), and Follistatin binds BMP 4 and BMP7, (although it also binds activin with high affinity) (Fainsod 1997). Other secreted proteins have been shown to antagonise BMP signalling in *Xenopus* embryos, such as Cerberus, DAN and Gremlin (for review (Balemans & Van Hul 2002)). In addition, the pseudo-receptor BAMBI (BMP and activin membrane bound inhibitor) competes with Type II receptors to form stable inactive complexes with type I receptors, preventing the formation of active receptor complexes upon ligand binding (Onichtchouck *et al.* 1999).

The first mediators of intracellular modulation are via the inhibitory smads, which are reviewed extensively in Derynk *et al.* (1998) and Massague *et al.* (2005). In brief, Smad6 competes with Smad4 to interact with receptor-activated Smad1, forming inactive Smad1–Smad6 complexes. Smad 7 competes with R-Smads in binding to activated TGF β and BMP receptors, preventing R-smad phosphorylation and activation. The expression of Smad 6 and 7 is induced by multiple stimuli, such as EGF and various TGF β family members that include BMP 7, suggesting the existence of a feedback signal. Two Smurfs (Smad ubiquitination regulatory factors) Smurf 1 and 2, modulate signalling by selectively targeting the activated type I receptors for degradation, and Smurf1 also regulates basal levels of Smad 1, for review see (Massague 2005).

A recently discovered means of pathway modulation that is discussed in more detail later links the MAPK pathway to BMP signalling via the R-Smad 1. The R-Smad linker region contains multiple serine and threonine consensus sites for ERK MAP kinases, and ERK1/2 mediated phosphorylation of Smad 1 has been demonstrated to inhibit nuclear accumulation *in vitro* (Kretzschmar *et al.* 1997).

Mechanisms of BMP mediated inhibition of neural induction and promotion of differentiation into non-neural ectoderm

Activated receptor smads mediate the transcriptional regulation of a plethora of genes in response to BMP signalling in a cell type specific manner, depending on the co-factors and transcriptional activators and repressors. Most of the BMP target genes identified to date encode homeobox transcription factors. Transcription factors that can mediate BMP responses identified in developing *Xenopus* include Msx1 (muscle segment homeobox 1), (Suzuki *et al.* 1997b), Msx2, Vent2 (or Xom) and Dlx5 (Miyama *et al.* 1999) , see review by Chang and Hemmati-Brivanlou and references therein (1998). Msx1 and Vent2 are reported to be directly regulated by BMP signalling in the absence of protein synthesis in *Xenopus*, and injection of Msx1 is able to counteract the neuralizing activity of a truncated BMP receptor to restore epidermal fate in intact animal caps (Ladher *et al.* 1996, Suzuki 1997b).

Homeobox genes Msx1 and Msx2 and Ids (inhibitors of differentiation) 1-3 have also

been reported by Hollnagel *et al.* (1999) to be direct targets of BMP signalling in the developing mouse, demonstrating transcriptional up-regulation in BMP treated mouse ES cells. Members 1-3 of the Id family function as negative regulators of pro-neural helix loop helix factors, (such as neurogenin), thus mediating a direct link between BMP signalling and inhibition of neural induction (Hollnagel *et al.* 1999). In addition, studies by Pera *et al.* (1999) in the developing chick also demonstrate that the homeobox gene *dlx5*, (that is expressed in prospective epidermis) is induced by BMP signalling, suggesting that these homeobox genes play an evolutionarily conserved role in mediating BMP induced inhibition of neural induction and promotion of epidermal fates.

4.1.3 Neural induction – simply a matter of BMP signalling inhibition?

Although the BMP default neural induction model has generally received support in *Xenopus*, work in other systems, particularly the chick, has revealed a key role for FGF and Wnt signals and questioned the importance of BMP signalling inhibition in neural induction, as reviewed in Wilson & Edlund (2001) and Stern (2005). They discuss key evidence that includes the observations that FGF could initiate ectopic expression of neural markers but Chordin and Noggin could not. In addition, intact lateral chick epiblast explants formed epidermis and did not neuralise in response to FGF or BMP antagonists. However, if Wnt signaling levels were lowered by treatment with Wnt antagonists, then both FGFs and BMP antagonists were able to induce neural differentiation in lateral epiblast cells.

Correspondingly, in *Xenopus* animal cap explants, Wnt antagonists are able to transiently induce neural markers and FGFs can function as neural inducers. Taken together, the available evidence implies that the 'default model' of neural induction is over-simplified and that multiple signalling pathways including Wnts and FGFs are involved in neural induction in vertebrates. However, there is also evidence that at least one means of the neural inducing effects of FGF and Wnt may be through antagonising BMP pathway activity: For example, during pre-gastrulation in the chick, neural fate choice is thought to involve FGF-mediated suppression of BMP transcription (Wilson *et al.* 2000). FGF expression in the epiblast leads to the downregulation of BMP transcripts, whereas in the presence of the FGF receptor inhibitor SU5402, this down-regulation is prevented and epidermal fate is restored. FGF mediated suppression of BMP transcription at this stage has also been demonstrated to require the inhibition of the Wnt pathway (Wilson *et al.* 2001).

Recently, Pera *et al.* (2003) reported another mechanism by which FGF signalling may mediate the inhibition of BMP pathway activity during development, through ERK1/2 mediated phosphorylation of Smad 1. Positively acting neural inducers such as FGF signal through Tyrosine kinase (RTK) trans-membrane receptors, and Pera *et al.* reported that both

FGF and IGF induce mitogen-activated protein kinase (MAPK) mediated phosphorylation of Smad 1 in the linker region at four conserved PXSP sites. This phosphorylation prevents nuclear translocation despite previous phosphorylation events on the C-terminal by activated receptors, thus inhibiting the action of Smad 1 independent of BMP receptor activation. Figure 4.4 outlines the pathway by which FGF signalling can mediate an inhibition of BMP pathway activity regardless of BMP signalling. In support of this mechanism of MAPK mediated BMP pathway inhibition, over-expression of Smad1 in *Xenopus* embryos has little ventralizing (pro-BMP) effect except in the case of mutant proteins that cannot be phosphorylated by MAPK (Pera *et al.* 2003, Sater *et al.* 2003). This indicates that MAPK signals are active in the developing embryo and this mechanism of inhibiting BMP pathway activity is important in dorsalising the *Xenopus* embryo.

In summary, studies in the chick have demonstrated the role of multiple pathways including Wnts and FGFs in neural induction, and although mechanisms may vary between species, the inhibition of BMP pathway activity remains a central event that precedes the acquisition of neural fates in developing vertebrates such as *Xenopus* and chick. This leads to the question of whether the BMP mediated inhibition of neural induction during patterning of the ectoderm is conserved in human development.

4.1.4 Using ES cells as *in vitro* models for the study of neural induction

As discussed previously, embryonic stem cells provide valuable tools in which to study multiple aspects of embryonic development, although for human embryonic stem cells the conditions for feeder free maintenance and propagation are still being established. As a cell line that shares key characteristics of human embryonic stem cells, and has been demonstrated to preferentially differentiate into ectodermal cell fates, TERA2.sp12 cells provide a useful model in which to address this question. Previous studies have demonstrated that human EC cells do respond and differentiate in response to BMP 4 treatment: Andrews first reported that BMP7 treatment induced differentiation in human EC NTERA2 cells distinct from that of RA treatment in 1994, and in more recent studies with the human EC lines GCT 27X-1 and NT2D1, Pera & Herszfeld (1998) and Caricasole *et al.* (2000) further reported that BMP treatment induced differentiation into endoderm or epithelial non-neural ectoderm lineages respectively. However no studies have yet evaluated the effect of BMP signalling on neural induction in either human EC or ES cells, although in mouse ES cells BMP treatment has been reported to inhibit neural differentiation in embryoid bodies (Finley *et al.* 1999).

The effect of dissociation and subsequent culture in suspension of TERA2.sp12 cells drew several parallels to the dissociation experiments with *Xenopus* animal cap explants (discussed previously), suggesting that the increased neural differentiation observed in RA

Schematic Depicting FGF Mediated Inhibition Of BMP Pathway Activity Via ERK1/2 Phosphorylation Of Receptor Smad 1

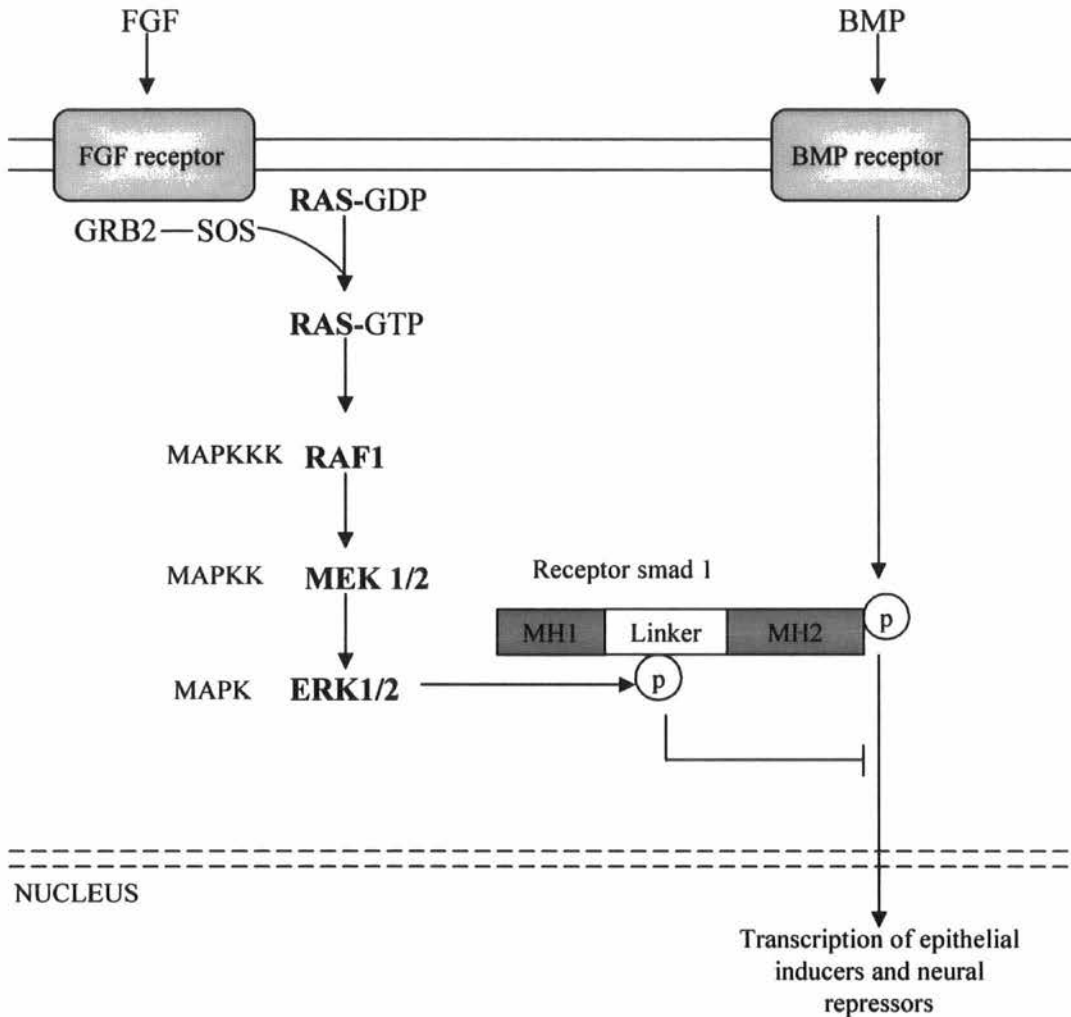
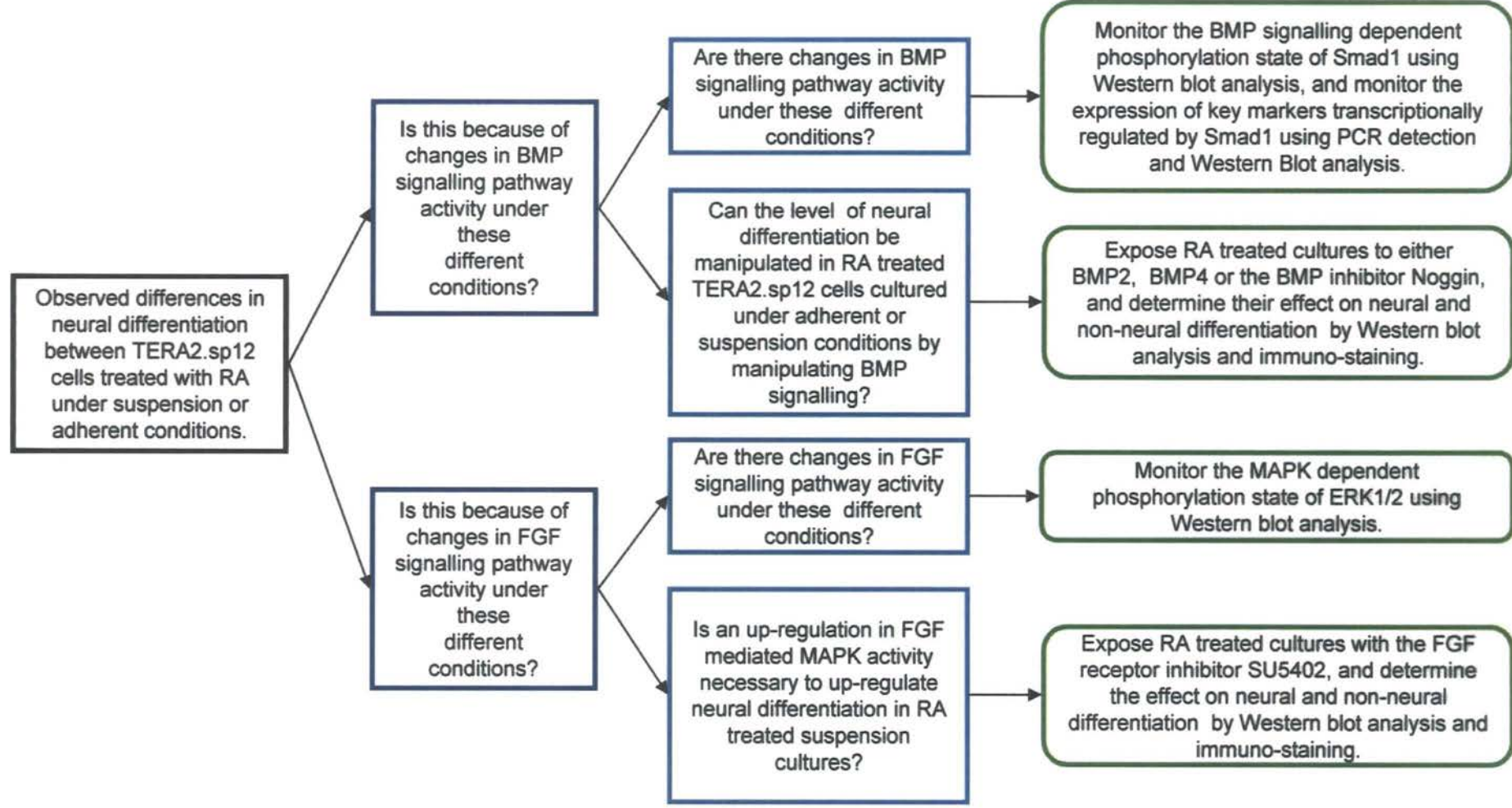


Figure 4.4 An Outline depicting FGF mediated inhibition of BMP pathway activity.

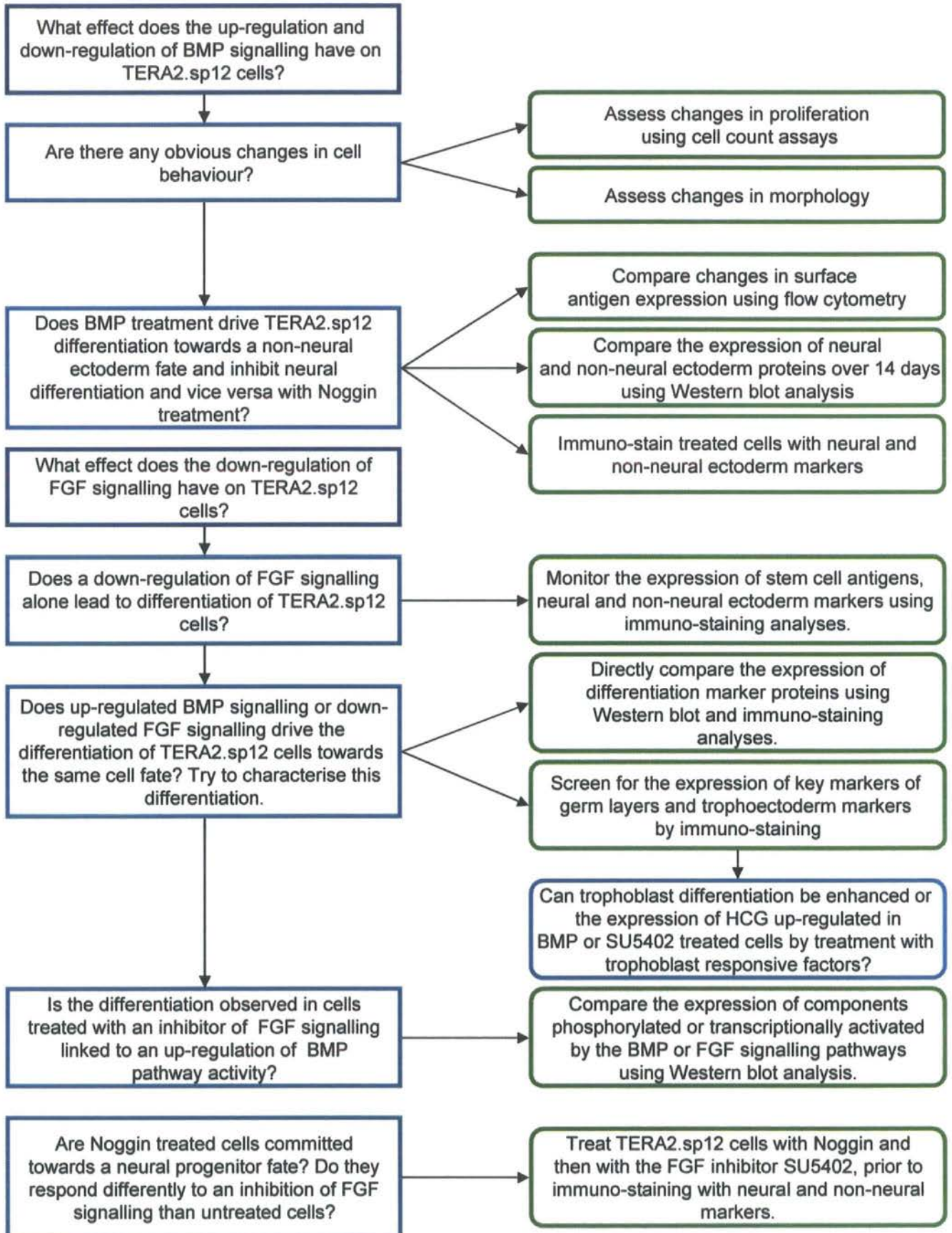
Ligands such as FGFs bind to the extracellular domains of Transmembrane receptor tyrosine kinases (RTKs) causing receptor dimerization, autophosphorylation and activation of the intracellular tyrosine kinase domains. This in turn leads to the activation of a number of intracellular signalling cascades, including the RAS-MAPK pathway. RTK activation leads to RAS activation which, in turn, induces sequential phosphorylation of the protein kinases RAF, MEK and ERK (MAPK). Activated ERK induces a variety of downstream responses including gene transcription, translation and cyto-skeletal rearrangement. Inhibitory phosphorylation of Smad1 in the linker region by RTK signals such as FGF, IGF, HGF, and EGF is mediated by activation of MAPK. MH1 and MH2 are evolutionarily conserved globular Mad-homology domains; MH1 contains the DNA-binding domain and MH2 multiple protein interaction sites. Phosphorylation of Smad 1 by MAPK prevents nuclear accumulation, regardless of activating phosphorylation events in the MH2 domain by activated BMP receptors. Figure adapted from Figure 5 in Pera et al. (2003).

Figure 4.5

Strategy Chart for Section A: Investigating The Role Of BMP And FGF Mediated Pathways In RA Mediated Differentiation Of TERA2.sp12 Cells Under Adherent Or Suspension Conditions



Strategy Flow Chart For Section B: Investigating The Effect Of Manipulations In BMP And FGF Signalling On TERA2.sp12 Cells In The Absence Of RA



treated aggregate cultures could be the consequence of a disruption in BMP signalling. In the following first section (Section A), I investigated whether a disruption in BMP signalling activity was causing the up-regulation in neural differentiation in RA treated suspension cultures and also whether this was linked to changes in FGF mediated signalling. Figure 4.5 presents an outline of the aims, objectives and experiments carried out in Section A of this chapter.

In order to more closely mimic early events in neural versus non-neural ectoderm specification, I also examined the effect of manipulating BMP and FGF signalling on the differentiation fate of TERA2.sp12 cells in the absence of RA. As treatment with BMPs or the FGF receptor1 inhibitor SU5402 induced not only non-neural ectoderm marker expression, but markers of mesoderm and trophoblast, I subsequently attempted to characterise this differentiation. Figure 4.6 presents an outline of the aims, objectives and experiments carried out in Section B of this chapter.

4.2 RESULTS

SECTION A

In order to determine whether the observed up-regulation of neural and corresponding down-regulation of non-neural ectoderm marker expression in TERA2.sp12 cells cultured under suspension as opposed to adherent conditions was due to changes in BMP and/or FGF signalling, in the following section I compared and manipulated these pathways in RA treated cultures under these conditions.

4.2.1 Monitoring the BMP signalling pathway in RA treated cultures

To detect changes in BMP signalling in TERA2.sp12 cells cultured under suspension or adherent conditions, the level of phosphorylation of the receptor Smads 1,5 and 8 proteins that occurs as a direct consequence of BMP-receptor activation was assayed using Western blot analyses with antibodies specific for Smad 1,5,8 and phosphorylated Smad 1,5,8 (P-Smad). TERA2.sp12 cells were cultured with 10 μ M RA under either suspension or adherent culture conditions for 4, 5, 7 or 14 days. At each time point protein was extracted from samples in each culture condition, and protein was also extracted from a confluent culture of cells grown under maintenance conditions (EC sample). Protein samples were then subjected to Western blot analysis and probed with an antibody to Receptor Smads 1,5,8 (Santa Cruz), an antibody to phosphorylated Smads 1,5,8 (Santa Cruz) or with an antibody to β actin (Sigma), following the procedure outlined in Section 2.4. Western blot detection of Smad 1,5,8 and p-Smad 1,5,8 proteins was repeated over multiple experiments using cultures at different passage numbers.

Phosphorylated Smad 1, 5 and 8 protein was detected in TERA2.sp12 cells cultured under maintenance conditions in multiple experiments, and the level of P-Smad protein was detected to consistently increase with RA treatment in both adherent and non-adherent conditions. However, the level of P-Smad 1, 5 and 8 proteins over different time-points and in comparisons with adherent versus non-adherent conditions appeared to fluctuate in repeated experiments, and no positive detection with the antibody to Smad 1, 5 and 8 was achieved. As a consequence BMP signalling was monitored by detecting levels of transcripts from genes transcriptionally regulated by active receptor smads 1, 5 and 8.

Following the protocol in Section 2.7 and using the primers listed in Table 4.1, PCR detection of transcripts from genes that are reportedly transcriptionally regulated through Receptor smad binding, such as *Id2*, *Id3* and *Msx 1* were used to monitor the level of BMP signalling in TERA2.sp12 cultures, as represented in Figure 4.7. RNA degradation was

PCR Detection Of Transcripts Regulated By BMP Signalling In TERA2.sp12 Cells Treated With RA Under Suspension Or Adherent Culture Conditions.

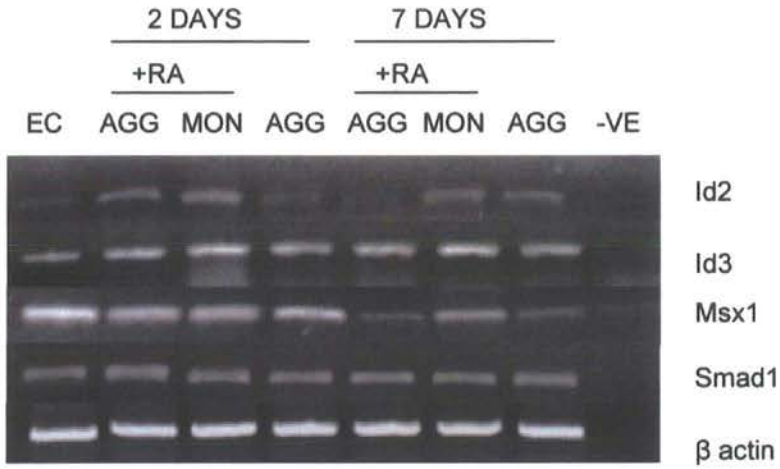


Figure 4.7 TERA2.sp12 cells were seeded at 20 000cells/cm² under either suspension conditions in bacteriological dishes (AGG) or adherent conditions in T75 culture flasks (MON) and treated from day one with 10 μ M RA (+RA) or without RA for 2–7 days. A confluent culture of TERA2.sp12 cells grown under maintenance conditions was used for the EC sample. At the selected time-points cultures were individualised with Trypsin, the RNA extracted using Tri-reagent, 1 μ g of RNA for each PCR sample was reverse transcribed and amplified with specific primers using a PCR machine. As a negative control (-ve) no reverse transcriptase enzyme was added prior to amplification. The resultant samples for each transcript detection were electrophoresed in 1% agarose gels and the bands visualised under ultra violet light. Id2 transcripts were up-regulated in RA treated cultures after 2 days, although in aggregate cultures this decreased to undetectable levels after 7 days. In aggregates cultured without RA little difference from the low expression levels in EC cultures was observed. Id3 transcripts were up-regulated in all treatments compared to EC cultures, and the strongest expression was detected in adherent cultures. Msx1 expression was markedly down-regulated from EC levels in aggregate cultures (with or without RA) after 7 days of treatment, contrasting with more sustained expression in adherent cultures treated with RA. Smad 1 transcript detection remained relatively equal in all treatments. Detection of β actin transcripts demonstrated equal loading.

assayed by running RNA samples on a 1% agarose gel as described in Section 2.7 and to ensure equal loading primers for β actin were used.

Table 4.1 Details of primers used for PCR analysis.

PRIMER FOR	PRIMER CODE	ANNEALING TEMPERATURE
Id2	Forward: 5-CAC CAT GGG GAG CGA AAA C-3	51.4 °C
	Reverse: 5-GAA ATA AAG CAG GCA ATC AC AT-3	
Id3	Forward: 5-AGC TCG CTC CGG AAC TTG TCA TC-3	61.4 °C
	Reverse: 5-CCG CCT TGG CAT AGT TTG GAG AG-3	
Smad 1	Forward: 5-AAGCGTTCATGCCTCCTC-3	54.7°C
	Reverse: 5-CTCCCCAGCCCTTCACAAA-3	
Msx 1	Forward: 5-GTGGCGCCCGTGGGACTCTACA-3	60.2°C
	Reverse: 5-CACCGATTCTCTGCGCTTTCTTG-3	
β actin	Forward: ATCTGGCACCACCTTCTACAATGAGCTGC-3	55°C
	Reverse: 5-CGTCATACTCCTGCTTGCTGATCCACATCTG-3	

Id2 and *Msx1* expression demonstrated marked regulation by RA treatment and exhibited differences in expression between suspension and adherent cultures of TERA2.sp12 cells. *Id2* transcripts were up-regulated after 2 days of RA treatment in both adherent and suspension cultures, although in suspension cultures *Id2* expression subsequently decreased to undetectable levels after 7 days. In cells cultured under suspension conditions without RA treatment, *Id2* expression remained at similar levels to in EC cultures. *Id3* transcripts were up-regulated in all treatments compared to EC cultures, with the strongest expression detected in adherent cultures. *Msx1* expression was markedly down-regulated from EC levels in cells cultured under suspension conditions (with or without RA) after 7 days of treatment, contrasting with more sustained expression in adherent cultures treated with RA, and this observation was consistent in repeated experiments. PCR detection also confirmed that *Smad 1* expression was equal between treatments at the transcriptional level. This pattern of down-regulation of BMP regulated genes is consistent with the hypothesis that BMP mediated pathway activity is markedly down-regulated in TERA2.sp12 cells differentiated under suspension conditions compared to adherent conditions.

To determine whether the changes in transcript expression of BMP regulated *Id2* and *Id3* genes were reflected by changes in protein expression, Western blot analysis was subsequently used to monitor changes in the levels of Id2 and Id3 protein in TERA2.sp12 cells cultured for 4-14 days with RA under either suspension or adherent culture conditions, following the procedure described in Section 2.4. Western blots were probed with antibodies to Id2, Id3 and β actin, and one of the two Western Blot analyses is presented in Figure 4.8.

Multiple bands corresponding to large molecular weights were detected in immunoblots probed with Id2 and Id3 over repeated experiments, although only a single band was present at the predicted size and this was taken as the representative protein. Id2 protein was detected in untreated cells and rapidly down-regulated after 4 days of RA treatment when cultured under suspension conditions, compared to a more gradual down-regulation in cells

Western Blot Detection Of Proteins Regulated By BMP Mediated Signalling In TERA2.Sp12 Cells Treated With RA Under Suspension Or Adherent Culture Conditions.

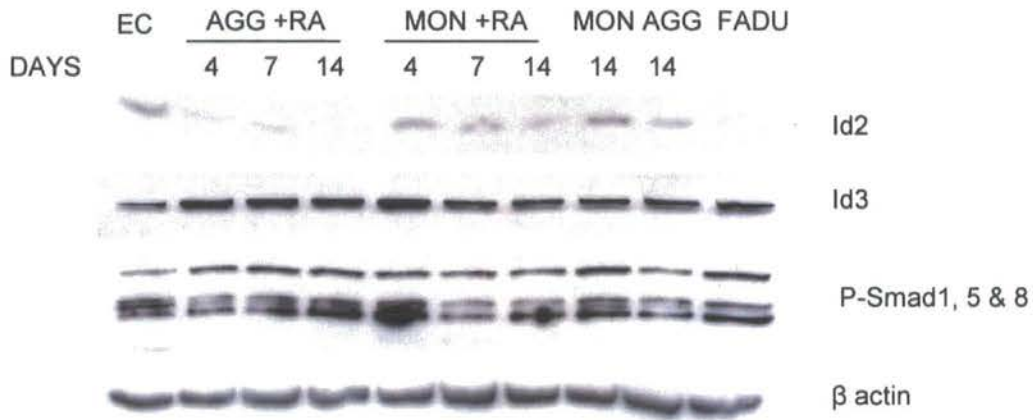


Figure 4.8 TERA2.sp12 cells were seeded at 20 000cells/cm² under either suspension conditions in bacteriological dishes (AGG) or adherent conditions in T75 culture flasks (MON) and treated from day one with 10 μ M RA, (+RA) or without RA for 4, 7 or 14 days. At the selected time-points protein was extracted and analysed by Western blot analysis. Blots were probed with antibodies to Smad1 regulated proteins ID2, ID3, to phosphorylated Smad1,5 and 8 (P-Smad), and equal loading was demonstrated by blotting with an antibody to β actin. A confluent culture of TERA2.sp12 cells grown under maintenance conditions was used for the EC sample, and protein extracted from human FaDU cells was used as a positive control for the expression of BMP regulated proteins. Id2 protein levels were observed to decrease to almost undetectable levels after 14 days in RA treated suspension cultures, compared to sustained levels in RA treated adherent cultures. In contrast, detected levels of Id3 protein did not markedly change between treatments, although a stronger band was detected at 4 days of RA treatment under adherent conditions. Detected levels of P-Smad1 protein varied between treatments. In suspension cultures, initially low levels of phosphorylated Smad1 were detected, although these increased over the 14 day period. In contrast, in RA treated adherent cultures phosphorylated Smad1, 5 and protein was initially detected at high levels, and decreased over the 14 day period. In cultures grown without RA the level of P-Smad1 5 and 8 protein detected was markedly lower in cells grown under suspension conditions compared to adherent cultures.

cultured under adherent conditions, such that Id2 protein was still at detectable levels after 14 days. Id2 was also down-regulated to some extent in suspension cultures grown without RA, whereas protein levels in corresponding adherent cultures were similar at 14 days to those of untreated cells. In contrast, levels of Id3 remained similar in all treatments. These observations were consistent over 2 repeated Western blot analyses. This data demonstrates that although no marked change in Id3 protein occur, the down-regulation of Id2 gene expression is also reflected in a down-regulation of protein activity.

4.2.2 Manipulating BMP pathway activity in RA treated cultures

To determine whether manipulations in BMP signalling would modify the differentiation behaviour of TERA2.sp12 cells cultured under adherent or suspension conditions, TERA2.sp12 cells were differentiated with 10 μ M RA as either suspension aggregates or adherent cultures with or without the addition of 25ng/ml BMP2 or 100ng/ml Noggin.

The predicted outcome was that Noggin treatment of adherent cultures would generate similar levels of neural differentiation to that observed in suspension cultures, and BMP2 treatment would down-regulate neural differentiation in suspension cultures.

As depicted in Figure 4.9, BMP and RA treated adherent cultures formed larger, less densely packed cells compared to RA treated cultures alone, and suspension cultures were slightly more irregular and smaller in shape than cultures treated solely with RA. In contrast, cultures treated with Noggin and RA combined were similar in morphology to cultures treated with RA alone, and showed markedly higher proliferation rates. In a flow cytometry analysis of adherent cultures, the down-regulation of stem cell markers TRA160 or SSEA3 upon RA treatment was not markedly affected by the addition of BMP or Noggin, although cultures treated with BMP4 or BMP2 and RA exhibited a reduced up-regulation of neural associated surface antigen A2B5 compared to cultures treated with RA alone. This indicates that the manipulation of BMP signalling is not affecting the rate of stem cell differentiation, and the up-regulation of A2B5 in BMP treated cultures is consistent with the hypothesis that neural differentiation is inhibited in cultures subjected to high BMP signalling.

Western blot comparisons of neural and non-neural ectoderm marker expression

Western blot comparisons of Neuronal β III tubulin and Keratin 8 protein expression in cultures of TERA2.sp12 treated with RA alone, with RA and BMP2, or with RA and Noggin demonstrated marked differences in the expression of these proteins in adherent cultures, in contrast to suspension cultures. At 5, 7 and 14 day time-points selected cultures were individualised and prepared for Western blot analysis as described in Section 2.4. Blots were probed with antibodies to β III tubulin, NSE, Keratin 8, Nestin and β actin. This experiment was repeated 3 times with cells grown at different passage numbers, measuring expression at

Changes In Morphology In TERA2.sp12 Cells Cultured Under Adherent Or Suspension Conditions And Treated With Either RA Alone Or Together With BMP 2 Or Noggin.

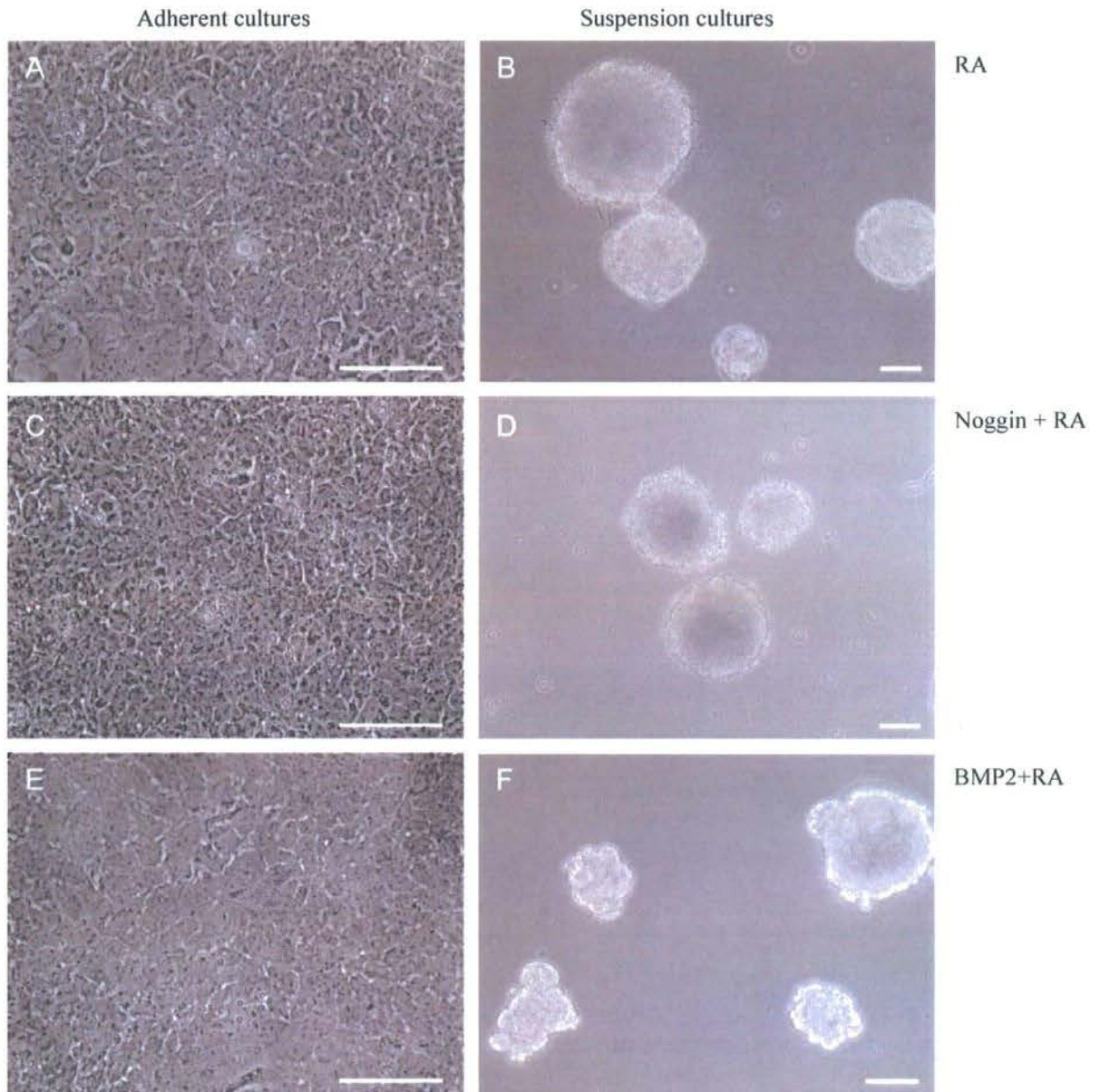


Figure 4.9 Representative phase contrast images of TERA2.sp12 cells seeded at a density of 20,000 cells/cm² into either adherent T25 culture flasks or non-adherent petri dishes and treated from day one with either 10 μ M RA (A-B), or with 10 μ M RA and 100ng/ml Noggin (C-D), or 10 μ M RA and 25ng/ml BMP2 (E-F). Note that Noggin and RA treated cultures demonstrated similar morphologies to RA treated cultures alone, whereas TERA2.sp12 cells treated with BMP and RA as adherent cultures formed larger, less densely packed cells and under suspension conditions formed more irregular aggregates. Scale bars = 50 μ m

time-points ranging from 5-14 days. A representative Western blot analysis is depicted in Figure 4.10. Consistent with previous observations, treatment of adherent cultures with RA alone up-regulated neuronal β III tubulin to a lesser extent than in suspension cultures, and also up-regulated Keratin 8 expression as opposed to the down-regulation observed in suspension cultures. However, treatment of adherent cultures with Noggin and RA combined up-regulated neuronal β III tubulin expression to levels comparable to suspension cultures, and down-regulated Keratin 8 expression. Thus inhibition of BMP signalling in adherent cultures was sufficient to increase neural differentiation and suppress non-neural differentiation to the same levels as that observed in suspension cultures. In contrast, the expression levels of neuronal β III tubulin and Keratin 8 in suspension cultures treated with Noggin and RA combined remained unchanged from the levels expressed in cultures treated with RA alone. One could hypothesise that BMP signalling is already attenuated to such a level that exogenous inhibition of BMP signalling has no effect. Adherent cultures also demonstrated a marked responsiveness to BMP2 treatment, strongly down-regulating neuronal β III tubulin and up-regulating Keratin 8 expression in cultures treated with BMP2 and RA combined. This effect was visible by 7 days of treatment. Suspension cultures also down-regulated neuronal β III tubulin expression and up-regulated Keratin 8 expression, but neuronal β III tubulin expression remained relatively high – often comparable to levels in adherent cultures treated with RA alone. This effect was reproduced in 4 different Western blot analyses over time-points ranging from 5-14 days. The marked responsiveness of adherent cultures to exogenous up-regulation and down-regulation of BMP signalling activity are in contrast to the more restricted response of suspension cultures. This could be explained by the presence of factors attenuating BMP pathway activity acting down-stream of BMP signalling which are active in suspension cultures and not in adherent cultures.

In a pilot experiment investigating the effect of changes in foetal calf serum (FCS) on neural differentiation, under low serum conditions (5% FCS) an up-regulation of β III tubulin and marked down-regulation of Keratin 8 expression in adherent cultures treated with RA was observed. Consistent with the hypothesis that this was due to reduced exposure to BMPs in the FCS, this effect was repressed in adherent cultures treated under low serum conditions with RA and BMP2 combined.

4.2.3 Inhibiting FGF mediated signalling prior to RA treatment

To investigate whether the up-regulation in neuronal markers observed in suspension cultures was linked to a temporary up-regulation in FGF signalling, similar to that observed in dissociated *Xenopus* explants (Kuroda *et al.* 2005), FGF mediated signalling was blocked in the first 24 hours prior to treatment with RA by exposure to SU5402, an FGF receptor

Western Blot Analyses Demonstrating The Effect Of Noggin Or BMP2 On Neural And Non-neural Ectoderm Marker Expression In TERA2.sp12 Cells Differentiated With RA

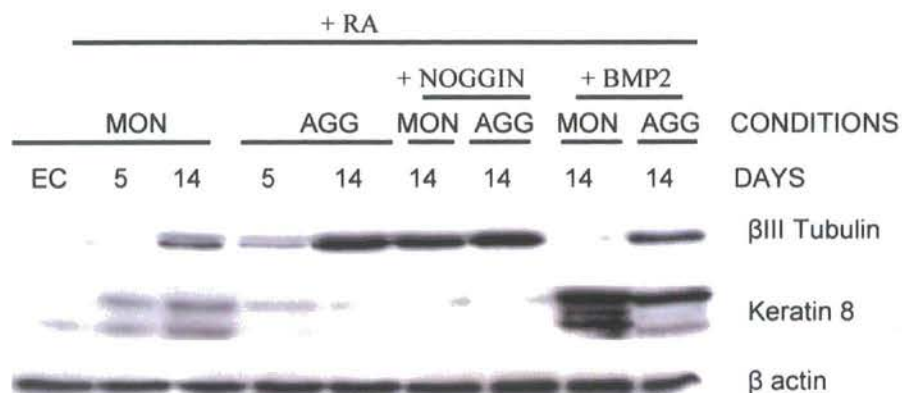


Figure 4.10 Western blot detection of neuronal β III tubulin and keratin 8 in protein extracted at 5 and 14 days from cells seeded at 20 000cells/cm² under either adherent conditions (MON) or suspension conditions (AGG) and treated from day 1 with either 10 μ M RA alone, 10 μ M RA and 25ng/ml BMP2 or 10 μ M RA and 100ng/ml of the BMP inhibitor noggin. For the EC sample, protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions, and to monitor equal loading blots were also probed with an antibody to β actin. In adherent cultures neuronal β III tubulin expression was detected to be markedly up-regulated by the addition of noggin, matching the levels of expression observed after 14 days of RA treatment in suspension cultures. In contrast, the level of β III tubulin up-regulation after 14 days remained similar in suspension cultures treated with either RA alone or a combination of noggin and RA. Similarly, in suspension cultures the addition of noggin was not detected to markedly change keratin 8 expression from the minimal levels observed after 14 days in RA treatments alone. In contrast, the addition of Noggin to RA treated adherent cultures prevented the up-regulation of keratin 8 observed in cultures treated with RA alone. Correspondingly, the addition of BMP2 was detected to inhibit the up-regulation of neuronal β III tubulin under both suspension and adherent conditions, although in suspension cultures β III tubulin expression remained relatively strong. In addition, keratin 8 levels were up-regulated by BMP2 treatment in both cultures, with adherent cultures exhibiting the strongest expression after 14 days.

inhibitor. Temporary treatment with the FGF inhibitor SU5402 induced distinct changes in morphology and differentiation in both adherent and suspension cultures, with the most marked effects on neuronal marker expression observed in cells cultured in suspension.

In adherent cultures pulsed with SU5402, plaques of large cells formed between 4-7 days in cultures treated either with or without RA. Under suspension conditions, treatment with SU5402 alone generated aggregates that were generally smaller and more irregular compared to mock treated aggregates, whereas in RA treated suspension cultures aggregates that had been initially treated with or without SU5402 were similar in size, although the total number of aggregates present in SU5402 treated cultures was lower.

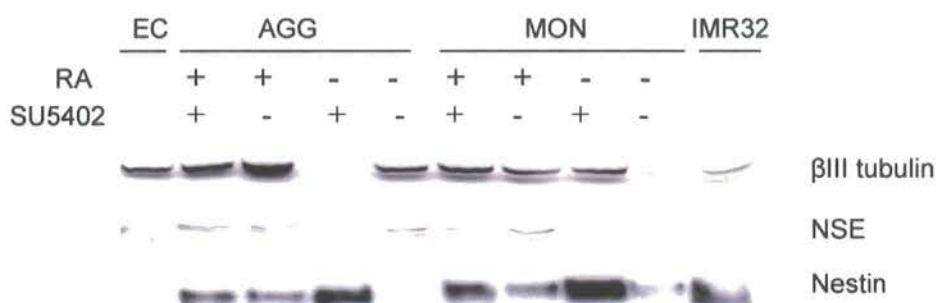
Western blot analyses demonstrated a marked effect of SU5402 exposure on neural and non-neural ectoderm marker expression in all conditions (see Figure 4.11). After 14 days of culture, the samples were individualised and prepared for Western blot analysis as described in methods Section 2.4. Blots were probed with antibodies to β III tubulin, NSE, Keratin 8, Nestin and β actin. This experiment was repeated twice with cells grown at different passage numbers. In both treatments 24 hour exposure to SU5402 increased levels of the neural progenitor Nestin detected after 14 days. However, under adherent conditions, prior SU5402 exposure was observed to up-regulate β III tubulin expression after 14 days, again with the most dramatic increase detected in adherent monolayer cultures grown without RA. The expression of simple epithelial keratins 8 and 18 was up-regulated by SU5402 exposure in all culture conditions (this effect was most marked in suspension cultures cultured without RA). These changes in protein expression were consistent in two repeated Western blot analyses, demonstrating that changes in FGF signalling generated marked effects on neural and non-neural ectoderm marker regulation in TERA2.sp12 cells cultured under both suspension and adherent conditions, and moreover that inhibition of FGF signalling in the first 24 hours of culture markedly inhibited neuronal β III tubulin up-regulation in cells cultured under suspension conditions.

Western blot mediated detection of MAPK pathway activity

To assess whether differences in FGF mediated MAPK pathway activity were present in TERA2.sp12 cells cultured under suspension or adherent conditions, levels of phosphorylation regulated MAPK pathway components ERK 1 and ERK 2 were compared. Protein samples from TERA2.sp12 cells cultured for 4-14 days with RA under either suspension or adherent culture conditions were subjected to Western blot analysis and probed with antibodies to ERK1/2, phosphorylated ERK1/2 and β actin following the procedure outlined in Section 2.4. The Western blot analysis data is presented in Figure 4.12. Total levels of ERK 1 and ERK 2 proteins increased with RA treatment in all cultures, although in suspension cultures, ERK 1 levels were very low after 4 days of treatment, and were up-regulated only after 7 days, as

Western Blot Comparison Of Neural And Non-Neural Ectoderm Protein Expression In TERA2.Sp12 Cells Pulsed With SU5402 Prior To Treatment With RA.

i. Expression of neural proteins after 14 days of treatment



ii. Expression of epithelial keratins and β actin after 14 days of treatment

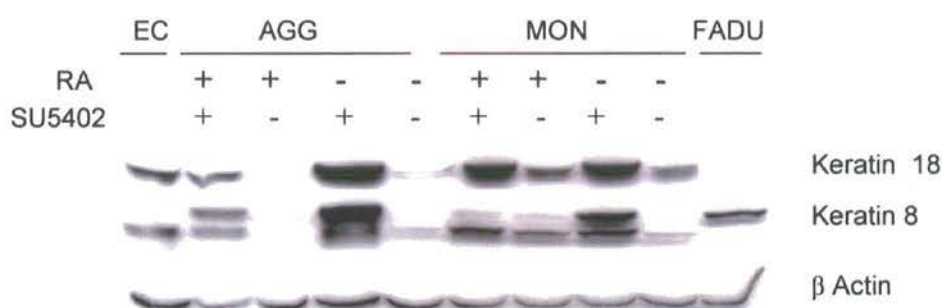


Figure 4.11 Western blot detection of neural markers (plate i) and epithelial keratins (plate ii) in TERA2.sp12 cells seeded at 20 000cells/cm² as either adherent (MON) or suspension (AGG) cultures and pulsed for 24 hours with 20μM SU5402 (+) or without SU5402 (-), prior to being cultured for 14 days with (+) or without (-) RA. After 14 days cultures were subjected to Western blot analysis and probed with antibodies to either neural proteins (i) or epithelial keratins (ii). Positive controls for neural proteins and epithelial keratins were extracted from IMR32 and FaDU cells respectively. For EC samples, protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions. In all conditions, detected levels of keratin 8 and keratin 18 protein were up-regulated by prior treatment with SU5402. Correspondingly, the level of neuronal βIII tubulin expression was detected to be markedly down-regulated by prior SU5402 treatment in suspension cultures, (in both RA treated and untreated cultures), whereas in adherent cultures the level of neuronal βIII tubulin expression was detected to be higher in cultures pre-treated with SU5402. Under all conditions prior treatment with SU5402 generated increased detected levels of nestin expression.

Monitoring MAPK Pathway Activity In Cultures Of TERA2.Sp12 Cells Treated With RA Under Either Adherent Or Suspension Conditions

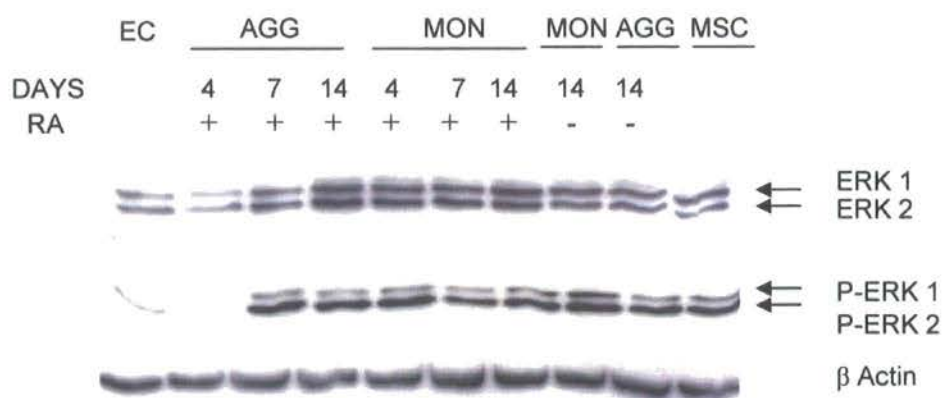


Figure 4.12 Western blot detection of active (phosphorylated) forms of MAPK pathway components ERK1 and ERK2 compared to total levels in TERA2.sp12 cells treated with RA as either adherent or suspension cultures. TERA2.sp12 cells were seeded at 20 000cells/cm² under either adherent (MON) or suspension conditions (AGG) and treated from day one with 10 μ M retinoic acid (RA). At 4, 7 and 14 day time-points culture samples were subjected to Western blot analysis and probed with antibodies to either the phosphorylated active forms of ERK1/2 or total ERK1/2 proteins. As a positive control for MAPK pathway activity, protein was extracted from human MSC cells grown on Laminin (MSC). For EC samples, protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions. Equal loading was determined by probing with an antibody to β actin. Total levels of ERK1 and ERK2 protein were detected to increase over 14 days of treatment in both suspension and adherent conditions. However, in suspension cultures a temporary decrease was initially detected. This pattern was mirrored to some extent in the detected levels of phosphorylated ERK1/2 protein (P-ERK1/2). However, no phosphorylated ERK1/2 was detected at all at day 4 in suspension cultures, in marked contrast to the relatively strong and steady expression detected from day 4-14 of adherent cultures.

opposed to a rapid increase detected after 4 days of treatment in adherent cultures. Phosphorylated ERK 1 and 2 was also up-regulated in treated cultures, although a temporary down-regulation was initially observed in suspension cultures such that at 4 days no phosphorylated ERK protein was detected. Thus MAPK pathway activity (as indicated by monitored levels of phosphorylated ERK1/2 protein) was not detected to be up-regulated in suspension cultures compared to adherent cultures, in fact exhibiting a marked down-regulation (at the earliest time-point of 4 days) in suspension cultures.

SECTION B

In the following section I characterised the differentiation of TERA2.sp12 cells generated as a result of direct manipulation of BMP or FGF signalling (in the absence of RA).

4.2.4 Manipulation of BMP signalling

To investigate the effect of manipulation of BMP signalling in the absence of a background of RA, TERA2.sp12 cells were seeded at 20 000 cells/cm² into electro-statically coated NUNC tissue culture flasks and cultured in DMEM media with 10% FCS, 2mM L-glutamine and 1µM pen/strep supplemented with either 25ng/ml BMP2, 25ng/ml BMP4, 100ng/ml Noggin, 10µM RA or vehicle treated with 0.25µM HCL.

General observations

Cells cultured under adherent conditions and treated with BMP2 or BMP4 rapidly lost their characteristic stem cell morphology and for a transient period 3-5 days after treatment cells demonstrated a shrunken and irregular morphology (depicted in plates E, G, Figure 4.13), with increased debris observed within the culture. However, by 7 days of treatment distinct patches of large plate-like cells with large nuclei and prominent nucleoli had developed, (plates F, H, Figure 4.13). After 14 days these large cells dominated the culture, occurring in extensive regions bordered by distinct clusters of small cells with densely packed nuclei. The formation of these plate-like cells was observed to occur in larger sheets and at an earlier stage in cultures treated with BMP4 compared to BMP2 treated cultures, such that large hexagonal cells were detected after approximately 7 days of treatment with BMP2 as opposed to after 4 days of exposure to BMP4. Increased levels of cell debris were consistently observed in early cultures of BMP treated cells, although there was some variation in the level of debris and the length of the stage in which shrunken morphologies were observed. In cells cultured for more than 14 days, increased debris was associated with the large celled populations, and the number of large hexagonal cells decreased. TERA2.sp12 cells mock treated with 0.25µM HCL used as a diluent for BMP2 and BMP4 did not develop any of the morphologies

Changes In Morphology Of TERA2.sp12 Cells Treated With RA, BMP2, BMP4, Or Noggin

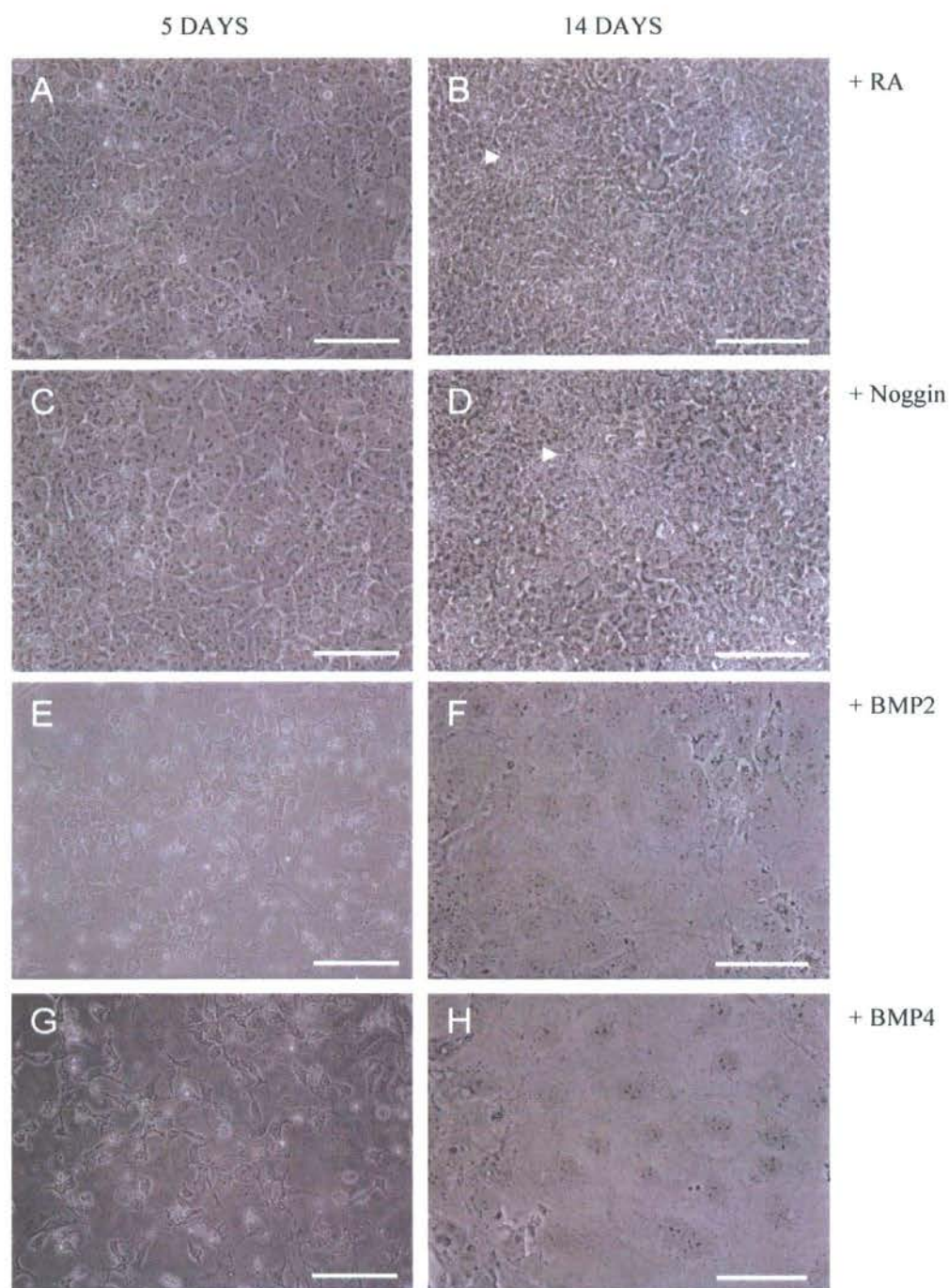


Figure 4.13 Representative phase contrast images of TERA2.sp12 cells seeded at a density of 20,000 cells/cm² and treated with either 10 μ M RA (A-B), 100ng/ml Noggin (C-D), 25ng/ml BMP2 (E-F) or 25ng/ml BMP4 (G-H) for 5 or 14 days. After 14 days Noggin treated cells formed densely packed cultures similar to RA treated cells, with rosette-like formations visible (arrow). After 5 days of BMP treatment cells exhibited a shrunken appearance and were sparsely populated. However, after 14 days of BMP treatment the culture was dominated by large flat plate-like cells with large nuclei that formed large continuous sheets and were bordered by small areas of densely packed cells. Scale bars = 50 μ m

described above, and behaved similarly to cells seeded at differentiation density and cultured in maintenance media, demonstrating that HCL was not linked to the observed differentiation behaviour in BMP treatments.

In contrast to BMP treated cells, cultures of TERA2.sp12 cells treated with the BMP inhibitor Noggin initially demonstrated a similar morphology to vehicle treated cultures. However, they were slower to form densely packed cultures and frequently formed rosette-like structures, similar to those observed in RA treated cultures (arrow heads in Figure 4.13).

A cell count assay was used to gauge the effect of these treatments on cell proliferation. After 7 and 14 days of treatment, cells within three culture flasks for each condition were individualised and counted with a haemocytometer following the protocol described in Section 2.2. The summarised results are displayed in Table 4.2.

Table 4.2 Mean number of cells (millions) in 3 T75 flasks harvested at 0, 7 and 14 days in cultures of TERA2.sp12 cells treated with RA, BMP2, BMP4 or Noggin.

Days	RA	BMP2	BMP4	Noggin
0	1.5	1.5	1.5	1.5
7	18.95	18.50	21.40	18.65
14	106.00	25.70	10.03	33.73

As demonstrated in the histogram summarising the cell count results in Figure 4.14, BMP or Noggin treatment resulted in a reduction in the rate of increase in cell number between 7 and 14 days compared to both vehicle treated and RA treated cultures, and this was most marked in BMP treated cultures. Vehicle (HCL) treated cultures behaved similarly to mock treated cultures, initially demonstrating slow proliferation and then between 7-10 days demonstrating an escalating increase in cell number such that the cultures became so dense that they were no longer viable. In both BMP2 and BMP4 treated cultures, little or no increase in cell number was observed between 7 and 14 days (in BMP 4 treated cultures the mean cell counts were actually lower than at 14 days, although this was not statistically significant). This stabilisation in cell number corresponds to the stage in which distinct populations of large plate-like cells and clusters of densely packed cells are established, compared to the initial 7 days when substantial cell debris was observed. This suggests that the stabilisation in cell number after 7 days in BMP treated cultures is due to cells exiting from the cell cycle and embarking on differentiation.

In the first 7 days of culture Noggin treated cells initially showed similar counts to that of vehicle or RA treated cells; however the increase in cell number remained steady between 7-14 days, in contrast to the exponential increases observed in RA or vehicle treated cultures. This suggests that Noggin treated cells are also exiting from the cell cycle and embarking on differentiation, albeit more slowly.

Histogram Depicting Mean Cell Counts Of TERA2.Sp12 Cells Cultured With Either RA, BMP2, BMP4, Or Noggin Over 14 Days

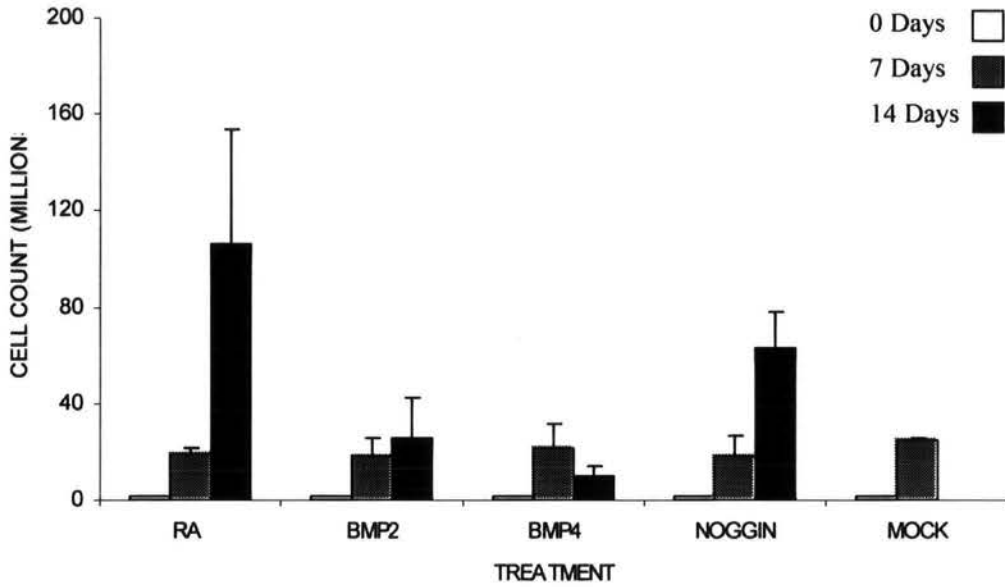


Figure 4.14 Histogram depicting mean cell counts (from three T75 flasks) over time of TERA2.sp12 cells seeded at 20,000 cells/cm² under adherent culture conditions (white bar) and treated with either RA, Noggin, BMP2, BMP4 or vehicle treated respectively. Cultures were individualised with Trypsin and counted using a haemocytometer at 7 days (patterned bars) or 14 days (black bars). Error bars represent the standard deviation of the mean (n=3 at the same passage number). Over the first 7 days all treatments demonstrated similar increases in cell number, however in the following 7 days BMP2 and BMP4 cultures demonstrated a relative stabilisation in cell number compared to RA treated cultures, which continued to increase at a rapid rate. Mock treated cultures proliferated so rapidly after 7 days that without splitting and re-seeding they became too dense and expired. Noggin treated cultures continued to increase in cell number, although to a reduced degree compared to RA or mock treated cultures.

Comparison of cell surface marker regulation

To compare the expression of surface antigens associated with the stem cell phenotype, (TRA 160, SSEA 3) or with the neural phenotype (VINIS 53, A2B5) in TERA2.sp12 cells treated with BMP2, BMP4, Noggin or RA, the remaining cells from the cell count assay at 7 and 14 days were used in a flow cytometry assay following the protocol outlined in Section 2.3. A confluent flask of undifferentiated TERA2.sp12 cells was used for the EC time point, and three replicates of cells from the same passage number were used for each treatment and processed in parallel. As measured by flow cytometry, BMP and Noggin treatment induced a down-regulation of stem cell marker expression in TERA2.sp12 cells, although to manifestly differing degrees (as depicted in Figure 4.15). To summarise, in BMP treated cultures SSEA 3 expression was rapidly down-regulated such that 20% of BMP2 treated cells and less than 10% of BMP4 treated cells were positive after 7 days of treatment. TRA 160 expression was down-regulated more gradually, with over 80% of cells still expressing TRA 160 after 7 days of BMP treatment, although in corresponding flow cytometry traces the intensity of TRA 160 expression in these cultures was markedly lower than in mock treated cells, and by 14 days TRA 160 was expressed in less than 20% of cells for both BMP treatments. This indicates that in the absence of a background of RA, exogenous activation of BMP signalling alone induces differentiation of TERA2.sp12 cells under these conditions.

Noggin treated cells also down-regulated stem cell surface marker expression to some extent, with less than 30% of cells positive for SSEA3 after 14 days, and although over 80% of cells were still positive for TRA 160 after 14 days of culture, corresponding flow cytometry traces (represented in plate ii of Figure 4.15) demonstrate that approximately 50% of the population had down-regulated the level of TRA 160 expression. Thus under these conditions, inhibition of BMP signalling in the absence of a background of RA signalling also induces differentiation of TERA2.sp12 cells.

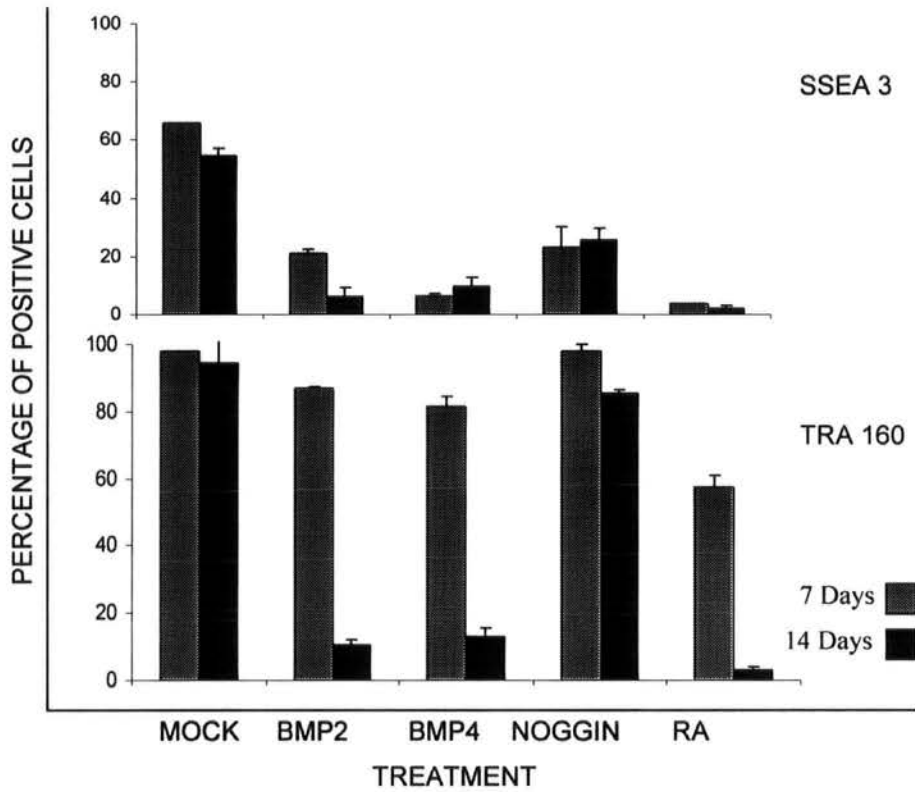
The expression of the neural associated marker A2B5 was also up-regulated to a certain extent by BMP or Noggin treatment, such that A2B5 expression was detected in 20-30% of cells treated with BMP2 or BMP4 and in roughly 10% of cells treated with Noggin. This unexpected result implies that up-regulation of BMP signalling in the absence of RA may induce some neural differentiation in TERA2.sp12 cells.

Comparing the differentiation fate of BMP versus Noggin treated cells: Testing for neural and non-neural ectoderm marker expression

The large cells dominating BMP treated cultures demonstrated some resemblance to the large cells in observed in RA treated cultures, and cultures exhibited no morphological evidence of neural differentiation, in contrast to the neural rosette-like formations observed in Noggin

Down-Regulation Of Stem Cell Surface Marker Expression In TERA2.sp12 Cells Treated With Either RA, BMP2, BMP4 Or Noggin

- i. Histograms Depicting Mean Percentage Of Cells Positive For Stem Cell Surface Markers SSEA3 Or TRA 160 After 7 Or 14 Days Of Treatment



- ii. Comparison Of Representative Flow Cytometer Traces For TRA 160 Detection In Noggin Or Mock Treated Cultures.

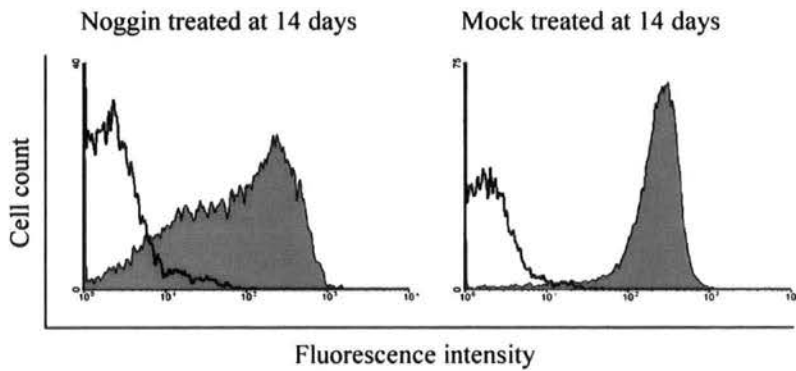


Figure 4.15 Detection of stem cell surface markers by flow cytometry. TERA2.sp12 cells were seeded at a density of 20,000 cells/cm² into T75 culture flasks and either mock treated (MOCK) or treated with 10 μ M RA, 25ng BMP2, 25ng/ml BMP4 or 100ng/ml Noggin. At 7 and 14 days 3 replicate cultures of each treatment individualised using Trypsin and immuno-labelled with antibodies to stem cell surface antigens SSEA3 or TRA 160 or with the control antibody P3X. The percentage of immuno-positive cells in each sample was detected using a flow cytometer, with the mean percentage for each treatment generated from 3 replicates. Histograms in plate i depict the mean percentage of positive cells at day 7 (patterned bars) and day 14 (black bars) after live immuno-labelling with stem cell surface markers SSEA3 or TRA 160. The error bars represent the standard deviation of the mean. Mock treated cultures were split and re-seeded at a ratio of 1:3 at 7 and 10 days of culture (only one flask from each original culture was maintained, the remaining cells were discarded). The percentage of cells immuno-positive for stem cell antigens SSEA3 and TRA 160 decreased rapidly in RA treated cultures and more steadily in BMP2 or BMP4 treated cultures, such that at 14 days approximately 20% and 15% of BMP treated cells were immuno-positive for SSEA3 and TRA 160 respectively. The proportion of cells immuno-positive for stem cell markers TRA 160 and SSEA3 also decreased in Noggin treated cultures to a certain extent, with approximately 30% and 90% of cells positive for SSEA3 and TRA 160 respectively after 14 days. Representative flow cytometry traces of cultures either mock treated or treated with Noggin and immuno-stained with either TRA 160 (grey trace) or with the control antibody P3X (clear trace) in plate ii, demonstrate that the fluorescence intensity for TRA 160 expression was markedly reduced in cells treated with Noggin.

treated cultures. To assay for neural differentiation and markers of non-neural ectoderm, cultures treated with either BMP2/4 or the BMP inhibitor Noggin were subjected to Western blot analyses. TERA2.sp12 cells were seeded as adherent cultures and treated with 25ng/ml BMP2, 25ng/ml BMP4, 100ng/ml Noggin, 10 μ M RA or mock treated with no additional supplement. After 7 and 14 days protein was extracted from cultures and used for Western blot analysis following the protocol in Section 2.4. Protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions for the EC sample, and protein extracted from IMR32 and FaDU cell lines was used as a positive control for neural and epidermal differentiation respectively. Blots were probed with antibodies to Nestin, the neuronal proteins β III tubulin and NSE, and Keratin 8 and 18. Equal loading was assessed by both ponceau staining and by probing with an antibody to β actin. These Western blot analyses were repeated using fresh cell samples at 7 and 14 day time points. As shown in a Western blot analysis in Figure 4.16 the expression of keratin 8 was markedly up-regulated in BMP treated cultures, with minimal up-regulation of neural proteins. In marked contrast, Noggin treated cultures demonstrated a strong up-regulation of the neural progenitor marker Nestin (even greater than in RA treated cultures), up-regulation of neuronal β III tubulin (more mature neural markers were not detected), and down-regulation of keratin 8 expression. These results are consistent with the hypothesis that an up-regulation of BMP signalling induces differentiation of TERA2.sp12 cells into non-neural ectoderm, whereas the inhibition of BMP signalling induces differentiation of TERA2.sp12 cells into neural ectoderm.

Immuno-staining analyses were subsequently used to characterise the differentiation of cells treated with either BMPs 2, 4 or Noggin. TERA2.sp12 cells were seeded as adherent cultures and treated with 25ng/ml BMP2, 25ng/ml BMP4, 100ng/ml Noggin, 10 μ M RA or mock treated with no additional supplement. After 14 days, cultures were fixed in 4% PFA and following the protocols outlined in Section 2.5 either immuno-labelled with the stem cell markers Oct4 and Nanog, or double immuno-labelled using the following combinations of antibodies: Nestin and Neuronal β III tubulin, MAP2 and Neuronal β III tubulin, Pan-epithelial keratin and Tp63, NF200 and Neuronal β III tubulin. This experiment was repeated and BMP treated cultures double immuno-labelled with antibodies to β III tubulin and Pan-epithelial keratin, Nestin and β III tubulin, Pan-epithelial keratin and Keratin 8.

Corresponding with the Western Blot analysis data, immuno-staining analyses demonstrated little supporting evidence for a neural population within BMP treated cultures. Nestin expression was barely detected and although some β III tubulin immuno-positive cells were identified, the localisation was distinctly atypical: Positive β III tubulin staining was detected in the cytoplasm of groups of large cells (demonstrated in Figure 4.17, plates A-B), with stronger staining surrounding the nucleus. Some co-localisation of β III tubulin and pan-epithelial keratin was also demonstrated (Figure 4.17 plates C-E). No cells displaying a neural

Western Blot Detection Of Neural And Non-Neural Ectoderm Proteins In Cultures Of TERA2.sp12 Cells Treated With Either RA, BMP2, BMP4 Or Noggin

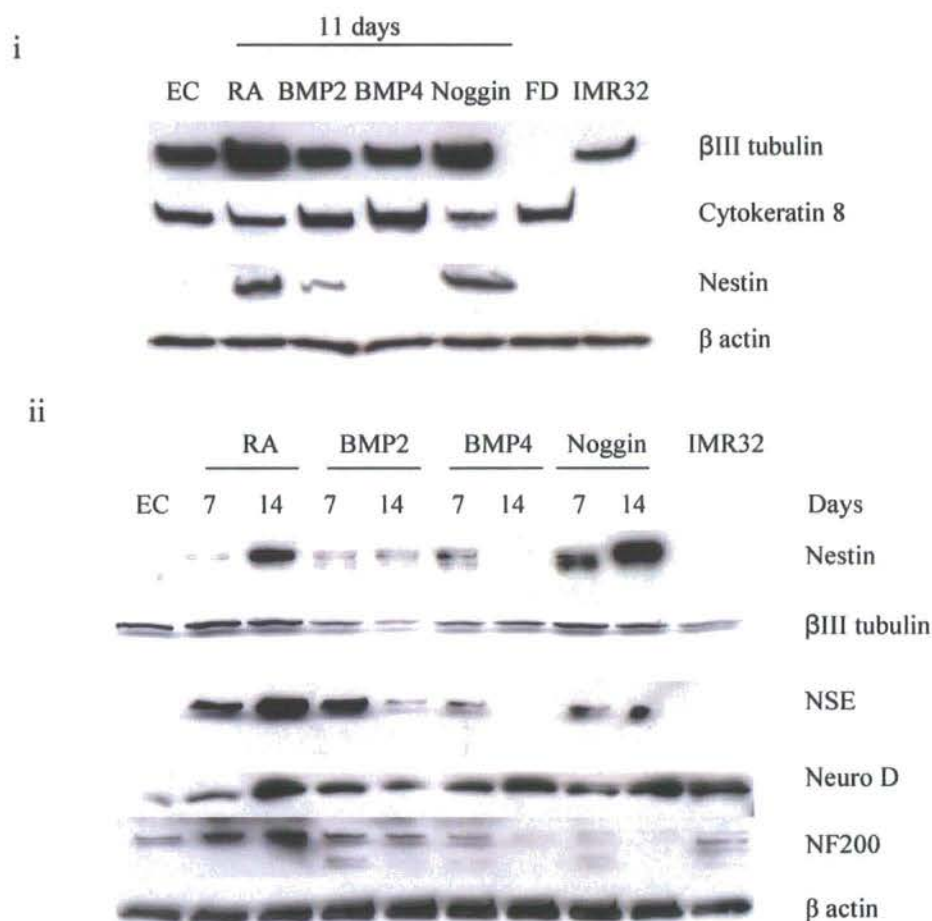


Figure 4.16 Western blot detection of neural and non-neural ectoderm markers from protein extracted at 7, 11 and 14 days from cells seeded at 20 000cells/cm² in culture flasks and treated with either 10μM RA (A), 25ng/ml BMP2 (B), 25ng/ml BMP4 (C) or 100ng/ml Noggin (D). Positive controls for neural protein and epidermal protein were extracted from IMR32 and FaDU (FD) cells respectively, and protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions for EC samples.

In BMP2 treated cultures, Nestin was slightly up-regulated such that a faint band was present at day 7 and 14, and after 7 days exposure to BMP4 Nestin was detected as a faint band at day 7 and then down-regulated to minimal detected expression by day 14. In contrast, the level of Nestin protein detected was markedly up-regulated by Noggin treatment after 14 days, even to a greater extent than the level of Nestin detected during RA treatment. Neuronal βIII tubulin expression followed a similar pattern, with BMP treatment decreasing βIII tubulin levels to a level lower than present in untreated cells. Noggin treatment correlated with slightly up-regulated levels of TUJ1 protein, yet not to a level comparable with RA treatment. Levels of NSE were detected to be temporarily up-regulated by BMP2 treatment, and this was mirrored to a smaller degree by BMP4 treatment. As predicted, RA treatment markedly up-regulated NSE levels, and Noggin treatment also appeared to up-regulate NSE levels to a small extent. Levels of the transcription factor NeuroD1 were up-regulated by RA treatment, and also up-regulated by BMP4 and Noggin treatment. The neurofilament protein NF200 was detected to be up-regulated by RA treatment, and also detected at low levels in BMP2 treated cells. Epithelial Keratin 8 was up-regulated by BMP2 or BMP4 treatment, and down-regulated in Noggin treated cultures.

Neuronal β III Tubulin And Pan-epithelial Keratin Immuno-staining Of Large Cells In BMP4 Treated cultures of TERA2.sp12 Cells

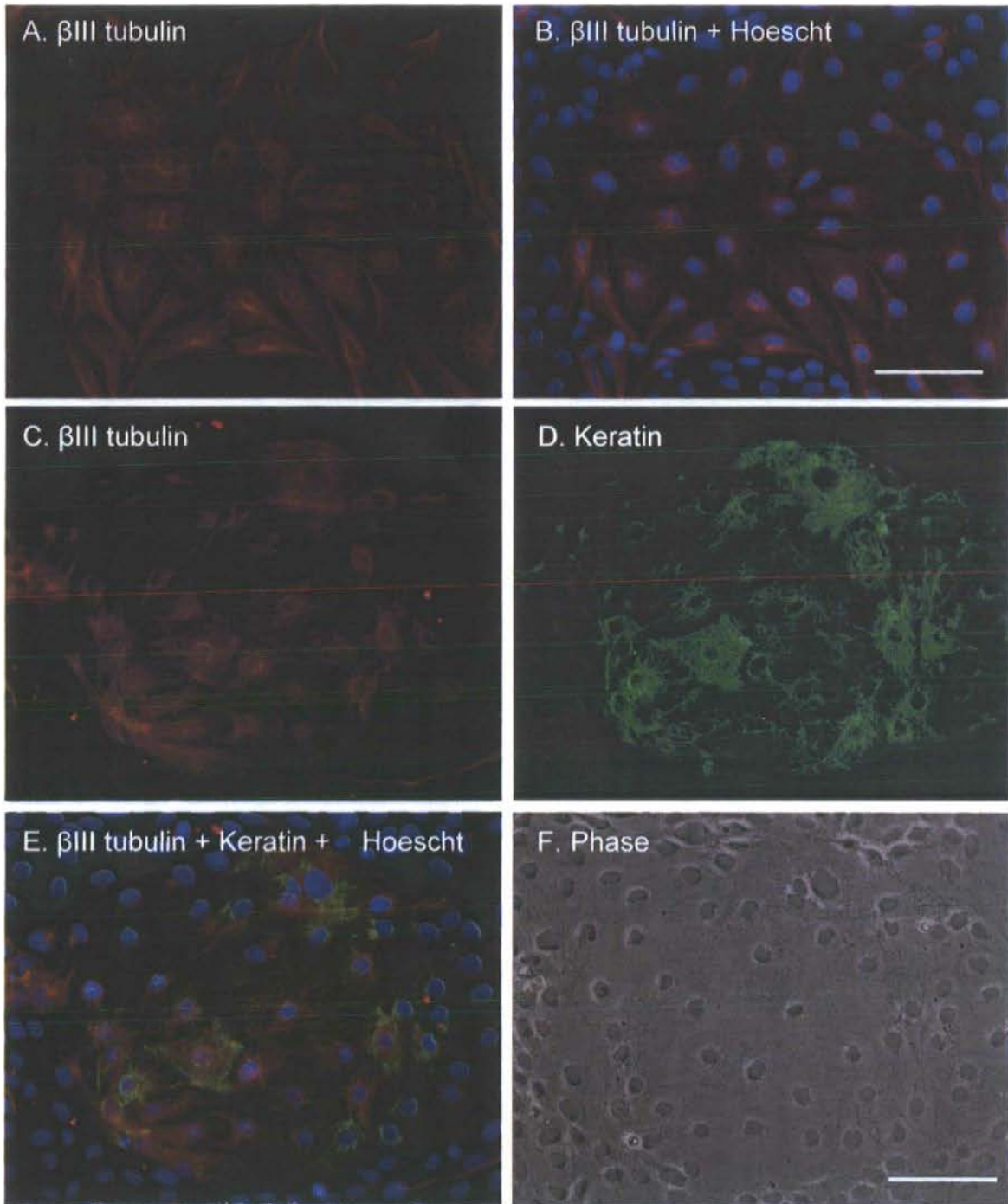


Figure 4.17 TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 25ng/ml BMP4 for 14 days prior to fixation in 4% PFA and immuno-labelling with neuronal β III tubulin, (A-B) or double immuno-labelling with pan-epithelial keratin (green) and neuronal β III tubulin (red), (C-F) and the nuclei stained with hoescht (blue). Large cells demonstrated atypical staining for neuronal β III tubulin (A with overlay in B), with increased staining intensity surrounding the nucleus. In some cases large cells immuno-labelled positive for both β III tubulin and pan-epithelial keratin (C, D with overlay including hoescht in E). The large cells are clearly defined in the corresponding phase contrast image (F). Scale bars = 100 μ m.

morphology and typical β III tubulin expression were detected. Taken together this confirms that neural differentiation did not occur in BMP treated cultures. Although these large cells showed some epithelial keratin expression, the level of expression was variable within large celled populations and in repeated experiments. Unlike the cells within large cell plaques generated by RA treatment, they did not typically express Keratin 8. Atypical Keratin 8 staining was also detected in these cultures, with small clusters of cells sometimes demonstrating solely nuclear localised staining with Keratin 8 antibody. This suggests that the non-neural differentiation occurring in BMP treated cultures is not similar to that seen in RA treated cultures and may not be non-neural ectoderm.

Noggin treated cultures demonstrated distinct and regional immuno-staining for Nestin, with organised orientated cells frequently forming neural rosettes that stained immuno-positive for Nestin, β III tubulin and MAP2, similar to those found in cultures of cells treated with RA (Figure 4.18). More mature markers of neural differentiation were not detected in these cultures, and additionally no epithelial keratin expression was detected. Noggin treated cultures also exhibited negative staining for the stem cell marker Nanog after 14 days of culture. This data is consistent with the hypothesis that inhibition of BMP signalling promotes neural differentiation of TERA2.sp12 cells, but suggests that inhibition of BMP signalling alone (under these conditions) may not be sufficient to induce widespread commitment to the neural lineage.

4.2.5 Inhibition of FGF mediated signalling

To investigate the effect of continuous inhibition of FGF mediated signalling in TERA2.sp12 cells, individualised cells were cultured under adherent conditions and treated with either 20 μ M SU5402 or vehicle treated with DMSO. Media was replenished every 3-4 days (or every 1-3 days as required for vehicle treated cultures). After 14 days cultures were fixed in 4% PFA and double immuno-labelled (using the protocol in Section 2.5) with antibodies to the following: β III tubulin and Nestin, β III tubulin and pan-epithelial keratin, Keratin 8 and 18, Tp63 and Pan-epithelial keratin, MAP2 and β III tubulin, GFAP and β III tubulin.

TERA2.sp12 cells cultured under adherent conditions and continuously treated with SU5402 rapidly developed morphologies similar to those observed in cultures treated with BMP2 or BMP4. Sheets of large plate-like cells interspersed with clusters of densely packed nuclei formed between 7-10 days, and dominated the culture after 14 days. Vesicle-like dots were also visible surrounding the nucleus of the large cells. A pilot immuno-staining analysis also revealed a similar expression pattern to that of BMP2 or 4 treated cells, as pan-epithelial keratin was frequently expressed in these large cells and similar patterns of non-neural β III tubulin expression were observed. This suggested that either inhibition of FGF signalling or

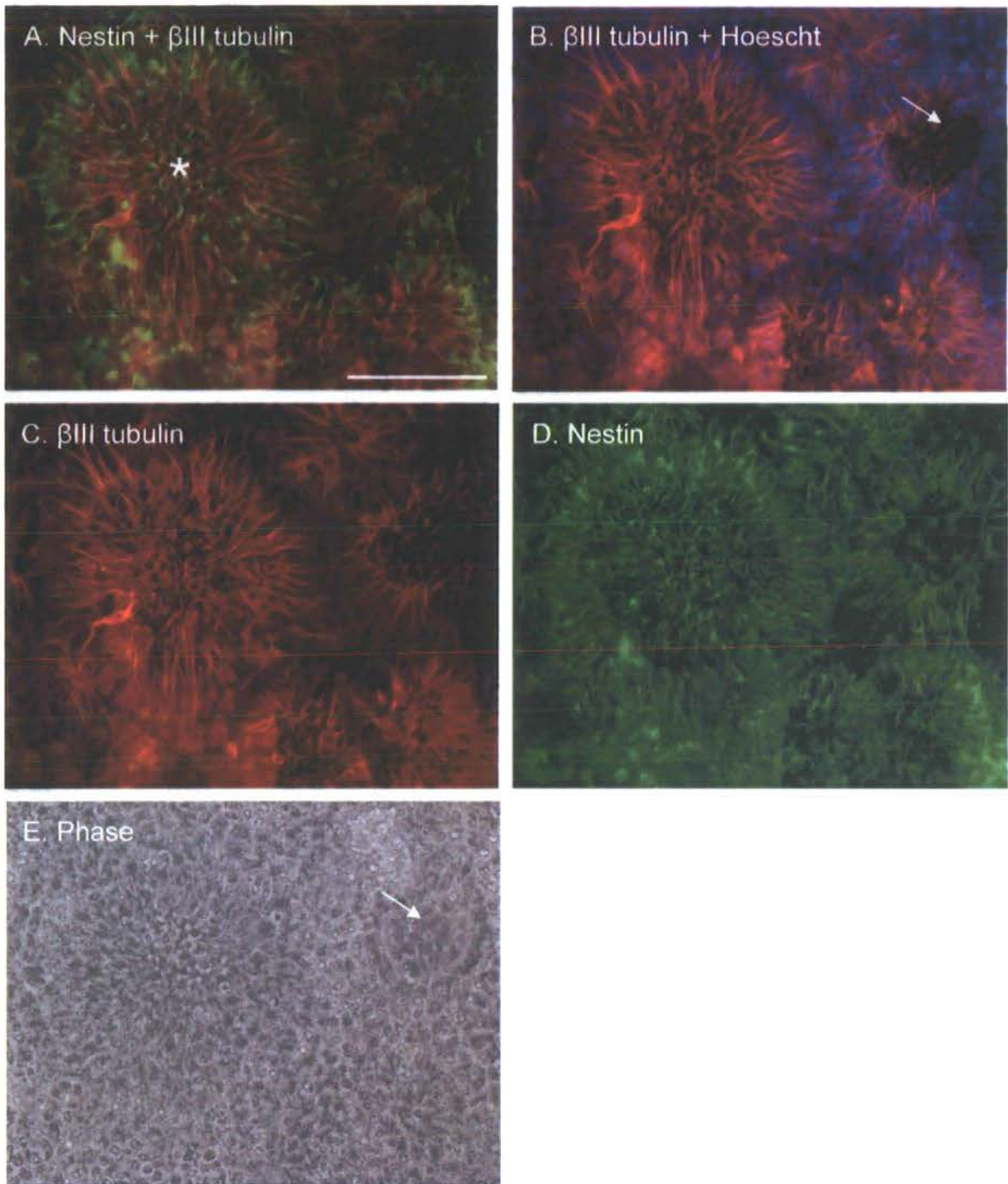
Nestin and β III tubulin expression in Noggin treated cultures of TERA2.sp12 cells

Figure 4.18 TERA2.sp12 cells were seeded at 20,000 cells/cm² into 12 well plates and treated with 100ng/ml Noggin for 14 days prior to fixation in 4% PFA and double immuno-labelling with Nestin (green) and neuronal β III tubulin (red), and the nuclei stained with hoescht (blue). Distinctive aggregate-like structures are visible (asterix), which are also visible under phase microscopy (E). Note that radially orientated β III tubulin or Nestin positive cells project out from this aggregate-like structure (C, D or overlays A, B) with Nestin expression strongest towards the edge of the structure, and neuronal β III tubulin towards the centre. Note that small groups of morphologically distinct cells under phase contrast (arrow in E and in overlay B) exhibited weaker staining for Nestin or β III tubulin expression, and were typically surrounded by radially orientated cells strongly positive for these markers. Scale bar = 100 μ m.

activation of BMP signalling induced the same effect on TERA2.sp12 cells.

4.2.6 Characterising cultures treated with BMP2, BMP4 or SU5402

Since cultures of TERA2.sp12 cells treated with the FGF inhibitor SU5402 or BMP2 or BMP4 exhibited similar morphologies, and pilot immuno-staining analyses of SU5402 treated cells demonstrated similar expression patterns of epithelial keratins and β III tubulin, the differentiation of TERA2.sp12 cell cultures in response to these treatments were together more comprehensively characterised and compared using both Western blot and immuno-staining analyses. TERA2.sp12 cells were cultured under adherent conditions and treated with 25ng/ml BMP2, 25ng/ml BMP4, 20 μ M SU5402 or vehicle treated. Media was replaced every 2-3 days, and after 7 and 14 days protein was extracted from cultures and used for Western blot analysis following the protocol in Section 2.4. Blots were probed with antibodies listed in Table 4.3.

Table 4.3. List of antibodies used in Western Blot analysis of samples from TERA2.sp12 cells treated for 14 days with BMP2, BMP4, SU5402 or RA.

Antigen	Molecular weight (kDa)	Antibody	Source	Dilution	Category
β actin	42	Mouse IgG	Sigma	1: 4000	House keeping gene
Nestin	220-240	Mouse IgG	Chemicon	1:200	Ectoderm- neural
β III Tubulin	50-54	Rabbit IgG	Covance	1:4000	"
NSE	45	Mouse IgG	Chemicon	1:200	"
Vimentin	58	Mouse IgG	Sigma	1: 200	"
Keratin 18	45	Mouse IgG	Sigma	1:400	Ectoderm- non neural
Keratin 8	55,45	Rabbit IgG	Abcam	1:400	"
TP63 / TP73L	73	Goat IgG	R&D systems	1:200	"
HCG	40	mouse IgG	Abcam	1:200	Trophoectoderm-Trophoblast
α smooth muscle actin	42	mouse IgG	Sigma	1:400	Mesoderm- smooth muscle

Protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions for the EC sample, and protein extracted from cultured IMR32, FaDU and MSC cells was used as positive controls for neural, epidermal and mesenchymal markers respectively. Protein extracted from day 19 human villous placental tissue was used as a positive control for the expression of HCG protein. Equal loading was assessed by staining the blot with Ponceau prior to blotting and also by probing with an antibody to β actin.

In Western blot analyses, the protein expression profile of cultures treated with BMP2, 4 or SU5402 was very similar (representative blots in Figure 4.19). Neural proteins β III tubulin, Nestin and NSE were not up-regulated after 14 days of treatment (with the exception of BMP2 treated cells, in which Nestin and NSE protein was detected at low levels after 14 days of culture). Vimentin, a protein typically expressed in neural cells, (although it is also expressed in mesenchymal cells) was also markedly down-regulated in contrast to the up-

Developmental Regulation Of Neural, Non-Neural Ectoderm, Trophoblast And Smooth Muscle Markers In TERA2.sp12 Cells Treated With Either BMP2, BMP4 Or The FGF Receptor Inhibitor SU5402.

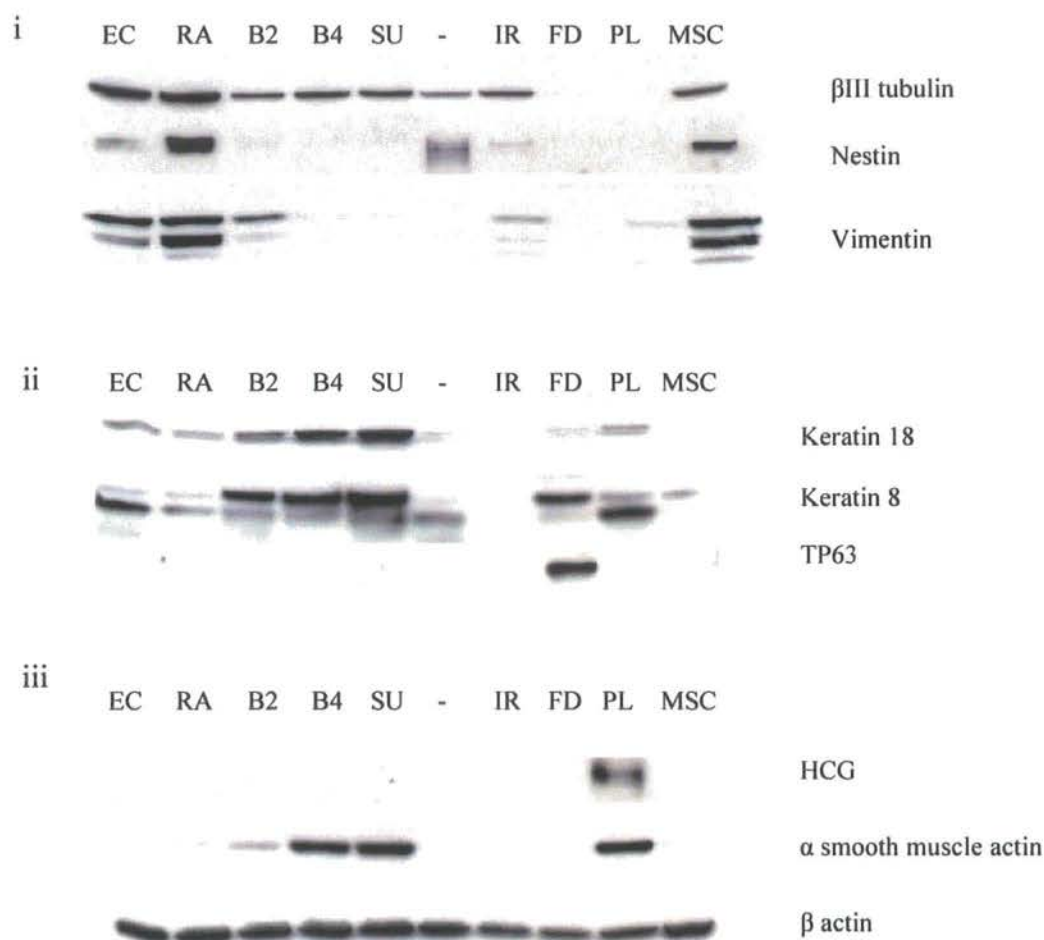


Figure 4.19 Western blot detection of a panel of neural (i), non-neural ectoderm (ii), trophoblast and smooth muscle (iii) markers from protein extracted at 14 days from TERA2.sp12 cells treated with either 10 μ M RA, 25ng/ml BMP2 (B2), 25ng/ml BMP4 (B4), 20 μ M FGF inhibitor SU5402 (SU), or untreated (-). As positive controls for the expression of neural proteins, epidermal proteins, the trophoblast hormone Human chorionic gonadotrophin (HCG) and α smooth muscle actin, protein was extracted from IMR32 cells (IR), FaDU cells (FD), day 9 human placental tissue (PL) and human MSC cells respectively. For the EC sample protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions. To determine equal loading blots were also probed with β actin. (i) In contrast to RA treated cultures, BMP2, BMP4 or SU5402 treated cultures down-regulated β III tubulin expression and did not up-regulate Nestin or Vimentin with the exception of BMP2 treated cultures, which minimally up-regulated both of these markers. Correspondingly Keratins 8 and 18 were markedly up-regulated with BMP or SU5402 treatment, with BMP2 treated cells and SU5402 treated cells demonstrating the least and most up-regulation respectively (ii). However, expression of the epidermal stem cell marker TP63 was not detected in any treatments, compared to strong detection in control FaDU cultures. As an indicator of trophoblast differentiation no HCG protein was detected in treated TERA2.sp12 cells. However HCG was only faintly detected in the human placental tissue used as a positive control. In contrast, α smooth muscle actin, expressed in smooth muscle, was strongly detected in cultures treated with BMP4 or SU5402, with low levels also detected in BMP2 treated cultures and trace detection in RA treated cells.

regulation observed in RA treated cultures. Simple epithelial keratins 8 and 18 were also up-regulated, with the strongest up-regulation in SU5402 treated cultures, and weakest in BMP2 treatments. However, the epidermal stem cell marker TP63 was negative in all treatments, including RA treated cultures.

During an immuno-staining survey for markers of differentiation into non-ectodermal lineages, positive expression of the smooth muscle marker α smooth muscle actin and the trophoblast hormone HCG was detected, (as described in detail later). To compare the expression of these markers between cultures the level of total protein was detected in Western blot analyses (plate iii in Figure 4.19). Smooth muscle actin protein was detected at increasing levels in cultures treated with BMP2, BMP4 or SU5402 respectively and unexpectedly also at trace levels in RA treated cultures. The trophoblast hormone HCG was not detected in Western blot analyses, despite multiple attempts. However HCG protein was only faintly detected in the positive control tissue (day 9 human placental villous tissue), indicating that the negative results may be due to difficulties with this technique of detection.

To more comprehensively characterise the differentiation of BMP or SU5402 treated TERA2.sp12 cells, cultures treated for 14 days with BMP2, BMP4 or SU5402 were immuno-labelled with a range of markers indicative of different germ layers and with markers to extra-embryonic endoderm and ectoderm. TERA2.sp12 cells were cultured under adherent conditions and treated with 25ng/ml BMP2, 25ng/ml BMP4, 20uM SU5402 or vehicle treated. Media was replaced every 2-3 days. After 14 days cultures were fixed in 4% PFA and double immuno-labelled using the protocol in Section 2.5 with antibody combinations listed in Table 4.4. As a positive control for markers of differentiation into multiple germ layers and for trophoblast differentiation, 11 day spontaneously differentiated H7 embryonic stem cells and sectioned 19 day human villous tissue were used respectively (as described in Sections 2.12 and 2.9 respectively).

Table 4.4 Details of the antibodies and antibody combinations used in multiple immuno-labelling and double immuno-labelling experiments.

Antibodies	Staining notes	Aim/question
β III tubulin and pan-epithelial keratin		Is β III tubulin and pan-epithelial keratin expression mutually exclusive?
Nestin and β III tubulin		Confirm that no neuronal differentiation is occurring
Gata 4		Assay for endoderm differentiation
Brachyury		Assay for mesoderm differentiation
TP63		Assay for epidermal differentiation
HNF3 β		Assay for endoderm differentiation
AFP	Pre-treat with ethanol	Assay for primitive endoderm differentiation
α smooth muscle actin	Fix in methanol with 5% acetic acid	Assay for smooth muscle differentiation
α smooth muscle actin and β III tubulin	Fix in methanol with 5% acetic acid	Are the large β III tubulin positive cells also expressing smooth muscle actin?
HCG	Pre-treat with Brefeldin A	Assay for trophoblast differentiation
HCG + Keratin 8	Pre-treat with Brefeldin A	Do HCG positive cells also express trophoblast marker nuclear localised Keratin 8 Ab staining?
HCG + β III tubulin	Pre-treat with Brefeldin A	Are cells positive for trophoblast markers also expressing non-neural β III tubulin?
HCG + Gata 4	Pre-treat with Brefeldin A	Is HCG (trophoblast marker) and Gata 4 (endoderm) marker expression mutually exclusive?
ID2		Is ID2 expressed at high levels in a morphologically distinct sub-population?
Vimentin		Assay for neural or mesenchymal differentiation

As indicated in the table, the standard staining protocol was adjusted for the detection of the certain markers and these modifications are described as follows:

1. To increase the detection of HCG, 4 hours prior to fixation in PFA 1.25 μ g/ml of Brefeldin A, (Sigma B7651) was added to the cell cultures to be immuno-stained with HCG antibody.
2. After PFA fixation, cells to be immuno-stained for AFP were treated for 1 min with ethanol, washed briefly in water and then PBS before continuing with the standard immuno-staining method.

In essence the same population profiles were observed within all treated cultures, with some variation in the frequency of each phenotype: Brief summaries of the immuno-staining phenotypes observed are described below.

1. Sub-population (10-15%) Densely clustered small cells

These clustered small cells were frequently immuno-positive for nuclear localised Keratin 8 antigen (the nuclear staining generated from the cross-reaction of a trophoblast antigen with Keratin 8 antibody), and a sub-population of cells within these clusters was immuno-positive for the trophoblast hormone HCG, indicating that these cells were differentiating along a trophoblast lineage (see Figure 4.20). Some of these HCG positive cells appeared to be multinucleate cells, containing multiple small clustered nuclei, (often arranged as 'strings') and a more or less continuous membrane. These cells were negative for AFP, Neuronal β III tubulin, pan-epithelial keratin and Gata4. In the case of Gata4, large cells immediately surrounding clusters of HCG positive densely packed cells were also negative (as depicted in



Trophoblast Marker Expression In TERA2.Sp12 Cells Treated With An Inhibitor Of FGF Signalling

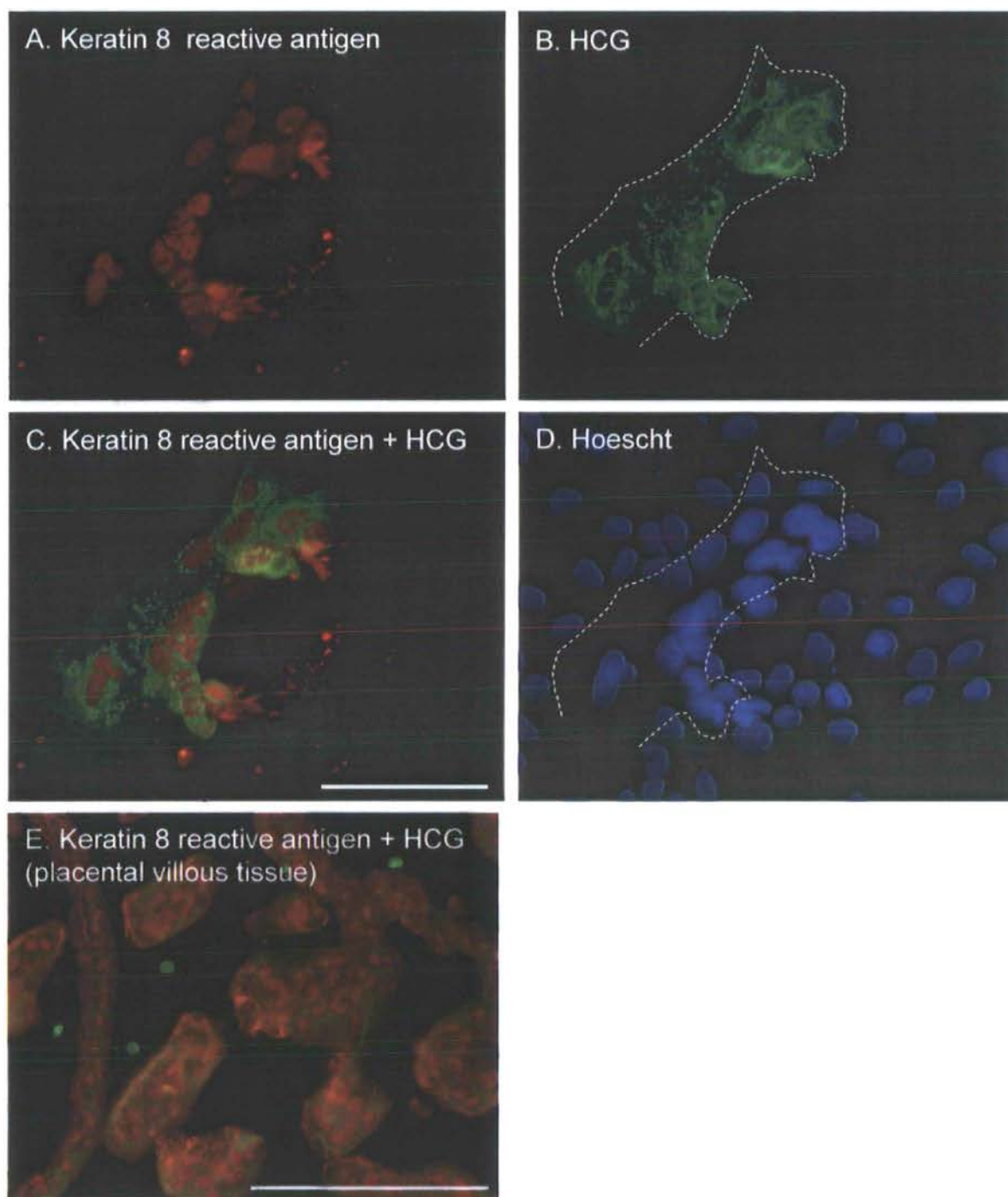


Figure 4.20 TERA2.sp12 cells were seeded into 12 well plates and treated with 20 μ M of the FGF receptor inhibitor SU5402 for 14 days. Four hours prior to fixation in 4% PFA cultures were pulsed with 1.25 μ g/ml Brefaldin A. Fixed cultures were double immuno-labelled with antibodies to HCG (green) and Keratin 8 (red), and the nuclei stained with hoescht (blue). Plates A-D depict the expression of trophoblast hormone HCG (green) and nuclear localised cross-reactive staining of a trophoblast antigen to Keratin 8 antibody (red) in a representative cluster of cells. Positive immuno-staining for HCG is outlined by white dashes in plate B, and the corresponding outline transposed to the hoescht stained image in D. Note that cells positive for HCG also exhibit nuclear localised Keratin 8 reactive antigen expression (A and overlay in C), and hoescht staining (D) reveals a distinctive morphology with 'strings' of nuclei clustered together. For comparison a section of day 19 villous placental tissue double immuno-labelled with Keratin 8 (red) and HCG (green) is presented (E). Note that nuclear localisation of Keratin 8 reactive antigen and HCG expression is visible in cells lining the villous tissue. Scale bars = 100 μ m.

A-D of Figure 4.21). Regions containing HCG positive cells were also highly distinctive under phase microscopy, with prominent arrangements of densely clustered nuclei. Brefaldin A treatment to disrupt the Golgi apparatus and reduce the secretion of HCG into the media did enhance the brightness of immuno-staining for HCG. However HCG positive cells were also identified in un-treated cultures, indicating that Brefaldin A treatment was not necessary for the immuno-detection of HCG. Taken together this provides supporting evidence that a sub-population of BMP or SU5402 treated cells formed trophoblast cells.

2. Remaining population: Mixture of large plate-like cells and less defined smaller cells forming a continuous sheet.

As previously described, neural marker expression was minimal in all treatments, with the exception of neuronal β III tubulin, which was often expressed in large hexagonal shaped cells. These large cells were also detected to weakly express pan epithelial keratin and Keratin 18, with co-localisation of neuronal β III tubulin and pan epithelial keratin demonstrated in some regions. However, unlike RA treated cultures, the expression of the epidermal stem cell protein TP63 was not detected in any cultures in all repeated experiments. Some very large cells with large nuclei were also observed, typically in close association with clusters of cells exhibiting similar morphologies to those positive for HCG. Taken together this suggests that non-neural ectoderm was not present at significant levels in these cultures.

Through-out the culture more densely packed cells with less defined borders were also observed and the frequency of these cells in comparison to the large hexagonal cells exhibited some variation in repeated culture experiments. In general these cells were negative for epithelial keratins and β III tubulin, and stained immuno-positive for the endoderm transcription factor Gata4. Nuclear localised Gata4 was detected in large patches through-out these cultures with the highest levels of Gata4 detected in BMP 4 treated cultures. A proportion of cells exhibiting negative staining for nuclear localised Gata4 was consistently observed in all culture treatments and in repeated experiments, with distinct patches of negative staining detected in cells associated with trophoblast differentiation or in larger hexagonal cells (see plates A-D in Figure 4.21). This suggests that a sub-population of BMP or SU5402 treated cells formed endoderm progenitors.

The smooth muscle marker α smooth muscle actin was also detected in large regions through-out the culture, typically associated with the plate-like large cells, suggesting that some cells had differentiated into smooth muscle. These cells were not similar to positively stained cells in cultures of spontaneously differentiated H7 ES cells, with smooth muscle actin positive cells in TERA2.sp12 cultures exhibiting a less mature differentiation morphology (see Figure 4.21 plates E-F), although this could be attributed to differences in cell density.

Expression Of Gata4, HCG And Alpha Smooth Muscle Actin In BMP2 Treated TERA2.Sp12 Cells

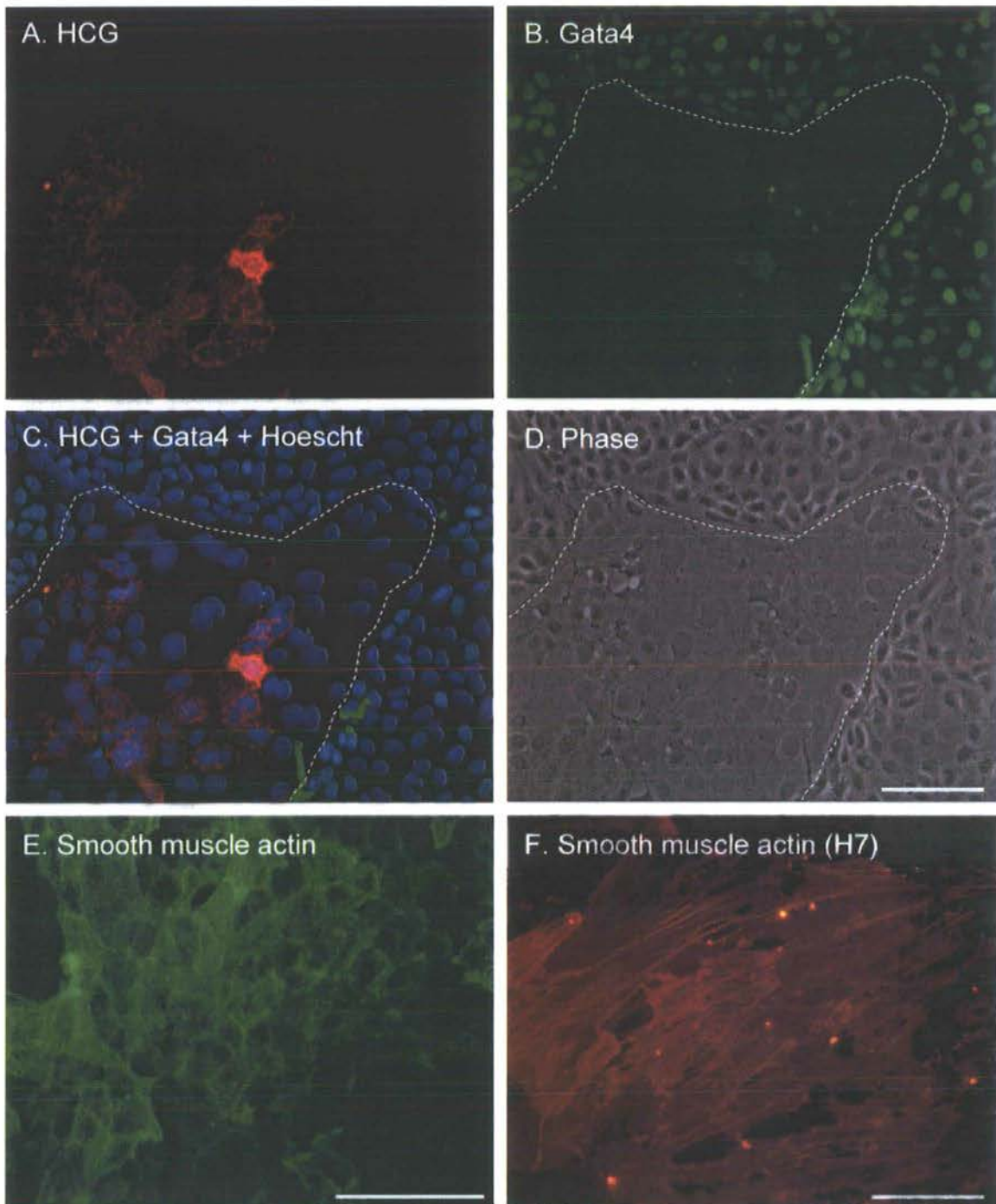


Figure 4.21 TERA2.sp12 cells were seeded into 12 well plates and treated with 25ng/ml of BMP2 or BMP4 for 14 days. Plates A-D depict a representative region of HCG positive and Gata4 negative cells in BMP2 treated cultures double immuno-labelled after fixation in 4% PFA with antibodies to Gata4 (green) and HCG (red), and the nuclei stained with hoescht (blue). Negative immuno-staining for Gata4 is outlined by white dashes in plate B, and the corresponding outline transposed to an overlay in C and the phase contrast image in D. Note that HCG positive cells were located within distinct regions of Gata4 negative expression, and Gata4 positive cells exhibited a more compact morphology than Gata4 negative cells. Plates E and F depict representative regions of positive α smooth muscle actin expression in either 14 day BMP4 treated cultures of TERA2.sp12 cells (E) or for comparison in 11 day cultures of H7 human embryonic stem cells grown in the absence of stem cell maintenance factors (F). Cultures were fixed in methanol with 5% acetic acid and immuno-stained with an antibody to α smooth muscle actin. Note that immuno-positive cells in BMP treated TERA2.sp12 cultures (E) exhibit a less differentiated morphology and remain relatively compact compared to immuno-positive cells in H7 cultures (F). Scale bars = 100 μ m.

Variations in levels of staining

Although cultures treated with either BMP2, BMP4 or SU5402 exhibited very similar immuno-staining expression patterns, some variation was observed between these treatments. To more qualitatively compare this variation, for each antibody immuno-stained cultures were scored according to staining intensity and frequency of positive staining (see Table 4.5). The tabulated scores are based on the number of immuno-positive cells, ranging from – (negative), slight positive [(+)] and increasing levels of positive staining indicated by more [+] symbols, such that very high levels of staining are scored with [+++]. The tabulated score is representative of three sets of individual scores for each culture condition at the same passage number.

Table 4.5 Scored levels of positive immuno-staining for a range of antibodies in TERA2.sp12 cells treated with BMP2, BMP4, SU5402 or vehicle treated for 14 days.

Cell type	Antibody	BMP4	BMP2	SU5402	Mock SU5402	Mock BMP
Neural progenitor	Nestin	(+)	(+)	(+)	++(+)	
Neural	β III tubulin (neural morphology)	-	-	-	(+)	
-	β III tubulin (non-neural morphology)	+	++(+)	++	-	
Non-neural ectoderm	Pan Epithelial Keratin	+	+	(+)	-	-
Non-neural ectoderm	keratin 18	(+)	+	(+)	-	-
Trophoblast	Keratin 8 Ab binding antigen (nuclear localisation)	+	+	(+)	-	-
Trophoblast	HCG	++(+)	++	+	-	-
Smooth muscle	α smooth muscle actin	++	+	++	-	-
Endoderm	GATA4	++(+) (70%)	++ (50%)	++ (50%)	(+)	
Endoderm	HNF3 β	(+)	(+)	(+)	-	-

To summarise, the level of CGB staining was variable in cultures treated with BMP2, BMP4 or SU5402, with the highest population of HCG positive cells occurring in BMP2 treated cultures, in distinct large patches. Gata 4 immuno-staining was very high in BMP or SU5402 treated cultures, with BMP4 treated cultures exhibiting the highest proportion of positive cells. The protein α smooth muscle actin was also detected at significant levels in BMP4 and SU5402 treatments, although BMP2 treated cultures exhibited slightly lower levels of smooth muscle actin expression. It was interesting to note that smooth muscle actin was also significantly present in placental villous tissue as detected by both immuno-staining and Western blot analyses.

Treatment with trophoblast stimulating hormones insulin and forskolin

To determine whether the subpopulation of clustered small cells exhibited behaviour typical of trophoblast cells, cultures previously treated for 14 days with BMP2, 4 or SU5402 were subsequently treated for 3 days with the trophoblast differentiation inducing hormones Insulin (200mg/litre insulin in 10mM HCL), Forskolin (10 μ M forskolin in ethanol), or mock treated. To determine whether any cells formed during RA differentiation were also responsive to these factors, 14 day RA treated adherent cultures were also treated with either RA alone or with the addition of Insulin or Forskolin for 3 days. After 17 days cultures were fixed for 15 minutes at room temperature with ice cold PFA, blocked with blocking buffer at 4°C overnight and then immuno-labelled with either HCG, Keratin 8, Neuronal β III tubulin or Nestin, following the protocol in Section 2.5. No HCG or nuclear localised Keratin 8 reactive nuclear antigen was detected in any cultures pre-treated with RA, consistent with previous observations that RA treatment does not induce trophoblast differentiation, and demonstrating that treatment with trophoblast stimulating hormones alone is not sufficient to induce trophoblast differentiation in TERA2.sp12 cells not previously exposed to BMP or SU5402. However, HCG and nuclear localised Keratin 8 antigen was detected in the remaining treatments, and Table 4.6 presents the scores for the level of positive immuno-staining in each condition. The tabulated scores are based on the number of immuno-positive cells range from – (negative), slight positive [(+)] and increasing levels of positive staining indicated by more [+] symbols, such that very high levels of staining are scored with [+++]. The tabulated score is representative of three sets of individual scores for each culture condition (at the same passage number). N=3 for only HCG. Note that no positive control was used in this experiment, as trophoblast cell lines were not available.

Table 4.6. Scored levels of positive immuno-staining for trophoblast markers in TERA2.sp12 cells treated with BMP2, BMP4 or SU5402 for 14 days (Treatment 1), prior to continued treatment with or without the addition of insulin or forskolin for 3 days (Treatment 2).

SET 1	Treatment 1	BMP4		
	Treatment 2	BMP4	INSULIN	FORSKOLIN
Antibody staining	HCG	++	++	++
	nK8	+	+	+
SET 2	Treatment 1	BMP2		
	Treatment 2	BMP2	INSULIN	FORSKOLIN
Antibody staining	HCG	++	++	++
	nK8	+	+	+
SET 3	Treatment 1	SU5402		
	Treatment 2	SU5402	INSULIN	FORSKOLIN
Antibody staining	HCG	+	(+)	+
	nK8	+	(+)	+

These results indicate that forskolin and insulin did not affect the expression of trophoblast markers HCG and nuclear localised antigen reactive to Keratin 8 antibody (nK8), and that under these conditions these cells were not responsive to Insulin and Forskolin. However, it is interesting to note that compared to previous experiments where cultures were grown for 14 days, both the expression of HCG and size of HCG positive clusters in all treatments cultured for 17 days (with or without Insulin or Forskolin) appeared markedly greater (Figure 4.22). This suggests that the trophoblast population in these cultures was still increasing and maturing between 14 and 17 days.

4.2.7 Monitoring BMP and FGF mediated pathway activity

To monitor the effect of FGF receptor inhibitor and BMP treatment on both BMP and MAPK pathway activity, in particular to determine whether inhibition of FGF signalling up-regulated the activity of the BMP pathway, the level of BMP regulated Id2 and MAPK regulated ERK1/2 phosphorylation were monitored using Western blot analysis. TERA2.sp12 cells were cultured under adherent conditions with the addition of 25ng/ml BMP2, 25ng/ml BMP4, 20 μ M SU5402 or vehicle treated. Media was replaced every 2-3 days, and after 7 and 14 days protein was extracted from cultures and used for Western blot analysis following the protocol in Section 2.4. Protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions for the EC sample, and protein extracted from cultured IMR32, FaDU, MSC cells or day 19 villous placental tissue was used as comparative controls for neural, non-neural ectoderm, mesodermal and trophoblast expression profiles. Blots were probed with antibodies to Id2, ERK1/2, phosphorylated ERK1/2 and equal loading was assessed by probing with an antibody to β actin. The results were consistent in two repeated Western blot analysis experiments, one of which is presented in Figure 4.23.

Levels of phosphorylated ERK1, (upper band) did not change markedly with treatment, in contrast to marked differences in levels of phosphorylated ERK2. Unexpectedly, levels of phosphorylated ERK 2 were reduced only slightly compared to mock treated cells, and EC cells showed only trace levels of P ERK phosphorylation. BMP2 or RA treated cells demonstrated marked or some up-regulation of phosphorylated ERK respectively compared to mock treated cultures, with a lower up-regulation compared to mock treated cultures observed in BMP4 treated cells. This pattern was consistent in both Western blot analyses. Total levels of ERK1/2 protein were highest in SU5402 treated cultures, and all treatments induced some up-regulation in ERK 1/2 expression. BMP regulated levels of Id 2 protein increased in RA, BMP2, BMP4 and SU5402 treated cultures to similar extents (with BMP2 treated cells up-regulating Id2 to the highest levels), indicating that BMP signalling was up-regulated in all of these cultures. This supports the hypothesis whereby FGF signalling in TERA2.sp12 cells mediates the attenuation of BMP pathway activity, such that inhibiting FGF signalling up-

Expression Of The Trophoblast Hormone HCG In 17 Day BMP Treated Cultures That Were Cultured In The Last 3 Days With Or Without Insulin

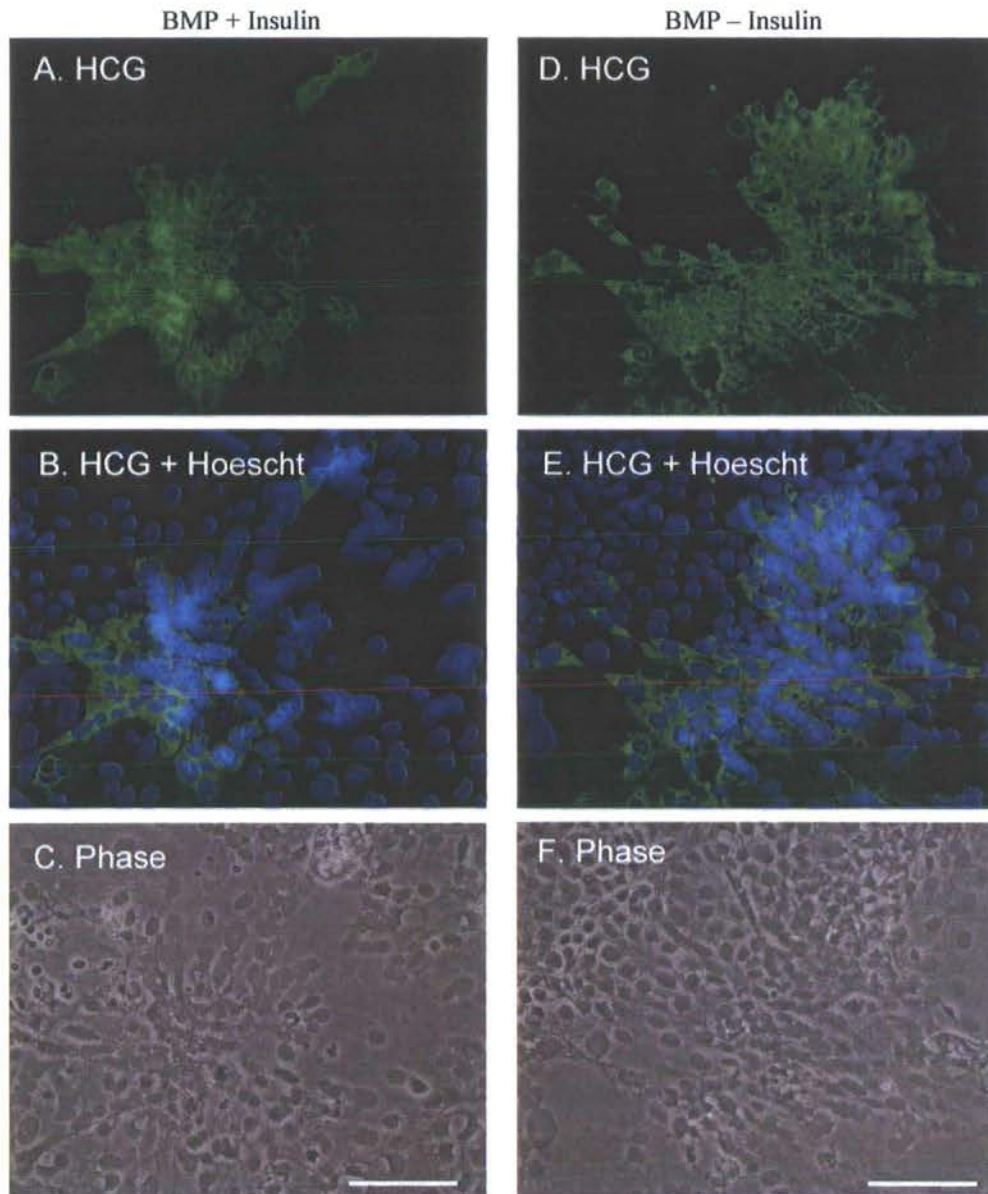


Figure 4.22 Expression of the trophoblast hormone HCG in 17 day BMP treated cultures that were cultured in the last 3 days with or without Insulin. TERA2.sp12 cells were seeded into 12 well plates and treated with either 25ng/ml of BMP2 or 25ng/ml BMP4 for 14 days, then cultured for a further 3 days with or without the addition of 200µg/ml Insulin. Cultures were treated for 4 hours with 1.25µg/ml Brefaldin A and then fixed in 4% PFA, prior to immuno-staining with an antibody to the trophoblast hormone HCG (green) and the nuclei counterstained blue with hoescht. Plates A-C depict a representative region of dense HCG staining in TERA2.sp12 cells cultured with BMP2 for 14 days and then BMP2 and insulin for a further 3 days. Note that densely packed 'strings' of nuclei are associated with bright HCG staining (overlay in B) and HCG positive cells exhibit a distinctive morphology under phase contrast (C). Plates (D-F), depict a representative region of dense HCG staining in TERA2.sp12 cells cultured with BMP4 continuously for 17 days. Note that similarly large and densely packed cells exhibit high staining for HCG (overlay in E) and demonstrate a distinctive morphology under phase contrast (F). Scale bars =100µm.

Regulation Of BMP And MAPK Pathway Activity In Cultures Treated For 14 Days With Either RA, BMP2, BMP4 Or The FGF Receptor Inhibitor SU5402

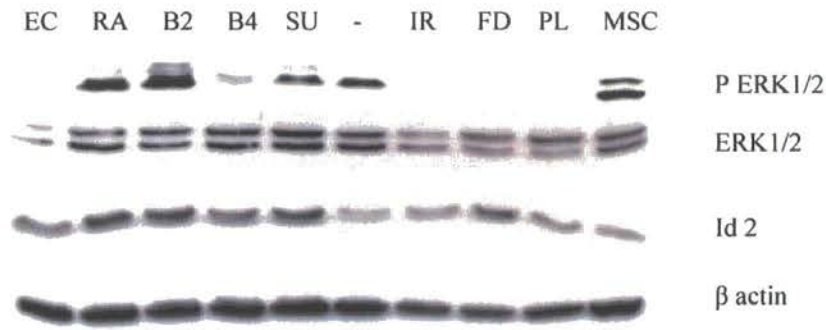


Figure 4.23 Western blot detection of phosphorylated ERK 1/2 (P ERK1/2) as an indicator of FGF mediated MAPK activity, compared with total levels of ERK1/2, and detection of Id2 as an indicator of BMP mediated Smad activity. Protein was extracted at 14 days from cells treated with either 10 μ M RA, 25ng/ml BMP2 (B2), 25ng/ml BMP4 (B4), 20 μ M FGF inhibitor SU5402 (SU) or mock treated (-). For comparison protein was extracted from IMR32 (IR), FaDU (FD), MSC cells and day 19 human placental tissue (PL) respectively. For the EC sample protein was extracted from confluent cultures of TERA2.sp12 cells grown under maintenance conditions. To determine equal loading blots were also probed with β actin. The expression of the Smad transcriptionally regulated protein Id2 was detected to increase in RA, BMP2, BMP4 and SU5402 treated cultures to similar extents (with BMP2 treated cells up-regulating Id2 to the highest levels), indicating that BMP signalling was up-regulated in all of these cultures. Levels of phosphorylated ERK1 (upper band) did not change markedly with treatment, in contrast to marked differences in levels of phosphorylated ERK2 (lower band). Unexpectedly, levels of phosphorylated ERK 2 were reduced only slightly compared to mock treated cells, and EC cells showed only trace levels of P ERK phosphorylation. In contrast, BMP2 or RA treated cells demonstrated marked or some up-regulation respectively compared to mock treated cultures, with a lower up-regulation compared to mock treated cultures observed in BMP4 treated cells. Total levels of ERK1/2 protein were highest in SU5402 treated cultures, and all treatments induced some up-regulation in ERK 1/2 expression.

regulates BMP pathway activity regardless of changes in BMP signalling.

4.2.8 Subsequent Noggin and FGF inhibitor treatment

To determine whether TERA2.sp12 cells initially treated with Noggin were committed to a neural fate and would respond differently to inhibition of FGF signalling (for example by differentiating further along a neural lineage), in a pilot study TERA2.sp12 cells were treated with Noggin for 14 days or mock treated and then trypsinised and reseeded at differentiation density prior to exposure to SU5402 for 7 days. The effect of SU5402 on Noggin treated cells that were not trypsinised and re-plated was also assayed. Cells pre-treated with Noggin and then re-seeded at low density and treated with SU5402 formed at least two distinct cell types. Large morphologically distinct flat cells expressing pan-epithelial keratin formed sheets of cells on which small numbers of highly differentiated neural β III tubulin positive cells were detected, exhibiting long axons and in some cases extensive branching (Figure 4.24). Cells treated without reseeding also formed neural cells, however these were less mature in morphology and extensive branching was not observed. This suggests that under these conditions, inhibition of BMP signalling using Noggin treatment is not sufficient to form committed neural progenitors from the majority of a population of TERA2.sp12 cells. However, a sub-population appears to have indeed responded as committed neural progenitors to FGF signalling inhibition and differentiated extensively into neural lineages.

Neural Versus Non-neural Differentiation In TERA2.Sp12 Cells After Sequential Inhibition Of BMP Signalling and FGF Mediated Signalling.

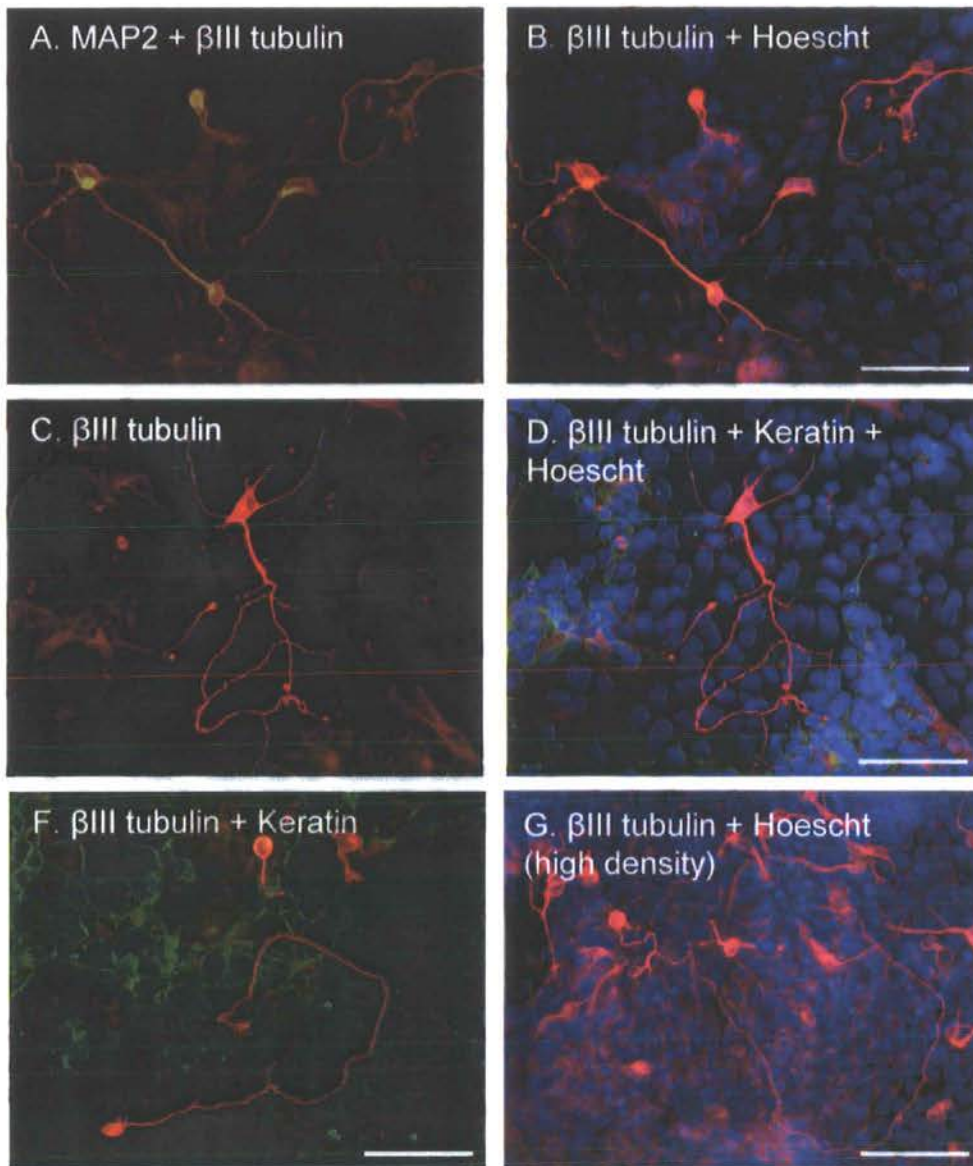


Figure 4.24 Neural vs Non-neural differentiation in TERA2.sp12 cells treated sequentially with inhibitors of BMP and FGF signalling. TERA2.sp12 cells were seeded into 12 well plates and treated with 100ng/ml Noggin for 7days, prior to being reseeded at a low density and treatment with 10 μ M SU5402 or 100ng/ml Noggin for 7 days. Cultures were fixed in 4% PFA prior to immuno-staining with antibodies to β III tubulin, β III tubulin and MAP2, β III tubulin and pan-epithelial keratin and the nuclei counterstained blue with hoescht. Plate A depicts a representative region of positive staining for neural markers β III tubulin and MAP2 in a culture treated with Noggin followed by reseeding at a low density and treatment with SU5402 (a corresponding image of β III tubulin staining alone with hoescht overlay is presented in B). In some cases highly mature and branched morphologies were observed (as depicted in the β III tubulin stained cell in C), and neural cells often developed in close proximity to epithelial keratin positive regions (see overlays in D and F). In cultures which were not subject to reseeding at low density, extensive neuronal differentiation was also observed (a representative region of positive β III tubulin staining is shown with hoescht overlay in G). Note that the highly branched morphologies observed in cultures reseeded at a low density were not observed in cultures maintained at a high density. Scale bars = 100 μ m

4.3 DISCUSSION

4.3.1 Summary

The initial aim of the investigations in this chapter was to determine whether the marked differences in neural differentiation between TERA2.sp12 cells differentiated with RA under either suspension or adherent conditions (Horrocks *et al.* 2003) were caused by changes in BMP signalling, and whether RA induced neural differentiation could be inhibited or promoted by manipulation of the BMP signalling pathway. As described in Section A, the manipulation of BMP or FGF signalling in RA treated cultures of TERA2.sp12 cells markedly affected the expression of neural and non-neural ectoderm markers, although differences were detected in the responsiveness of cells cultured under adherent or suspension conditions. Cells cultured under suspension conditions were less responsive to either BMP or Noggin treatment compared to adherent cultures, and PCR and Western blot detection of markers transcriptionally regulated by BMP signalling indicate that BMP signalling was reduced in cells differentiated under suspension conditions compared to adherent conditions. As an initial increase in FGF mediated signalling was proposed in a report by Kuroda *et al.* (2005) to mediate the inhibition of BMP pathway activity and the adoption of a neural fate in dissociated *Xenopus* explant tissue, the effect of early inhibition of the FGF mediated signalling pathway on neural differentiation was investigated. In this study a temporary pulse with the FGF receptor 1 inhibitor SU5402 prior to RA treatment markedly inhibited the up-regulation of neuronal β III tubulin in suspension cultures, whereas in adherent cultures the up-regulation of neuronal β III tubulin was not inhibited. This provides supporting evidence for a mechanism by which up-regulated FGF signalling and MAPK activity as a result of dissociation and culture in suspension inhibits BMP pathway activity in TERA2.sp12 cells, resulting in increased neural differentiation in response to RA treatment.

In the second part of this chapter (Section B), the effect of manipulations in BMP and FGF mediated signalling on TERA2.sp12 cells differentiation in the absence of RA was investigated, in order to more closely mimic signalling events of the embryonic ectoderm. Continuous treatment of TERA2.sp12 cells with either BMP2, 4 or the FGF receptor inhibitor SU5402 resulted in the down-regulation of stem cell marker expression and the formation of sheets of large cells that expressed epithelial keratins but were also detected by immunostaining to express Gata 4, neuronal β III tubulin and α smooth muscle actin, interspersed with dense clusters of multinucleate cells that expressed trophoblast markers HCG and nuclear localised Keratin 8 antigen. This demonstrated that TERA2.sp12 cells are not limited to ectodermal derivatives *in vitro* and are similar in their response to BMP treatment to human ES cells. In contrast, Noggin treated cultures formed densely packed cells which slowly down-

regulated the expression of stem cell surface markers TRA 160 and SSEA3, and after 14 days exhibited marked up-regulation of the neural progenitor protein Nestin. In addition, immunostained cultures contained many aggregate-like structures that were positive for Nestin and β III tubulin.

In preliminary experiments which aimed to monitor BMP pathway activity in these treatments, both SU5402 and BMP treatment were detected to up-regulate the expression of Id2, a transcription factor that is transcriptionally up-regulated by BMP pathway activity. This provides supporting evidence that both treatments drive the differentiation of TERA2.sp12 cells into similar fates by up-regulating BMP pathway activity. One mechanism by which inhibition of FGF signalling could up-regulate BMP pathway activity is through inhibiting MAPK mediated phosphorylation and inhibition of Smad1.

SECTION A

4.3.2 BMP and FGF mediated signalling in RA treated cultures

As RA induced neural differentiation was indeed enhanced in TERA2.sp12 cells cultured under suspension conditions, I postulated that changes in the activity of signalling pathways involved in neural induction could be occurring in differentiating TERA2.sp12 cells cultured as aggregates in suspension compared to adherent cultures. As previously discussed, both FGF and BMP signalling are both reported to play key roles in regulating neural induction in vertebrates, and previous studies have demonstrated their sensitivity to changes in cell-cell contact.

Monitoring BMP signalling

Initial attempts to monitor BMP signalling in TERA2.sp12 cells were made by detecting levels of phosphorylated Receptor Smad1 (p-Smad1) and total levels of Smad 1 using phosphorylation specific and non-specific antibodies to this protein. Unfortunately due to difficulties with the antibody no data was collected on levels of Smad 1 protein expression, although PCR detection showed stable expression of Smad 1 transcripts in all treatments. RA treatment of TERA2.sp12 cells up-regulated the level of p-Smad 1 protein in all cultures, and this corresponds with studies by Caracisole *et al.* (2000) in which they reported a transcriptional up-regulation in BMP production upon RA treatment in NT2D1 cells. Levels of detected p-Smad 1 varied over the measured time-points during repeated Western experiments. This variation may be due to experimental errors or reflect marked fluctuations in p-Smad levels over time. Previous studies have demonstrated that receptor smads are susceptible to rapidly regulated negative feedback loops (such as inhibitory smad activation, see review Massague 2005), so the level of p-Smad detected at these time-points may reflect

very transient variations and not the overall level of BMP pathway activity.

As markers that would more stably reflect BMP pathway activity, I assessed the expression of *Msx1*, *Id2* and *Id3*; factors reported to be directly transcriptionally activated by BMP signalling activity (Hollnagel 1999, Suzuki 1997b) using PCR and Western blot detection. The expression of these genes as detected by PCR analysis was manifestly different in RA treated cultures grown under adherent or suspension conditions: *Msx1* and *Id2* transcripts detected by PCR were both present in undifferentiated cultures, and were markedly down-regulated after 7 days of treatment in TERA2.sp12 cells cultured with RA under suspension conditions compared to adherent cultures. Corresponding Western blot detection of *Id2* protein also demonstrated a marked down-regulation in expression during RA treatment in suspension cultures compared to adherent cultures. The down-regulation of transcripts reported to be directly regulated by BMP signalling does imply that BMP pathway activity is down-regulated in RA treated suspension cultures compared to adherent cultures.

Msx1 transcript expression in undifferentiated TERA2.sp12 cells suggests that this transcription factor is not solely associated with non-neural ectoderm differentiation in this culture system, or that a low level of epidermal differentiation may be occurring in maintenance cultures. However, the protein levels of *Msx1* were not assayed, so *msx1* transcript expression may not reflect active protein levels. *Msx1* expression has also been reported to inhibit epithelial progenitor differentiation through maintenance of the cell cycle regulators such as cyclin D (Hu 2001), and from this one could speculate that *Msx1* may also play an (as yet un-reported) role in cell cycle maintenance in other cycling populations such as stem cells.

Manipulating BMP signalling

To investigate whether the manipulation of BMP signalling could regulate RA induced neural differentiation of TERA2.sp12 cells cultured as suspension or adherent cultures, cells were cultured under these conditions and treated for 14 days with RA alone or with the addition of either BMP2 or the BMP signalling inhibitor Noggin, and the expression of neural and non-neural ectoderm proteins detected by Western blot analysis.

Combined Noggin and RA treatment up-regulated the expression of neuronal β III tubulin and down-regulated Keratin 8 in adherent cultures to levels comparable with suspension cultures, demonstrating that inhibition of BMP signalling in adherent cultures could generate an expression phenotype similar to that observed in suspension cultures. In contrast, no difference was detected in neuronal β III tubulin or Keratin 8 expression between treatments with RA alone or RA and Noggin combined in suspension cultures, indicating that the exogenous inhibition of BMP signalling in suspension cultures had no effect. This corresponds with similar observations in a study by Finley et. al. where Noggin was shown to

have no effect on the neural differentiation of aggregates of mouse ES cells when added alone or in combination with RA or its precursor retinyl acetate (Finley 1999). These results are consistent with the hypothesis that BMP pathway activity is down-regulated in suspension cultures as opposed to adherent cultures, such that exogenous inhibition of BMP signalling in adherent cultures mimics the suspension culture phenotype, and has no detectable effects on suspension cultures.

Exogenous BMP treatment markedly inhibited RA induced β III tubulin up-regulation in cultures grown under either suspension or adherent conditions, although this effect was most marked in adherent cultures. Similar observations were made in previous studies by Hoodless and Hemmati-Brivanlou (1997) in both adherent and suspension cultures of mouse embryonal carcinoma P19 cells, where BMP4 treatment was shown to interfere with RA mediated neural induction and promote the expression of epithelial keratins. Finley *et al.* (1999) also demonstrated that BMP4 exposure of aggregate cultures of mouse embryonic stem cells treated with RA or its precursor retinyl acetate markedly inhibited neural and glial differentiation.

As the most marked effects of BMP inhibition on neural and non-neural ectoderm protein expression were observed in adherent as opposed to suspension cultures of TERA2.sp12 cells, this implies that either the up-regulation of neural differentiation in suspension cultures was not entirely due to attenuated BMP signalling or that BMP pathway activity was still being attenuated in suspension cultures exogenously treated with BMP2. As previously discussed, FGF signalling has been extensively reported to play a key role in neural induction, and recent studies have identified a mechanism by which FGF signalling can directly inhibit BMP pathway activity (Pera 2003, Sater 2003). This occurs through MAPK mediated phosphorylation of receptor smad 1, which prevents smad 1 binding to smad 4 and subsequent localisation to the nucleus, regardless of previous BMP receptor mediated phosphorylation events. The importance of this phosphorylation mediated inhibition of BMP signalling was recently demonstrated by Kuroda *et al.* (2005) in a fresh evaluation of the classic *Xenopus* dissociation experiments carried out by Hemmati-Brivanlou and Wilson (1995). In summary, they demonstrated that dissociated *Xenopus* explants generated neural ectoderm whereas intact explants generated epidermis, and this effect could be rescued by exogenous BMP treatment. In support of the default neural induction theory, neuralisation was thought to occur in dissociated explants as a result of reduced BMP signalling from a dilution effect of endogenous BMPs in the media. However, Kuroda *et al.* showed that BMP signalling activity and Smad 1 phosphorylation remained high in dissociated tissues. They also observed that *Xenopus* explant dissociation induced an up-regulation of FGF mediated signalling, which resulted in inhibitory phosphorylation of Smad 1 via MAPK activity, and thus inhibition of

BMP mediated transcriptional regulation.

Dissociation-induced up-regulation of FGF signalling and MAPK mediated inhibition of BMP pathway activity provides a potential mechanism by which BMP pathway activity could be inhibited and neural differentiation promoted in suspension cultures as opposed to adherent cultures of RA treated TERA2.sp12 cells. To investigate whether an initial up-regulation of FGF mediated signalling was necessary for the increased neural differentiation observed in RA treated suspension cultures, the FGF receptor inhibitor SU5402 was used to temporarily down-regulate FGF signalling during the first 24 hours of culture (prior to treatment with RA) and the expression of neuronal proteins and epithelial keratins monitored by Western blot analysis.

Treatment with FGF inhibitor SU5402

Western blot analyses demonstrated that in all treatments, early exposure to SU5402 increased levels of Keratin 8 and 18 proteins detected after 14 days, suggesting that temporary inhibition of FGF signalling was promoting non-neural ectoderm lineage acquisition. However the effect of SU5402 exposure on neuronal β III tubulin expression differed in cells cultured under suspension or adherent conditions. Under suspension conditions, the RA induced up-regulation of neuronal β III tubulin expression was inhibited in SU5402 treated cells compared to untreated cells, and a dramatic reduction in neuronal β III tubulin expression upon SU5402 exposure was observed in cells cultured in suspension without RA, such that they expressed lower levels than EC cells. However, under adherent conditions, prior SU5402 exposure did not markedly affect the expression of β III tubulin expression after 14 days, and actually increased the expression of β III tubulin in adherent cultures treated without RA.

In the case of suspension cultures, the sensitivity of aggregate cultures to SU5402 mediated inhibition of FGF signalling during the first 24 hours in terms of neuronal β III tubulin and Keratin 8 expression is supporting evidence that early FGF signalling plays a key role in the up-regulation of neural differentiation markers. However, in adherent cultures, one would expect some down-regulation or little change in the expression of neuronal β III tubulin as a result of early inhibition of FGF mediated signalling; whereas in adherent cultures treated with SU5402 alone, β III tubulin expression was actually up-regulated to some extent. However, subsequent staining analyses demonstrated atypical, non-neuronal localisation of β III tubulin in TERA2.sp12 cells pulsed with SU5402, indicating that the up-regulation of neuronal β III tubulin detected in Western blot analyses did not reflect an up-regulation in neural differentiation. More extensive markers of neural differentiation besides β III tubulin and NSE are needed to clarify the effect of SU5402 pulsed exposure on neural differentiation in these culture systems.

Detection of FGF mediated signalling

As ERK phosphorylation has previously been utilised to measure FGF receptor mediated MAPK pathway activity (Christen & Slack 1999, Gotoh & Nishida 1996), antibodies to ERK1/2 and phosphorylated ERK 1/2 were used to monitor MAPK pathway in TERA2.sp12 cultures. In summary, levels of ERK1/2 and phosphorylated ERK1/2 proteins were up-regulated upon RA treatment to similar levels after 14 days in both adherent and suspension cultures in Western blot analyses. However in suspension cultures expression of P-ERK1 and P-ERK2 proteins was barely detectable levels after 4 days of culture, in contrast to in RA treated adherent cultures and EC cultures. Taken at face value this implies that although RA induced an up-regulation of FGF mediated MAPK pathway activity, this was temporarily disrupted in the initial phase of suspension cultures.

However, one key flaw to these experiments is the time-points at which MAPK signalling was monitored. SU5402 treatment indicated that FGF mediated signalling was necessary for up-regulated neuronal differentiation in the initial 24 hours of culture. Thus the level of FGF signalling through detection of phosphorylated ERK should also have been monitored in the initial 24-48 hours of culture. The detected down-regulation of phosphorylated ERK in 4 day aggregates could well be a subsequent negative feed back effect of high levels of ERK phosphorylation. To determine whether up-regulated FGF mediated signalling is causing the up-regulation of neural differentiation in RA treated suspension cultures, several further investigations are necessary: If FGF mediated phosphorylation of ERK1/2 is detected to be up-regulated in the initial 24-48 hours of culture, one would also need to determine whether the up-regulation in FGF mediated signalling correlated with an inhibition of BMP signalling via inhibitory phosphorylation of Smad 1. Western blot analyses could be used to compare the inhibitory phosphorylation status of Smad 1 under suspension and adherent conditions and in cultures treated with SU5402 or FGF. Additionally, one could assess whether inhibition of MAPK activity is able to prevent the up-regulation of neural differentiation in RA treated suspension cultures. However, due to time restrictions these experiments were not carried out in the course of this study.

Conclusions

In summary, BMP and FGF manipulation analyses provide some preliminary evidence supporting the hypothesis that FGF signalling mediated inhibition of BMP pathway activity is occurring in suspension cultures of TERA2.sp12, accounting for the increased neural differentiation observed. This draws parallels to the signalling events and behaviour of *Xenopus* animal cap explants cultured as intact or dissociated tissue. However, no conclusive evidence was garnered from these investigations, and further experiments are necessary to compare the level of FGF signalling in adherent versus suspension cultures of TERA2.sp12,

and to determine whether up-regulated FGF signalling is causing MAPK mediated attenuation of BMP pathway activity and consequentially an up-regulation in neural differentiation in suspension cultures.

These preliminary experiments have demonstrated the importance of FGF and BMP signalling in the differentiation of TERA2.sp12 cells, with active manipulations of these pathways markedly modulating the response of these cells to RA. Although these experiments have been used in part to model the effect of BMPs and FGF on neural induction, the use of RA as a differentiation inducer is a complicating factor and does not model early events in the developing ectoderm. RA does play key roles in the dorsal/ventral patterning of the neural tube and later in the differentiation and specification of motor neuron subtypes, but is absent in the early stages of neural induction (for review see Wilson & Maden 2005). Therefore to more closely model the early signalling events during ectoderm development, in the remaining studies BMP and FGF signalling was manipulated in TERA2.sp12 cells in the absence of RA.

SECTION B

4.3.3 Manipulation of BMP signalling

To investigate the effect of manipulating BMP signalling on TERA2.sp12 cells, cultures were treated with either recombinant BMP2, BMP4 or the BMP inhibitor Noggin.

BMP treated cells – changes in morphology and down-regulation of stem cell markers

BMP treated cells rapidly exhibited shrunken morphologies, and the increased cell debris observed indicates some cell death was occurring. However the formation of large sheets of plate-like cells between 5-7 days of treatment was associated with a stabilisation in cell number and a reduction in cell debris. This suggests that in the first stage cell proliferation and some cell death was occurring as cells responded to BMP signalling by either beginning to differentiate or embark on cell death, whereas in the later stage the observed stabilisation in cell number was predominantly due to differentiation and exit from the cell cycle as opposed to increased cell death.

No increase in propidium iodide intake was detected in BMP treated cells prepared for flow cytometry between 7 and 14 days. However, media changes and initial PBS washes prior to individualisation with Trypsin and may have removed any apoptotic cells as they detached from the culture surface. Recently Gambaro *et al.* (2005) demonstrated that the BMP4 induced epidermal differentiation of mouse ES cells concomitantly induced apoptotic cell death in Sox1 positive neural precursors, suggesting this functioned as an additional mechanism by which BMPs promote epidermal as opposed to neural differentiation. To investigate whether

BMP treatment is inducing apoptotic cell death in cells committing to a neural fate in TERA2.sp12 cells, the expression of apoptotic marker C-JUN in BMP treated TERA2.sp12 cells, in conjunction with early markers of neural differentiation (Sox1, Neuro D1) as opposed to non-neural ectoderm differentiation could be assayed by immuno-staining. However this was not undertaken in the course of this investigation. If apoptosis was induced in some TERA2.sp12 cells as a result of BMP treatment, this would also imply that a sub-population of cells was already committed to early stages of neural differentiation in these cultures, or that seeding at a low density had triggered the differentiation of a proportion of cells towards early stages of neural differentiation.

The down-regulation of stem cell marker expression in BMP treated TERA2.sp12 cells is concordant with the observations of Andrews *et al.* in 1994 that BMP 7 treatment of NTERA2 cells induced the down-regulation of SSEA3 and SSEA4, a reduction in proliferation and the formation of cells that were morphologically distinct from either EC or RA treated cultures (Andrews *et al.* 1994). Caricasole *et al.* also described a similar pattern of stem cell marker down-regulation and the formation of sheets of large cells in embryonal carcinoma NT2D1 cells treated with either BMP2, BMP4 or BMP7 (Caricasole 2000). Specifically, they detected a down-regulation of stem cell markers TRA 160, SSEA3, SSEA4 and the expression of proliferative antigen PCNA, and the formation of epithelial-like sheets of cells in cultures treated with 25ng/ml of BMP 2, 4 or 7.

It is also interesting to note that TERA2.sp12 cells treated with RA down-regulated surface stem cell markers SSEA3 and TRA 160 more rapidly than BMP treated cells, yet demonstrated markedly high levels of proliferation even after 14 days. So although RA treated cultures more rapidly depart from the EC stem cell phenotype, the proliferation assay suggests that they subsequently remain in an earlier stage of differentiation than BMP treated cultures, as the data implies that BMP treated cells exit the cell cycle and embark on more advanced stages of differentiation, albeit in a different direction. This corresponds with the observation that RA treatment induces rapid differentiation into neuro-progenitors; in immuno-staining analyses of cultures treated for 14 days with RA treatment the majority of the cells expressed markers of proliferating neural progenitors such as Nestin and Musashi.

Noggin treated cultures – stem cell marker down regulation and reduced proliferation

Noggin treatment also appeared to reduce cell proliferation compared to untreated cells, as little evidence of increased cell death was observed. In addition, treated cells slowly down-regulated surface stem cell surface antigens TRA 160 and SSEA3 and stem cell markers Oct4 or Nanog such that after 14 days of treatment the majority of cells were negative for SSEA3, Oct4 and Nanog and a substantial population had down-regulated expression of TRA 160.

Combined with the Western blot detection and immuno-staining detection of increased Nestin expression, this indicates that many Noggin treated cells were differentiating into early neural progenitors. Pera *et al.* (2004) demonstrated that treatment of human HES-2 and HES-3 embryonic stem cells with Noggin (at 100-500ng/ml) transcriptionally down-regulated Oct4 expression and up-regulated Nestin and Pax6 expression. After subsequent culture under neural stem cell maintenance conditions these cells demonstrated increased capability of forming Nestin immuno-positive neurospheres compared to those initially cultured without Noggin treatment.

It is possible that Noggin treatment of TERA2.sp12 cells did not sufficiently inhibit BMP signalling for neural differentiation to occur throughout the culture. To determine whether a greater attenuation of BMP signalling is necessary for neural induction in these cells, one could treat TERA2.sp12 cultures with a higher concentration of Noggin or in combination with other BMP inhibitors, (such as Chordin, Follistatin or with a recombinant BMP receptor). It would also be pertinent to determine whether FGF and Noggin treatment combined can induce neural differentiation, such that all cells within the culture express a neural progenitor phenotype. This would support the hypothesis that both FGF signalling and inhibition of BMP signalling are necessary for neural induction in the human ectoderm.

Non-neural ectoderm differentiation in BMP2 or BMP 4 treated cultures?

As previously discussed, BMP signalling has been demonstrated to promote epidermal differentiation of the naive ectoderm in the *Xenopus* embryo (Suzuki 1997a, Suzuki 1997b). Western blot and immuno-staining analyses demonstrated an up-regulation of simple epithelial keratins such as keratins 8 and 18, and a corresponding inhibition of neural differentiation in BMP treated cultures. This concurs with the characterisation of BMP2 or 4 induced simple epithelial differentiation marked by the up-regulation of epithelial keratins and repression of pro-neural differentiation markers in the human embryonal carcinoma NT2D1 cell line by Houldsworth *et al.* (2001), Caricasole *et al.* (2000) and more recently Chadalavada *et al.* (2005). Coraux *et al.* (2003) have also successfully derived keratinocyte progenitors from murine embryonic stem cells by seeding on matrix derived from human normal fibroblasts and treatment with BMP4.

However, in BMP2 treated TERA2.sp12 cells no expression of the epidermal stem cell marker TP63 was detected, in contrast to positive staining in RA treated 'large-celled plaques'. In addition, immuno-staining analyses revealed abnormal neuronal β III tubulin expression in some of these large cells, in some cases co-localising with epithelial keratin expression. As the expression of keratins 8 and 18 is not restricted to that of simple epithelia, (as previously discussed), the expression analyses left open the possibility of other types of differentiation.

4.3.4 Similar effects of BMP or FGF inhibitor treatment

In initial studies TERA2.sp12 cells treated with the FGF receptor 1 inhibitor SU5402 displayed a similar morphology to BMP4 or BMP2 treated cells and this was confirmed by immuno-staining analyses. Comparisons of differentiation by Western blot analysis and immuno-detection revealed marked similarities in marker expression between these cultures, with SU5402 treatment bearing the most potent effect on the up-regulation of markers of non-neural ectoderm and inhibition of neural differentiation. In addition, large neuronal β III tubulin positive cells similar to those identified in BMP treated cultures were observed, and markers of further epidermal differentiation such as TP63 were not detected.

The detection of the mesoderm transcription factor Brachyury and endoderm transcription factor Gata4 in TERA2.sp12 cells cultured under suspension conditions to form aggregates in previous experiments indicates that unlike previously thought, TERA2.sp12 cells retain the ability to differentiate into non-ectodermal derivatives. As previously discussed, BMP signalling plays multiple roles in development, and in developing vertebrates in addition to promoting the formation of the prospective epidermis, BMP signalling also plays an important role in specifying dorsal mesoderm. This corresponds with data from a pilot assay for the effects of 8 different growth factors on the differentiation of embryoid bodies cultured from the human embryonic stem cell line H9 clone 1 by Schuldiner *et al.* (2000), in which BMP 4 treatment was detected to be permissive for both ectoderm and mesoderm differentiation.

However, two key studies indicate that direct treatment of human embryonic stem cells with BMPs appears to induce differentiation into extra-embryonic lineages, with Xu *et al.* (2002a) reporting trophoblast differentiation, and Pera *et al.* (2004) reporting the formation of extra-embryonic endoderm in ES cells treated with BMP2 or BMP4. As a consequence, BMP2, BMP4 or SU5402 treated cultures were screened with markers for lineages from all germ layers, particularly focussing on markers of mesoderm, extra-embryonic and definitive endoderm and trophoblast differentiation.

In summary, after 14 days of treatment the BMP responsive endoderm transcription factor Gata4 was highly expressed in all cultures, as well as α smooth muscle actin. However, the early mesoderm transcription factor Brachyury was not detected in cultures after 14 days of treatment. Markers of trophoblast differentiation were detected in all cultures, whereas no evidence of extra-embryonic endoderm differentiation such as cyst formation or AFP production was observed.

Trophoblast differentiation

The detection of a sub-population of cells displaying trophoblast characteristics; forming

multi-nucleate cells, exhibiting nuclear localised Keratin 8 antigen and producing HCG, draws several parallels to work by Xu *et al.* (2002b) on BMP4 treated human embryonic stem cells. They described a synchronous wave of differentiation characterised by flattened enlarged cells and reduced proliferation, with changes obvious on day 2 for cell colonies treated with BMP4 at 100ng/ml, days 3-4 for 10ng/ml and days 4-5 for 1ng/ml. BMP2, BMP7 and GDF5 induced similar changes. This corresponds somewhat to the observations made in BMP or SU5402 treated cultures of TERA2.sp12 cells, however the proportion of cells expressing HCG after 14 days of treatment was markedly lower than described for ES cells, as in the study by Xu *et al.* flow cytometry data demonstrate a remarkably uniform shift upon 100ng/ml of BMP4 treatment, such that the majority of the population were detected to express the HCG hormone after 7 days. It would be interesting to investigate whether treatment of TERA2.sp12 cells with higher concentrations of BMP4 (such as 100ng/ml) would up-regulate trophoblast differentiation and HCG production in TERA2.sp12 cells. However a difference in the proportion of cells differentiating along a trophoblast lineage as opposed to other lineages in cells treated with lower concentrations of BMP4 was not reported, instead Xu *et al.* described a slower rate of differentiation. In their study ES colonies treated with BMP4 formed mononuclear cells, however when individualised cells were plated at low density and treated with BMP4, syncytial cells containing multiple nuclei formed after 2 weeks (44 in 622 cells). Cells bearing similarities to syncytial cells were also observed in TERA2.sp12 cultures, consistent with the fact that all cultures were seeded from individual cells. One possible method of extending this investigation would be to seed TERA2.sp12 cells at a range of lower densities prior to BMP treatment, to determine whether the proportion of HCG positive cells or syncytial cells formed is directly correlated to the original seeding density, and subsequently compare the activity of key signalling pathways in these cells seeded at different densities, to identify candidate promoters of syncytial cell formation.

Characterising the remainder of the population generated by BMP or SU5402 treatment

The widespread expression of the endoderm transcription factor Gata4 corresponds with reports that Gata 4 is up-regulated by BMP signalling (Rojas *et al.* 2005). Gata4 is primarily expressed in developing endoderm, although it is also expressed in mesoderm derived myocardial cells (Arceci *et al.* 1993, Heikinheimo *et al.* 1994, Laverriere *et al.* 1994). Together with the absence of positive Brachyury expression, this data suggests that early endoderm differentiation may be occurring in these cultures, although this cannot be fully asserted until further staining analyses are carried out with more specific markers of endoderm and also other markers of mesoderm differentiation.

Taken at face value, the presence of smooth muscle actin positive cells in treated cultures could represent a mesoderm derivative. However, it is known that during

development BMPs play a role in the formation of the neural crest, which subsequently has the potential to differentiate into multiple cell types, including smooth muscle (Luo *et al.* 2003, Mizuseki *et al.* 2003). These results correspond somewhat with observations of smooth muscle differentiation in BMP2 treated NT2D1 cells (Chadalavada 2005). As well as markers of differentiation for prospective epidermis, Chadalavada *et al.* detected the expression of smooth muscle actin by immuno-staining and a panel of markers associated with smooth muscle differentiation by micro-array analysis. No significant Brachyury expression was detected, or other key markers of mesoderm differentiation. Since smooth muscle differentiation was detected to correlate with an earlier up-regulation of markers of neural crest, they proposed that the observed smooth muscle differentiation neural crest derived. To support their hypothesis they detected an early induction of SNAI2 that has been associated both with the epithelial to mesenchymal transition and in the diversification of neural crest lineage from naïve ectodermal cells through BMP signalling (Hemavathy *et al.* 2000). It is interesting to note that no evidence of trophoblast differentiation was observed in their study.

The consistent non-neural localisation of neuronal β III tubulin in these cultures and detection of some co-localisation with epithelial keratins could (as previously discussed), be a result of unusual β III tubulin expression accrued under prolonged cell culture. However, non-neural populations documented to express neuronal β III tubulin include mesenchymal stem cells (Tondreau *et al.* 2004), which also express Vimentin and α smooth muscle actin. Thus differentiation along the mesenchymal stem cell lineage is also a possible explanation for the expression of this panel of markers in TERA2.sp12 cells. However, the liver (endoderm) derived HepG2 cell line also stained immuno-positive for neuronal β III tubulin, (as well as Gata 4) which together suggests that early endoderm derivatives could also express β III tubulin.

To summarise, in the light of other reports and previous observations, the current evidence suggests both endoderm differentiation and possibly neural crest derived smooth muscle differentiation is occurring in BMP or SU5402 treated cells. However, in order to more definitively characterise the differentiation of BMP or SU5402 treated cells, more extensive staining analyses with further markers of endoderm (both primitive and definitive), mesoderm and neural crest differentiation are necessary.

BMP mediated induction of differentiation - trophoblast or extra-embryonic ectoderm?

It is interesting to note that BMP2 or BMP4 treatment of TERA2.sp12 cells was detected to induce trophoblast differentiation and the expression of markers or characteristic morphologies specific to extra-embryonic endoderm differentiation were not observed. This corresponds with the report of trophoblast differentiation in BMP2 or BMP4 treated human

ES cells by Xu *et al.* (2002), in contrast to the report by Pera *et al.* (2004) of BMP2 or BMP4 induced extra-embryonic endoderm differentiation (with minimal trophoblast differentiation) in HES cells. However, there are several key differences in the materials and methods used between these two studies, which are outlined and compared with this study in Table 4.7.

Table 4.7 Comparison of the materials and methods used in studies by Xu *et al.* and Pera *et al.*

with this study, in which BMP 2 or 4 treatment induces either trophoectoderm or extra embryonic endoderm. (Italicised markers are also expressed in other germ layers)

Studies:	Xu <i>et al.</i> 2002	Pera <i>et al.</i> 2004	This study
Cells used	HES cell lines H1, H7, H9 and H14	HES cell lines H2, H3 and human EC cell line GCT27X-1.	TERA2.sp12 cells
Culture conditions	Serum free mouse embryonic fibroblast conditioned media supplemented with 4ng/ml bFGF.	Media with 20% serum, grown on mouse embryonic fibroblasts	DMEM media with 10% serum.
Recombinant BMP proteins tested	BMP4 (1-300ng/ml) BMP2, (300ng/ml), BMP7 (300ng/ml), GDF5 (30ng/ml)	BMP2 (25ng/ml) BMP4 (25ng/ml)	BMP2 (25ng/ml) BMP4 (25ng/ml)
Morphology of cells	Flattened, enlarged cells formed within a few days, cells seeded individually at low density formed large multi-nucleate cells	Formed flat, squamous epithelial cells with abundant eosinophilic intracellular material. Cells sometimes formed fluid filled cysts, occasionally observed foci resembling trophoblast precursors (less than 5%)	Flattened, enlarged cells formed after 7-14 days, similar in appearance to BMP4 treated ES cells in the study by Xu <i>et al.</i> In approx 10-15% of the population cells with multiple densely packed nuclei were observed.
Detection of Epithelial markers		Stained positive to combined <i>Keratin 8, 18</i> and 19 antibody and to Laminin	Large cells stained positive to Pan-epithelial keratin, <i>Keratin8/18</i>
Detection of trophoblast differentiation	HCG immuno-positive, also progesterone, estradiol and HCG production increased in media. Microarray and PCR detection of up-regulated expression of placenta associated genes	Negative for HCG	Densely packed cells stained positive for HCG and nuclear Keratin 8 antigen, also evidence of multiple nucleated cells.
Detection of primitive endoderm differentiation	No up-regulation of endoderm markers compared to controls detected by microarray analysis	Cyst formation and AFP production (especially at edges of cysts) Expression of HNF3 α , HNF4, <i>Gata4</i> and <i>Gata6</i>	No HNF3 β detected. No AFP detected. No cyst formation observed. <i>Gata 4</i> positive.
Differentiation characterised as..	Majority of cells are trophoblast, no evidence of other lineages.	Extra-embryonic endoderm	Endoderm, smooth muscle, approximately 10-15% form trophoblast cells

One possible explanation for the alternate differentiation pathways induced by BMP treatment in the studies by Pera *et al.* and Xu *et al.* is based on the different ES cell lines used. Individual variation between ES cell lines could be the cause of their differential response to BMP treatment. In support of this theory, Xu *et al.* reported that as detected by micro-array analysis, many trophoblast associated markers were expressed at high levels in the undifferentiated ES cell lines used. However an alternative explanation could be based on the different culture conditions used between these studies. In both studies the HES cells are maintained either in media conditioned from mouse fibroblasts or co-cultured with mouse fibroblasts. However Xu *et al.* used serum free media supplemented with bFGF whereas Pera *et al.* used media supplemented with high levels of serum. One possibility is that a factor in

the serum could be promoting the differentiation of HES cells into primitive endoderm as opposed to trophoblast differentiation in response to BMP treatment. In support of this observation one would expect some evidence of primitive endoderm differentiation in this study, since TERA2.sp12 culture media is supplemented with 10% serum. However, observations of primitive endoderm differentiation reported by Pera *et al.*, such as cyst formation or AFP production, were not detected in treated TERA2.sp12 cells. This may be because TERA2.sp12 cells require treatment for longer time periods or with higher concentrations of serum for cyst formation or AFP production to be detectable. It would be interesting to investigate whether factors in the serum regulate the response of TERA2.sp12 cells to BMP induced differentiation (into either trophoblast or primitive endoderm lineages) by comparing the differentiation of cultures treated with BMPs in media supplemented with a range of serum concentrations.

Niwa *et al.* demonstrated that differentiating mouse ES cells can undergo either trophoblast or primitive endoderm differentiation depending on whether Oct4 levels rise or fall (Niwa *et al.* 2000). Since rapidly lowered Oct4 is linked to trophoblast differentiation, the serum free conditions used by Xu *et al.* could theoretically have destabilised Oct4 levels such that BMP treatment rapidly down-regulated Oct4 expression and consequentially induced trophoblast differentiation under their culture conditions. To investigate this further one could monitor the initial expression level and rate of down-regulation of Oct4 in TERA2.sp12 cells treated with BMPs under high and low serum conditions and determine whether this correlates with differing levels of endoderm or trophoblast differentiation.

4.3.5 Converging FGF and BMP mediated signalling pathways

The similar differentiation response of TERA2.sp12 cells to either an up-regulation in BMP signalling or inhibition of FGF signalling indicates that both treatments act on the same mechanism to induce differentiation. As discussed previously, FGF signalling can directly inhibit BMP pathway activity through MAPK mediated inhibitory phosphorylation of Smad 1, preventing translocation of Smad1 to the nucleus (Pera 2003). Thus I hypothesised that in TERA2.sp12 cells cultured under maintenance conditions, either autocrine FGF signalling or FGFs present in the serum were playing important roles in attenuating BMP signalling to prevent BMP pathway mediated differentiation. According to this hypothesis, treatment of cultures with recombinant BMPs or with the FGF receptor inhibitor SU5402 was overcoming FGF mediated attenuation of BMP pathway activity, and the resultant up-regulation in activity driving TERA2.sp12 differentiation. To account for the effect of Noggin on TERA2.sp12 cultures one could also hypothesise that very low levels or a complete abrogation of BMP pathway activity induces neural differentiation, in accordance with the default theory of neural induction. A schematic model of this hypothesis is presented in Figure 4.25. This theory

Proposed Mode Of Action For BMP And FGF Signalling On The Regulation Of BMP Pathway Activity And TERA2.Sp12 Differentiation

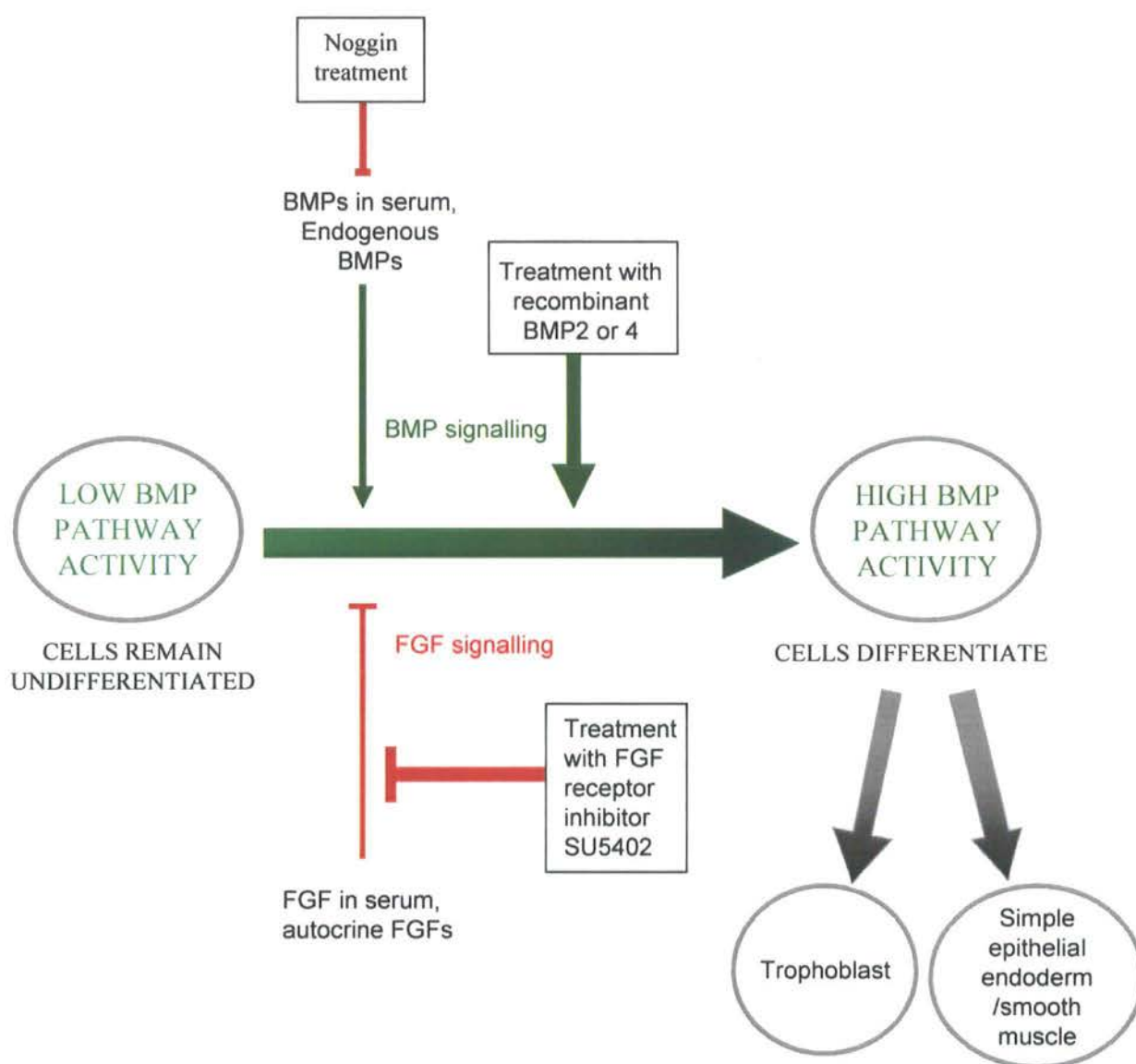


Figure 4.25 Model for the action of BMP and FGF signalling on the differentiation of TERA2.sp12 cells.

Bone morphogenetic protein (BMP) signalling proteins such as BMP2 and BMP4 activate the BMP pathway, whereas fibroblast growth factor (FGF) mediated MAPK activity inhibits BMP pathway activity in TERA2.sp12 cells. In the presence of a balance of FGFs and BMPs present in the serum, which induces low levels of BMP pathway activity in TERA2.sp12 cells, cultures remain undifferentiated. However, if cultures are treated with BMP2 or BMP4, which activate BMP signalling, or cultured in the presence of FGF receptor inhibitor SU5402, which inhibits FGF signalling, BMP pathway activity is up-regulated and cells differentiate into a heterogeneous population of trophoblast, simple endoderm and smooth muscle cells. Conversely, treatment of TERA2.sp12 cells with Noggin down-regulates BMP signalling to minimal levels, which encourages TERA2.sp12 differentiation towards a neural fate (neural differentiation not depicted in diagram).

corresponds with a recent report by Xu *et al.* (2005). In this report they demonstrate that human ES cells which are normally dependent on conditioned media from mouse fibroblasts for undifferentiated culture, can be maintained undifferentiated in unconditioned media supplemented with 100ng/ml Noggin and 40ng/ml bFGF or high concentrations of bFGF alone, and they attribute this effect to attenuated BMP signalling.

To investigate whether inhibition of FGF signalling by SU5402 treatment increased BMP pathway activity in a similar manner to exogenous BMP treatment (in support of my hypothesis) and also to investigate whether changes in BMP pathway activity in turn could regulate ERK1/2 phosphorylation, the levels of Smad 1 regulated Id2 and MAPK regulated phosphorylated ERK1/2 were assessed by Western blot analysis in protein extracted from 14 day cultures of TERA2.sp12 cells treated with RA, BMP2, BMP4 or SU5402. Id2 expression was up-regulated by BMP or SU5402 treatment. Taken together with the observed up-regulation of Gata4 in BMP and SU5402 treated culture in immuno-staining analyses, this indicates that SU5402 mediated suppression of FGF signalling was indeed generating an up-regulation of BMP pathway activity. Unexpectedly, levels of phosphorylated ERK 1 did not markedly change with any treatments, whereas levels of phosphorylated ERK2 were in general up-regulated from trace EC levels in all treatments, with BMP2 treatment inducing the highest up-regulation, and RA also inducing marked up-regulation. In addition, substantial P-ERK 2 phosphorylation was still observed in cultures continuously treated with the FGF receptor 1 inhibitor SU5402. SU5402 treatment has been used extensively to inhibit FGF mediated signalling in many culture systems, and also recently in embryonic stem cells (Dvorak *et al.* 2005, Vallier *et al.* 2005). However this raises an important point: As a downstream component of the MAPK pathway, ERK1/2 is phosphorylated not only by FGF mediated receptor activation but also as a result of signalling via a range of other growth factors, including EGF, NGF, BDNF, PDGF. Thus although inhibition of FGF mediated signalling through inhibition of the FGF receptor may attenuate MAPK pathway activity, MAPK activity can also be modulated by a host of other factors. Whether all of these factors can also mediate BMP signalling inhibition through ERK1/2 activation is yet to be determined. An alternative method of measuring FGF mediated signalling such as monitoring the level of FGF receptor activation could provide a means of monitoring specifically FGF mediated MAPK pathway activity. Otherwise a more simple means of investigating whether FGF mediated signalling inhibits BMP pathway activity in TERA2.sp12 cells would be determine whether BMP mediated differentiation is inhibited when cultures are treated with BMPs in the presence of high levels of FGFs (such as recombinant bFGF). If this is the case, to determine whether ERK1/2 is necessary for this inhibition, one could assay whether exposure to a specific ER1/2 phosphorylation inhibitor is able remove the inhibitory effect of FGF exposure on BMP induced differentiation.

4.3.6 Subsequent inhibition of BMP and FGF signalling

As Noggin treated cells expressed a phenotype similar to neural progenitors after 14 days of treatment, in a pilot experiment I assessed whether these cells had committed to a neural progenitor lineage and would now respond differently to an inhibition of FGF signalling through SU5402 treatment. Down-regulated FGF signalling (by bFGF withdrawal) is frequently used to differentiate neural progenitor cells in culture, and consequentially this was used as an assay to determine whether Noggin treated cells were committed towards a neural progenitor phenotype (differentiating into neural lineages) or whether they would respond similarly to previously untreated TERA2.sp12 cells. Cultures treated for 14 days with Noggin followed by 14 days with SU5402 generated a heterogeneous population of cells, with a small population exhibiting strikingly mature neuronal/glial morphologies, and the remaining population differentiating into similar fates observed in cells that had not previously been treated (expressing markers such as HCG and epithelial keratins). This indicates that most of the population had not committed towards a neural progenitor phenotype and retained the ability to differentiate into non-neural phenotypes. However the presence of a distinctive sub-population of cells that differentiated extensively along a neuronal/glial phenotype indicates that a small proportion of cells had committed towards a neural progenitor fate. Further investigations in which – prior to treatment with SU5402, cells are cultured for prolonged periods with Noggin, with higher concentrations of Noggin, or with Noggin in combination with FGF, would be of interest here. As a comparison it would also be valuable to investigate the effect of SU5402 treatment on TERA2.sp12 cells treated for 14 days with RA, as the majority of these cells demonstrate marked up-regulation of neural progenitor markers and should respond as neural progenitors.

4.3.7 Conclusions

The effect of BMP and FGF signalling on TERA2.sp12 cells was initially investigated with the aim of characterising their role in neural and non-neural ectoderm differentiation decisions as an *in vitro* model of human ectoderm. These investigations did demonstrate the marked inhibitory effect of increased BMP signalling or decreased FGF signalling on neural differentiation. However, during these studies TERA2.sp12 cells demonstrated their capacity to differentiate not only into ectoderm lineages but also into putative tropho-ectoderm, endoderm (and possibly mesoderm). Supporting evidence was also generated for a convergent effect of up-regulated BMP signalling or down-regulated FGF signalling on the pathway of differentiation, possibly via the regulation of BMP pathway activity through Smad 1 phosphorylation events.

Since the findings of this study show that TERA2.sp12 cells respond to BMP treatment

similarly to human ES cells, this suggests that TERA2.sp12 cells are not restricted in their differentiation potential as previously thought, and further characterisation is required to determine their full differentiation potential.

CHAPTER 5

CHARACTERISING TERA2.SP12 DIFFERENTIATION IN VIVO

5.1 INTRODUCTION

The initial premise of the previous chapters was that TERA2.sp12 cells were limited in their differentiation capacity to ectodermal lineages, thus providing a suitable *in vitro* model in which to investigate neural versus non-neural transitions in the developing human ectoderm. As discussed previously, this was based on both previous *in vitro* studies and histological observations that tumours derived from TERA2.sp12 cells transplanted into immune-deficient mice contained only ectodermal derivatives (Przyborski *et al.* 2004, Stewart *et al.* 2003).

The formation of tumours from transplanted stem cells

Teratomas have fascinated clinicians and researchers for centuries, mainly by their ability to generate extensively differentiated and morphologically identifiable complex tissue structures including bone, tooth, hair and gut that very closely resemble tissues found in normal development, but within a grossly disorganised context, for reviews (Andrews 2002, Andrews *et al.* 2001).

As previously discussed, embryonal carcinoma cell lines were first isolated from teratomas and characterised as the stem cell component by their ability to form new tumours upon transplantation into immuno-compromised mouse hosts (for review see Andrews 2002). Subsequent investigations to identify an embryonic counterpart in normal development which also exhibited teratoma forming capability led to the first isolation of mouse embryonic stem cells from the inner cell mass of developing blastocysts in 1981, and subsequently the derivation of human embryonic stem cells from developing human blastocysts in 1998 (Martin 1981, Thomson *et al.* 1998).

Although embryonic stem cell lines can differentiate into derivatives of all three germ layers *in vitro*, complex tissue formation, pattern formation or organogenesis does not occur to a significant degree. In contrast, the highly differentiated tumours formed upon transplantation of human embryonic stem cells into immune-deficient mice frequently exhibit complex structure formation. In this context, many of the normal features of tissue architecture are reproduced. For example, epithelia exhibit polarity, are enveloped by a basement membrane and are surrounded by mesenchyme, and composite tissue structures such as hair follicles, teeth, and gut are also formed (for review see Oderico 2001). Human embryonic stem cells injected into immune-deficient mice form benign teratomas with advanced differentiated tissue types representing all three embryonic germ layers, and transplantation into immune-deficient mice is now routinely used to demonstrate the full differentiation potential of newly derived embryonic stem cell lines, (Heins *et al.* 2004, Reubinoff *et al.* 2000, Stojkovic *et al.*

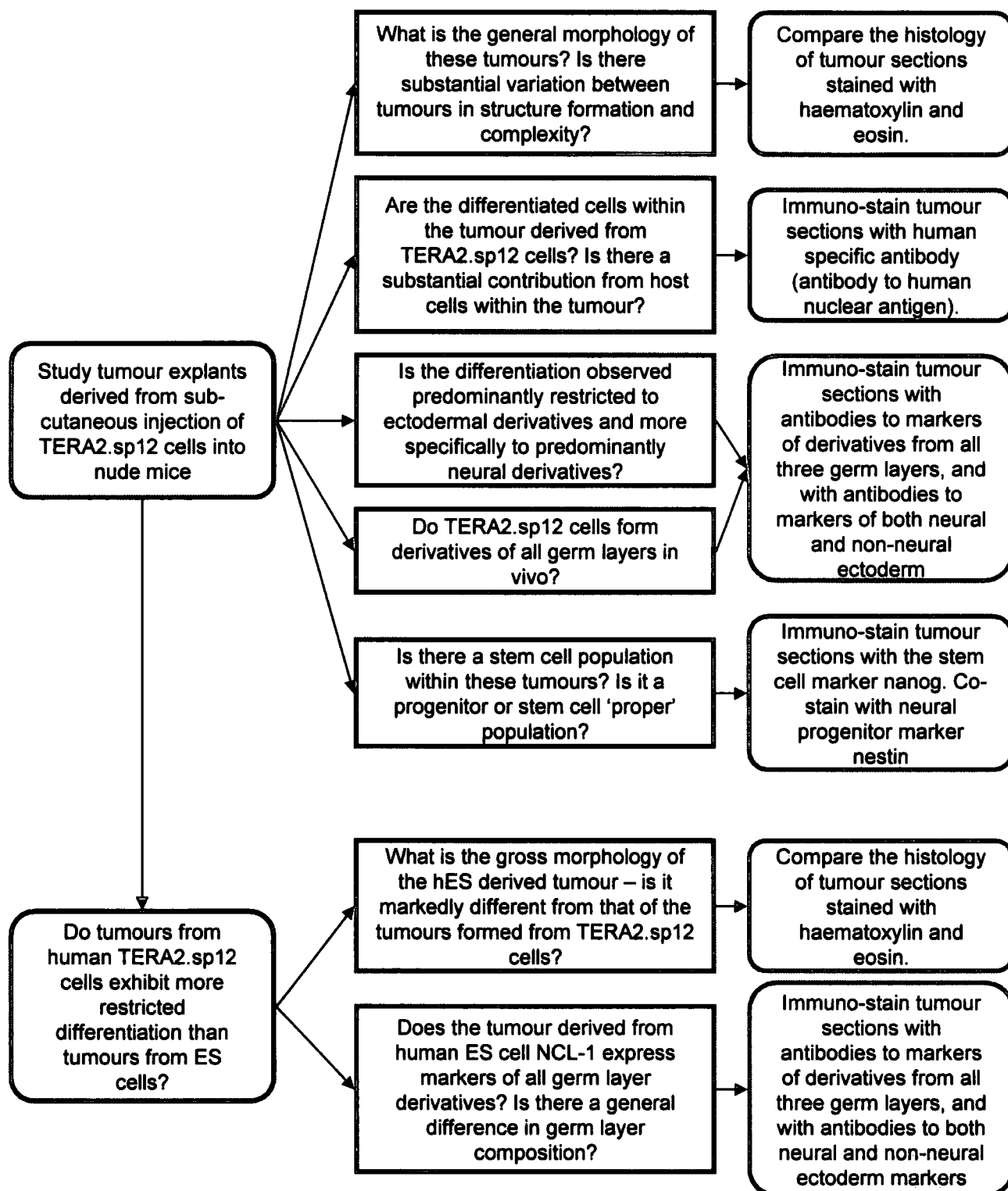
2004a, Thomson 1998).

Tumour formation from TERA2.sp12 cells – a means of fully testing their developmental potential

Although earlier studies of TERA2.sp12 differentiation had only revealed evidence of differentiation into ectodermal derivatives (Horrocks *et al.* 2003, Przyborski 2004, Stewart 2003), during investigations described in the previous two chapters I demonstrated that TERA2.sp12 cells were capable of differentiating into cells expressing markers of trophoectoderm, endoderm and mesoderm differentiation. This led to the questioning of earlier observations that TERA2.sp12 cells differentiated into solely ectodermal derivatives after transplantation into immune-deficient mice.

As a means to investigate the full developmental potential of TERA2.sp12 cells, and examine whether these cells exhibit exclusively or predominantly ectodermal differentiation *in vivo*, in the following chapter I characterise the differentiation of TERA2.sp12 cells within tumours formed from transplantation into immune-deficient mice using both histological and immuno-staining techniques, and compare this differentiation with a tumour derived from the human embryonic stem cell line NCL-1. An outline of the aims, objectives and experiments in this chapter is presented as a strategy flow chart in Figure 5.1.

Strategy Flow Chart For The Characterisation Of TERA2.sp12 Differentiation In Vivo



5.2 RESULTS

As described in Section 2.8, batches of prepared TERA2.sp12 cells were injected subcutaneously on the left flank into nude (*C57BL/6J-Hfh1/nu*) adult male mice. Tumours were isolated on the basis of identifying an obvious palpable growth that was not too large to affect the welfare of the animal (maximum dimensions up to 1.5cm in length). At this time the mice were sacrificed and tumours surgically removed, fixed in 4% PFA, processed and sectioned as described in Section 2.6.4. Although for each batch of injections 3-4 animals were used, I only had access to some of these tumours, the details of which are listed in Table 5.1.

Table 5.1 Details of tumours derived from sub-cutaneous TERA2.sp12 injection into immune-deficient mice.

Tumour	Date of injection	Age of tumour	PFA fixation	Embedded and processed in
1.	12.04	6-7 weeks	Post-dissection overnight 4°C	Wax
2.	14.12.05	Isolated 31.3.05 ~ 14 weeks	Post- dissection 40-60 mins	Wax
3.	14.12.05	Isolated 8.3.05 ~11 weeks	Post- dissection 40-60 mins	OCT
4.	1.6.06	Isolated 20.9.05 ~ 17 weeks	Perfusion fixed prior to dissection	OCT

Gross morphology – Histological staining

After 11-17 weeks readily palpable, solid tumours ranging from 40-50mm³ in diameter had formed at the site of injection. These were surrounded by a layer of fat and in some cases hair, and were heavily vascularised. Fixation, sectioning and histological staining of these tumours revealed large regions of loosely packed disorganised cells of mixed morphology interspersed with some distinctive organised formations, typically composed of an organised layer or layers of cells surrounding a cavity such that cross-sections appeared like 'islets' or tubular-like. Sections from tumours fixed post-extraction in PFA and embedded in OCT exhibited poorer morphology preservation than those from tumours embedded in wax or perfusion fixed prior to OCT embedding. However in all cases a similar range of phenotypes was observed in consecutive sections through the tumour.

Immuno-detection of markers of germ layer derivatives

To characterise the differentiation of cells within the TERA2.sp12 derived tumours, serial wax and cryostat sections of the tumours were immuno-stained with antibodies to a panel of markers detailed in Table 5.2. Since several of the available antibodies were raised in mouse hosts (see Table 5.2), in many cases non-specific staining occurred as a result of non-specific

interactions between the anti-mouse secondary antibody and host antigens. In order to minimise these non-specific interactions, sections to be immuno-stained with primary antibodies that had been raised in mouse hosts were first blocked for 1 hour with a monovalent Fab fragment of anti-mouse IgG, (goat anti-mouse Ig H+L from Jackson Labs), diluted to 1:50 in blocking buffer, prior to incubation with the primary antibody overnight and immuno-staining following the protocols outlined in Section 2.5.

Table 5.2 Details of antibodies used in immuno-labelling screen for markers of differentiation

ANTIGEN	ANTIBODY	SOURCE	DILUTION	Pre-treatment	CATEGORY
Nestin	Mouse IgG	Chemicon	1:400	Block with mouse Fab fragment	Ectoderm – neural progenitor
β III Tubulin	Rabbit IgG	Covance	1:400		Ectoderm – neuronal
NF160 or NFM	Mouse IgG	Chemicon	1:100	Block with mouse Fab fragment	"
NF200 or NFH	Rabbit IgG	Chemicon	1:400		"
GFAP	Mouse IgG	Sigma	1:100	Block with mouse Fab fragment	Ectoderm – glial
S100	Rabbit IgG	Abcam	1:200		"
TP63 / TP73L	Goat IgG	R&D systems	1:50		Ectoderm – epidermal
Keratin 8	Rabbit IgG	Abcam	1:100		Epithelial
Keratin epithelial	Mouse IgG	Chemicon	1:400	Block with mouse Fab fragment	"
Gata4	Goat IgG	R&D systems	1:50		Endoderm (and mesoderm derived myoblasts)
AFP	Mouse IgG	Sigma	1:100	PFA then treat for 1 minute with ethanol, then block with mouse Fab fragment	Endoderm and primitive endoderm
HNF3 β	Goat IgG	R&D systems	1:50		Primitive streak and endoderm
Brachyury	Goat IgG	R&D systems	1:50		Mesoderm
A smooth muscle actin	mouse IgG	Sigma	1:100	Block with mouse Fab fragment	Mesoderm or ectoderm (neural crest derived)- muscle
HCG	mouse IgG	Abcam	1:50		Trophoblast of trophoectoderm

Non-specific staining generated from antibodies raised in a mouse host

Of the range of primary antibodies used in immuno-staining analyses that were raised in mouse hosts, under the standard immuno-staining protocol most generated non-specific staining in negative controls of mouse tissue and in tumour explants. However for some antibodies (such as Nestin, β III tubulin and epithelial keratin), positive immuno-staining was easily distinguishable from non-specific background staining because of the marked difference in staining intensity. In order to reduce background staining caused by host antigens interacting with the mouse secondary antibody, prior to the normal blocking step tumour sections were incubated with a mouse Fab fragment. This reduced the background staining intensity in many cases but not in all. Alpha-smooth muscle actin for example still

exhibited high levels of non-specific staining.

Ideally it would be preferable to completely remove this background effect from staining analyses by using primary antibodies raised in a different host (such as rabbit or goat), although in several cases these were not available.

A comment on the quality of immuno-staining, morphology and tumour processing techniques

In addition to non-specific staining generated from using antibodies raised in mouse hosts, other difficulties with background staining were encountered. Some antibodies functioned poorly or not at all on sections from wax-embedded tissue, and many antibodies exhibited high back-ground despite extensive antigen retrieval, blocking and prolonged primary incubation steps. As a result the remaining tumours were embedded in OCT instead of wax. However, although these antibodies functioned better on sections from OCT embedded tumours, the preservation of tumour morphology (from tumours fixed after removal from the host), was reduced in those embedded in OCT compared to wax, and substantial background staining was still observed. However, the last tumour, which was perfusion fixed prior to surgical removal, exhibited a high level of morphology preservation, stained positively for the range of antibodies tested and exhibited much lower background staining, including in sections immuno-stained with primary antibodies raised in the mouse host.

Detection of multiple germ layer derivatives

In all TERA2.sp12 derived tumours surveyed, section samples exhibited extensive immuno-positive staining for the neuro-progenitor marker Nestin, and large areas were also immuno-positive for neuronal markers β III tubulin and MAP2. The expression pattern of these neural markers throughout the tumour was heterogeneous and associated with discrete patches of tissue. Neural rosette like structures displaying immuno-positive staining for Nestin and surrounded by β III tubulin positive areas were also observed. Although Nestin and β III tubulin expression was closely associated in these areas, cellular co-localisation was not detected. Figure 5.2 depicts neural marker expression in different regions and increasing magnifications in a representative section of the PFA perfusion fixed tumour. Some evidence of more mature neuronal differentiation was also observed, demonstrated by the expression of mature neurofilaments and substantial staining for the early glial marker S100 (and in rare cases the astroglial marker GFAP was detected), in some instances in cells exhibiting a branched glial morphology (Figure 5.2 plates E-F). It is interesting to note that one of the tumours examined exhibited a general trend whereby regions of neural marker expression were typically detected closer to the outer edge of the tumour.

Pan-epithelial keratin and simple epithelial markers Keratin 8 and 18 were also

Neuronal and Glial Differentiation In TERA2.sp12 Derived Tumours

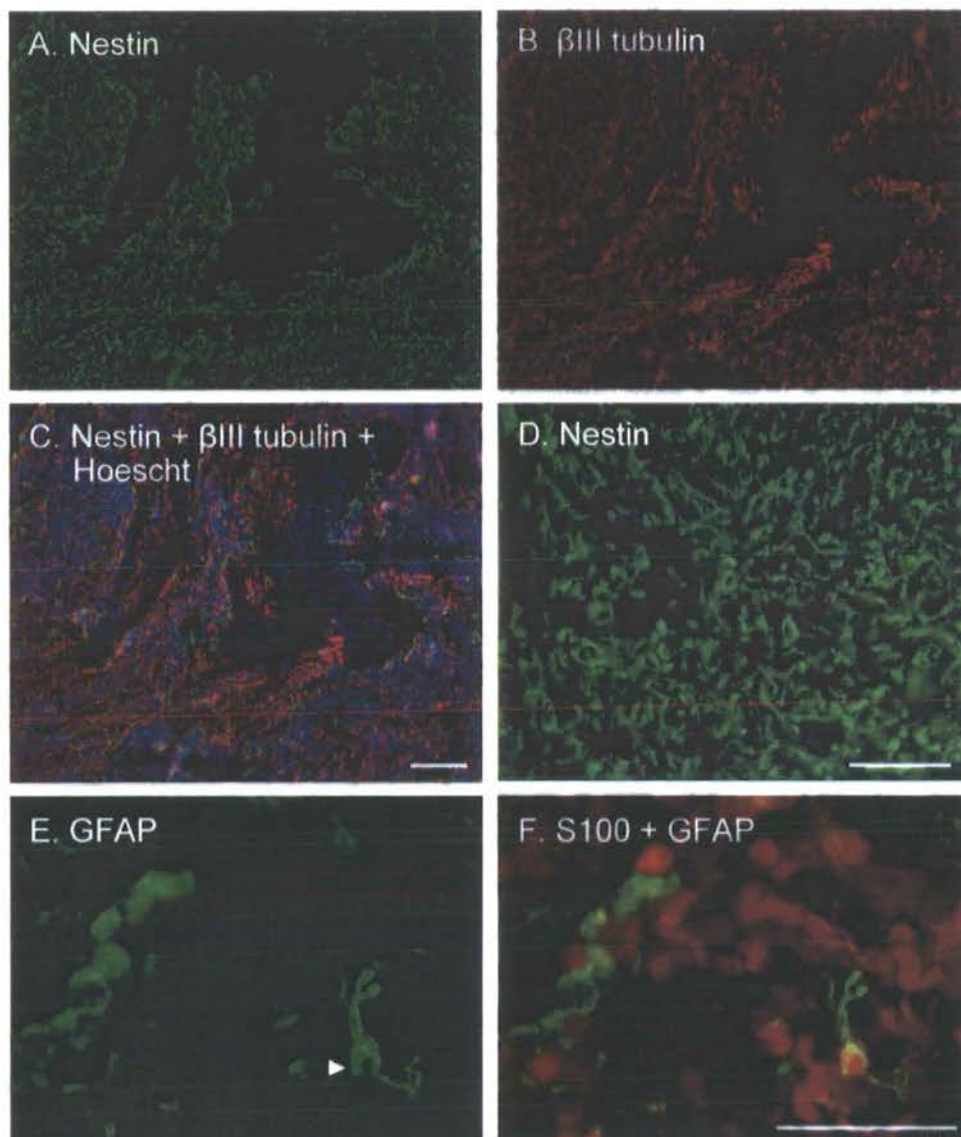


Figure 5.2. Neural marker expression in representative sections from a TERA2.sp12 derived tumour generated from injection into an immune-deficient mouse host. TERA2.sp12 cells were injected sub-cutaneously into a nude mouse and after a period of 17 weeks the tumour was perfusion fixed in 4% PFA, embedded in OCT and 10 μ m cryosections double immuno-labelled with either Nestin (green) and β III tubulin (red), (plates A-C and D), or double immuno-labelled with GFAP (green) and S100 (red), (plates E-F). Cells were counterstained with hoescht (blue) prior to mounting under coverslips. Note the extensive regions exhibiting early neural differentiation as demonstrated by positive expression of Nestin (A and in another region at higher magnification in D) and β III tubulin (B with overlay of Nestin, β III tubulin and hoescht in C). Some glial differentiation indicated by the expression of GFAP was detected (as depicted in E), with some GFAP immuno-positive cells exhibiting a branched glial morphology (arrow head) and demonstrating nuclear localisation of the astrocytic marker S100 (overlay in F). Scale bar for plates A-C = 100 μ m. Scale bar for plates D-F = 50 μ m

expressed within tumour sections (although not at comparable levels with Nestin), and epithelial keratin expression was typically associated with ordered arrangements of cells that formed a simple epithelial or stratified cell layer around a small cavity (see Figure 5.3). No-co-localisation was observed between epithelial keratins and the neural antigens Nestin and β III tubulin. The majority (although not all) of these epithelial keratin positive structures exhibited nuclear localised expression of the epidermal stem cell marker TP63. In cross sections of predominantly single-cell lined (epithelial keratin positive) tubular structures TP63 expression was detected in only a few adjacent cells, and this was observed in regions where the lining was no longer composed of single cells. Tubular epithelial keratin positive structures within sections that were composed of multiple cell layers exhibited the highest frequency of TP63 positive nuclei, and TP63 positive cells were localised to the outer edge or basal layer of the structure (as shown in Figure 5.3).

A minority of these epithelial keratin positive structures exhibited strong staining for the nuclear localised endoderm transcription factor HNF3 β (typically expressed in gut), and these were typically more complex and often branched, comprised of multiple layers of cells. Unlike the more restricted pattern of expression observed for the epidermal stem cell marker TP63, almost all of the epithelial keratin positive cells within this structure stained positive for HNF3 β . It is important to note that other epithelial keratin positive islets within the same sections exhibited no positive immuno-staining for HNF3 β , even when surrounding cells were immuno-positive. In some regions exhibiting positive HNF3 β and epithelial keratin expression, the pattern of epithelial keratin expression and arrangement of cells were more loosely organised. Figure 5.4 presents the two main patterns of HNF3 β expression observed in tumour sections. No positive HNF3 β staining was observed in the absence of positive staining for epithelial keratin. Laminin (the main extra-cellular matrix component of basement membranes), was detected to line both the outer edge of cells forming the organised branched structures and also to surround the large regions of more loosely organised HNF3 β positive cells.

The endoderm transcription factor Gata4 was detected in extensive regions throughout tumour sections, (also closely associated with areas of positive epithelial keratin staining). Different regions within the same tumour sections exhibited marked variations in staining intensity (see Figure 5.5). Ordered arrangements of epithelial keratin positive cells around a cavity such as those exhibiting positive staining for HNF3 β or TP63 were not typically positive for Gata4, although cells exhibiting high Gata4 expression frequently surrounded these structures.

Little expression of the mesoderm transcription factor Brachyury was detected in sampled tumour sections, and this was not associated with any morphologically distinct

Non-Neural Ectoderm Differentiation In TERA2.sp12 Derived Tumours

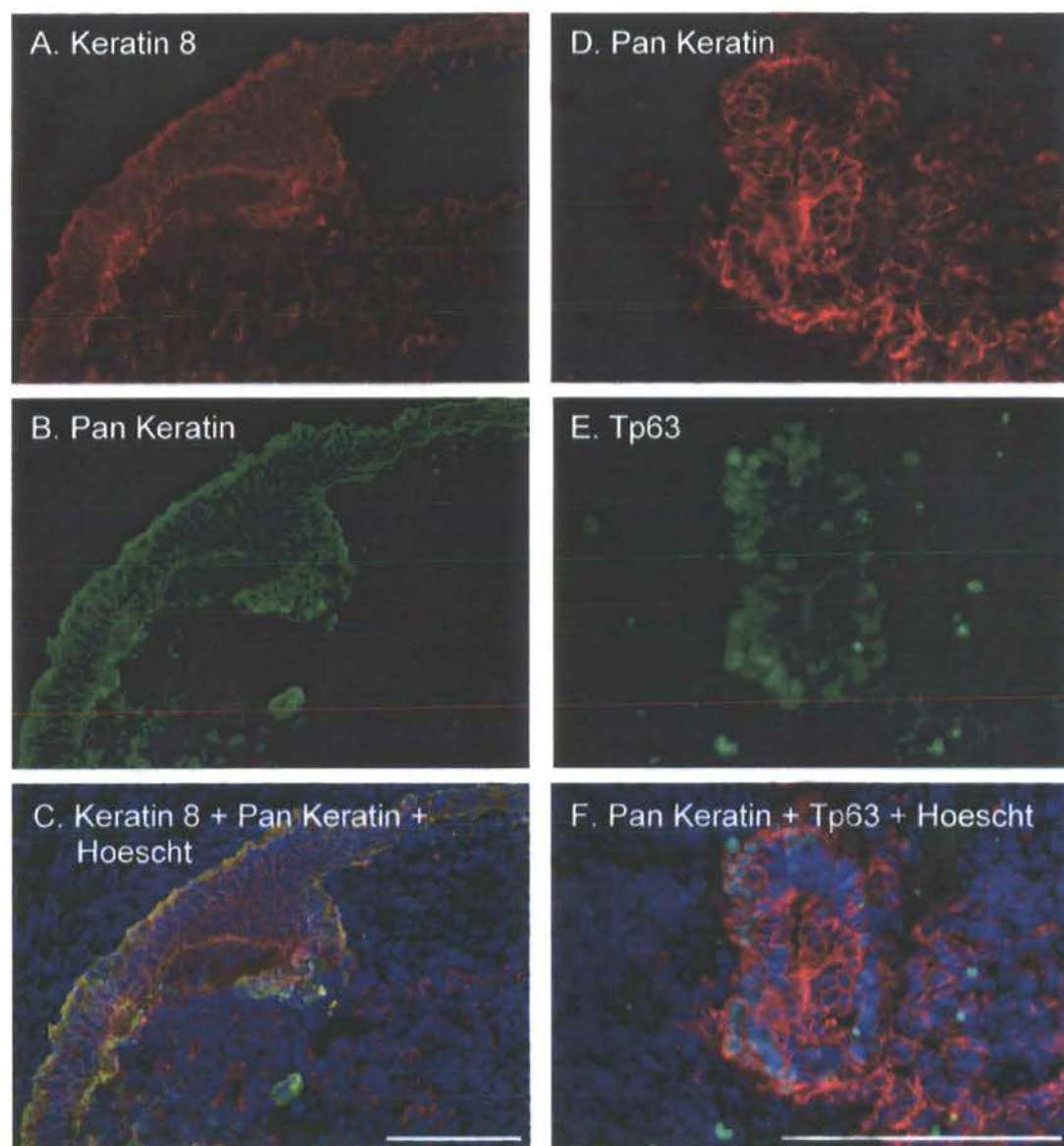


Figure 5.3. Epidermal antigen expression in representative sections from a TERA2.sp12 derived tumour generated in an immune-deficient mouse. TERA2.sp12 cells were injected sub-cutaneously into the left flank of a nude mouse. After a period of 17 weeks the tumour was perfusion fixed in 4% PFA, embedded in OCT and 10 μ m cryo-sections double immuno-labelled with either epithelial keratin (green) and Keratin 8 (red), (A-C), or epithelial keratin (red) and TP63 (green), (D-F). Cells were counterstained with hoescht (blue) prior to mounting under coverslips. Plates A-B (and overlay with hoescht in C) depict a structure composed of relatively organised layers of cells expressing epithelial keratins. As depicted in (D-F) morphologically distinct islet shaped structures were observed, comprising of layers of cells which organised around a small central cavity and were immuno-positive for epithelial keratin and the epidermal stem cell transcription factor Tp63. Note that some cells demonstrate nuclear polarity (overlay with hoescht in G). Scale bars=100 μ m.

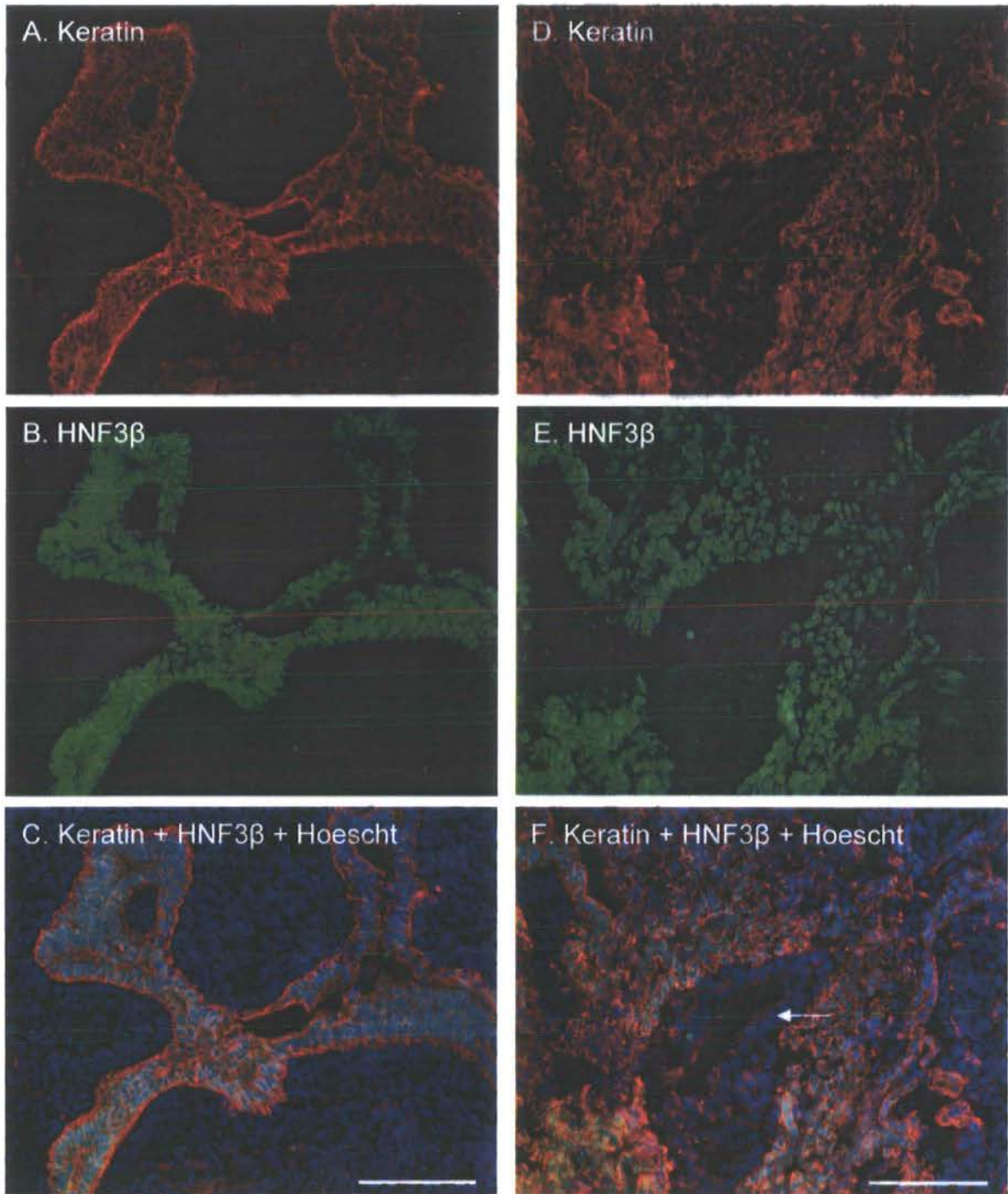
Expression Of The Endoderm Marker HNF3 β In TERA2.sp12 Derived Tumours

Figure 5.4. Expression of the endoderm transcription factor HNF3 β and epithelial keratin in a representative section from a TERA2.sp12 tumour generated in an immune-deficient mouse host. TERA2.sp12 cells were injected sub-cutaneously into a nude mouse and after a period of 17 weeks perfusion fixed in 4% PFA, embedded in OCT and 10 μ m cryo-sections double immuno-labelled with epithelial keratin (red) and the endoderm transcription factor HNF3 β (green). The nuclei were counterstained with hoescht (blue) prior to mounting under coverslips. Plates A-B (with overlay including hoescht in C) depict a relatively organised branched structure comprised of a multi-cellular lining of cells immuno-positive for HNF3 β and pan-epithelial keratin. Plates D-E (with overlay including hoescht in F) depict HNF3 β positive staining in a less organised arrangement of cells that surround epithelial keratin positive cells organised around a cavity (arrow). Scale bars=100 μ m.

Expression Of The Endoderm Transcription Factor Gata4 In TERA2.sp12 Derived Tumours

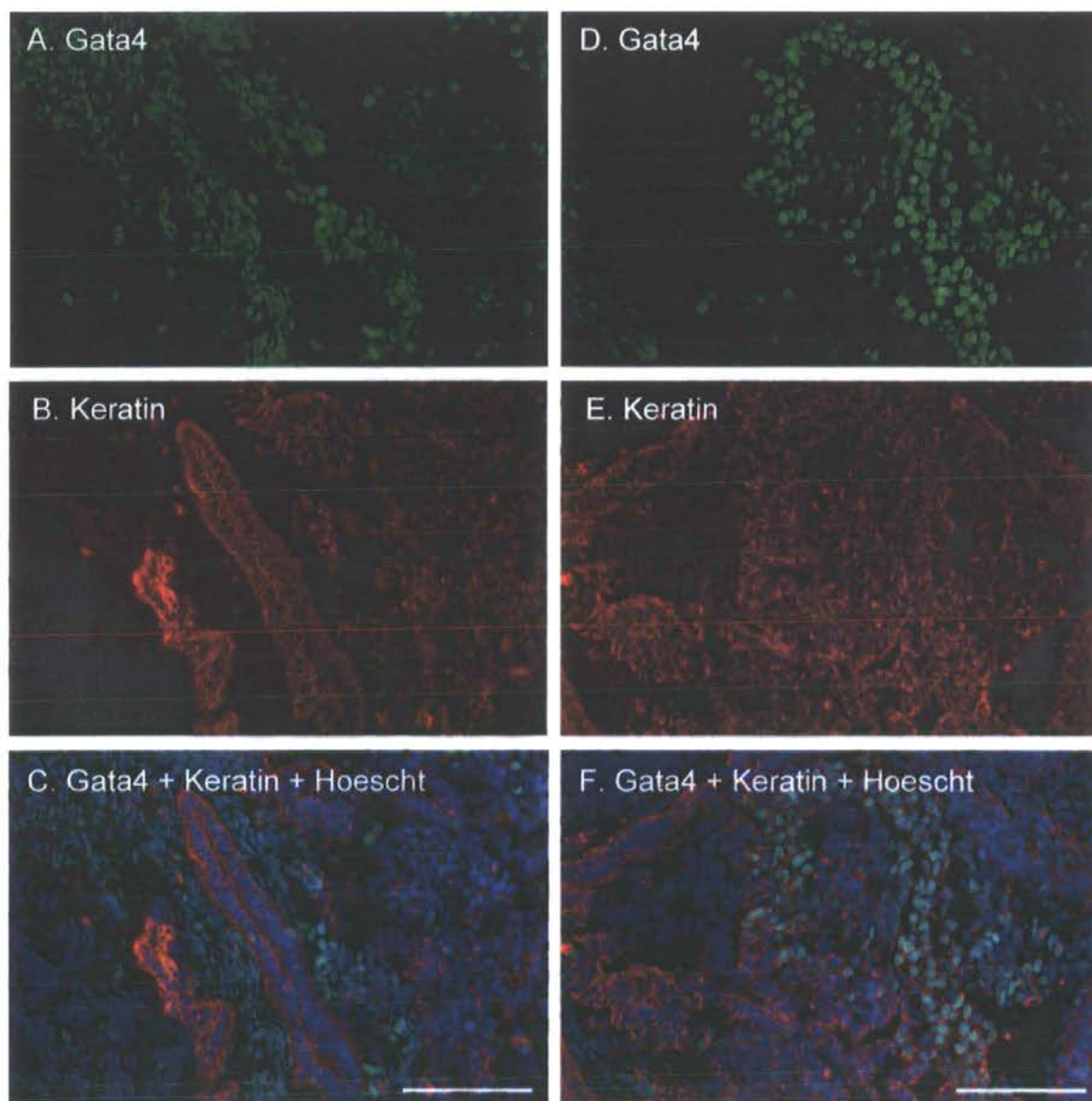


Figure 5.5. Epithelial keratin and Gata4 expression in representative sections from a TERA2.sp12 derived tumour. TERA2.sp12 cells were injected sub-cutaneously into a nude mouse and after a period of 17 weeks perfusion fixed in 4% PFA, embedded in OCT and 10 μ m cryo-sections double immuno-labelled with epithelial keratin (red) and the endoderm transcription factor Gata4 (green). Cells were counterstained with hoescht (blue) prior to mounting under coverslips. Sections A-C and D-F depict the two main patterns of Gata4 and epithelial keratin expression observed within the tumour. As depicted in A-B (with overlay in C), in morphologically distinct and organised 'eyelet' structures immuno-positive for epithelial keratin, no Gata4 expression was detected, such that adjacent cells exhibited distinct patterns of Gata4 or epithelial keratin expression. In other regions, as demonstrated in D-E (with overlay in F), co-localisation of Gata4 and epithelial keratin was frequently observed, (typically in less organised areas of epithelial keratin expression). Note that in these images taken from the same section Gata4 expression is much higher in cells within plates D-F than within plates A-C. Scale bars=100 μ m.

Detection Of Human Nuclear Antigen Within TERA2.sp12 Derived Tumours

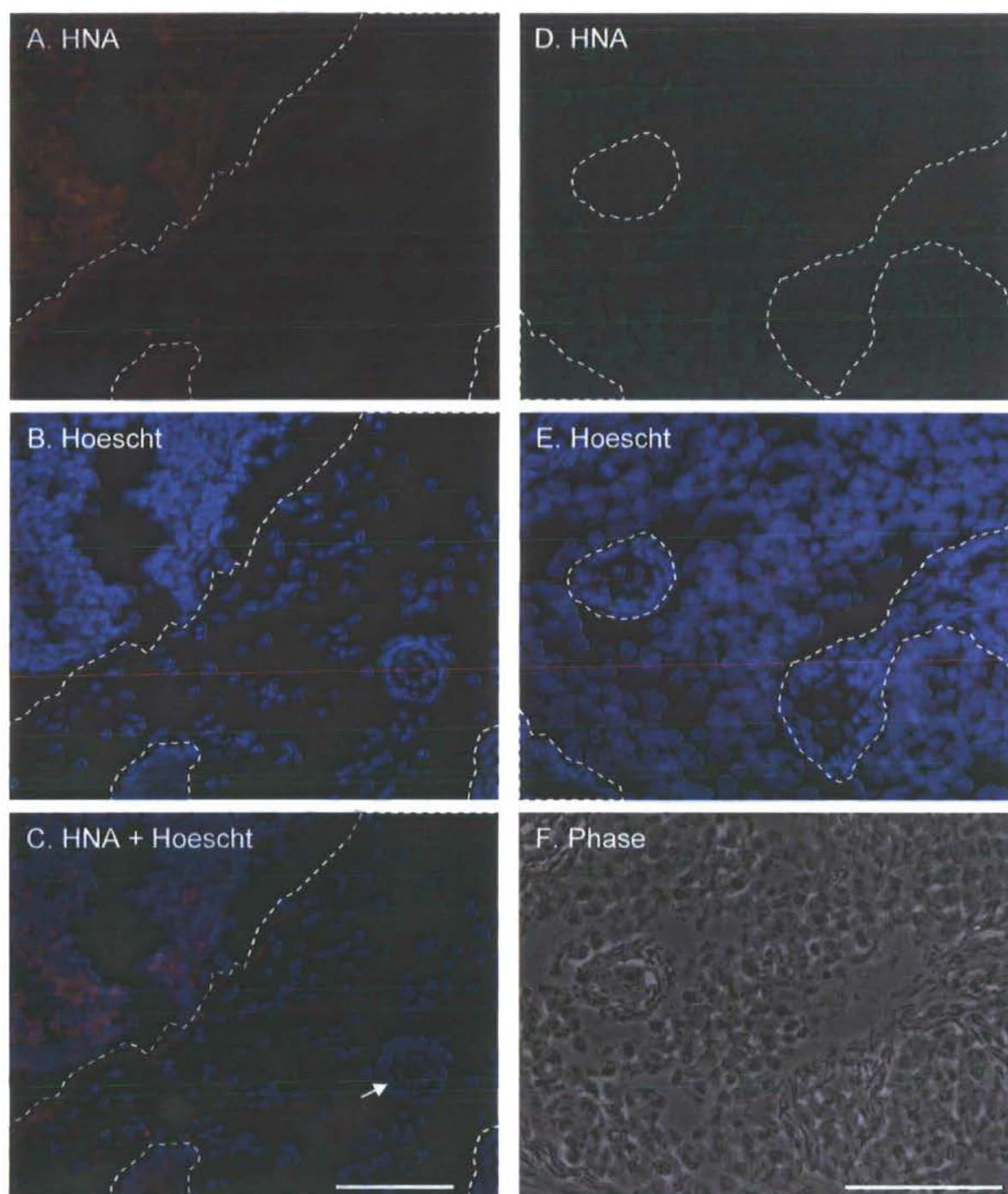


Figure 5.6. Human specific nuclear antigen expression in two representative sections from different TERA2.sp12 derived explants. TERA2.sp12 cells were injected sub-cutaneously into nude mice and after a period of 14-17 weeks fixed in 4% PFA, embedded in Wax (A-C) or OCT (D-F) and 10 μ m cryo-sections immuno-labelled with an antibody to Human nuclear antigen (red or green) and counterstained with hoescht (blue) prior to mounting under coverslips. Close to the edge of a tumour section in plates A-C, a layer of tissue containing a hair follicle-like structure (arrow) exhibits no nuclear staining for human nuclear antigen, compared to adjacent tissue which exhibits bright nuclear localised staining (positive staining outlined in white dashes). As depicted in plates D-F nuclear localisation of human nuclear antigen within the tumour is exhibited in the majority of cells, although regions negative for human nuclear antigen are present (negative staining outlined with white dashes). These negative regions were typically tubular structures, containing cells with a more compact elongated nucleus (D and E) and a distinctive morphology under phase contrast (F) indicative of vascular tissue. Note that although relatively high background staining was exhibited, non-specific staining was weaker than in surrounding tissue and did not specifically label the nuclei. Scale bars=100 μ m.

Detection Of A Stem Cell Population Within TERA2.sp12 Derived Tumours

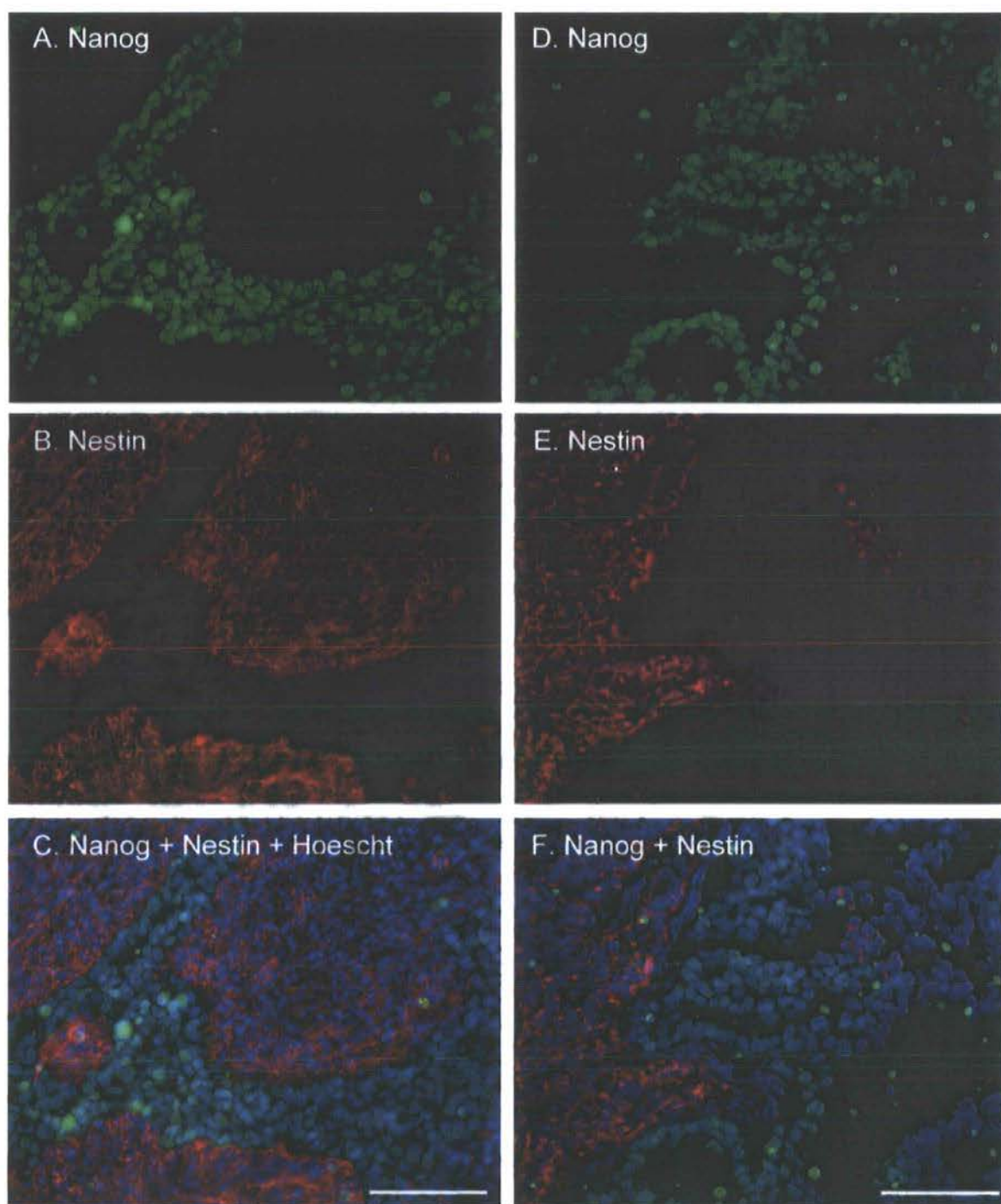
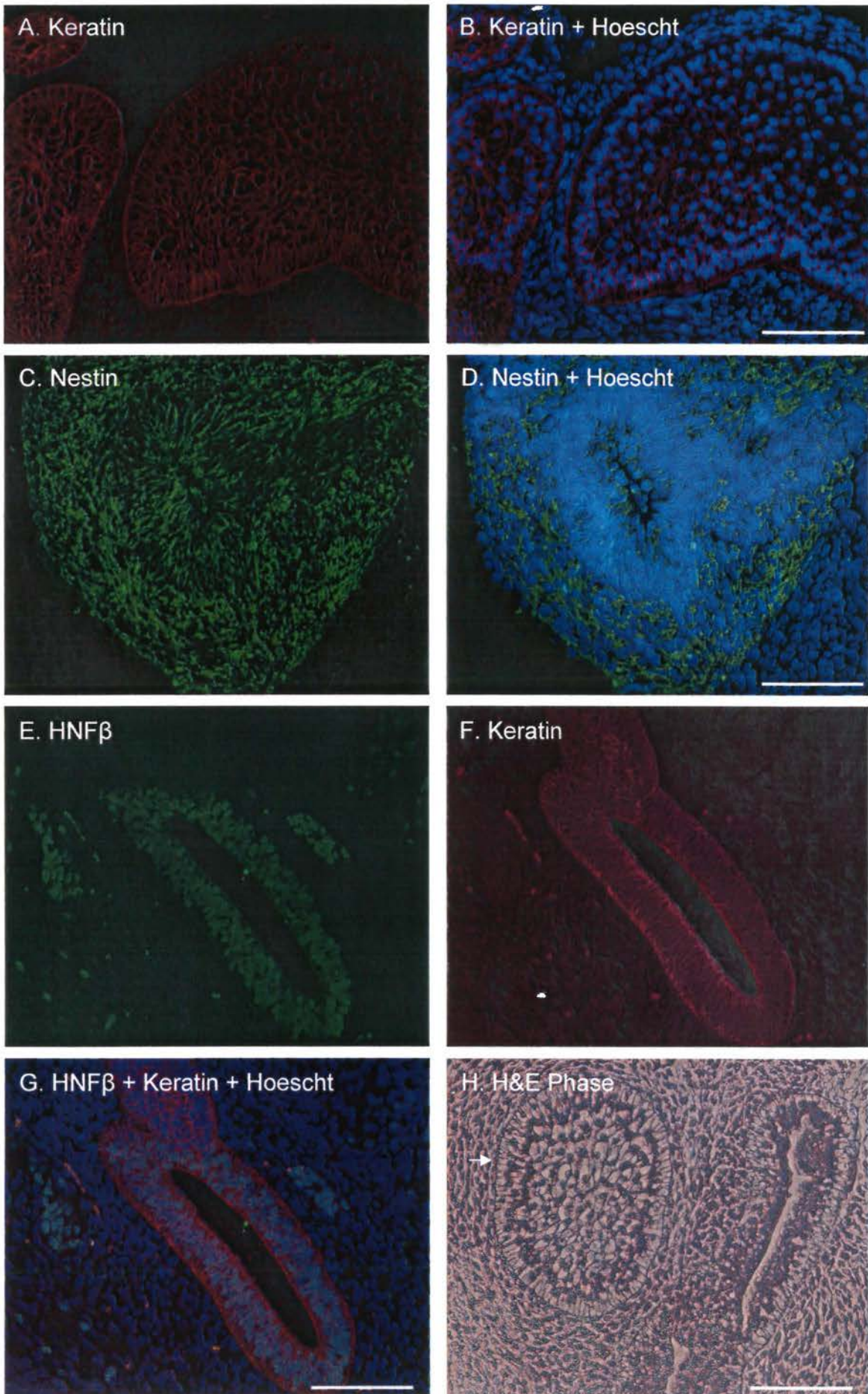


Figure 5.7. Nestin and Nanog expression in two representative sections (A-C and D-F) from a TERA2.sp12 derived tumour. TERA2.sp12 cells were injected sub-cutaneously into a nude mouse and after a period of 17 weeks the tumour was perfusion fixed in 4% PFA, embedded in OCT and 10µm cryo-sections double immuno-labelled with Nestin (red) and the stem cell transcription factor Nanog (green). The nuclei were also counterstained with hoescht (blue). Two different sections in plates A-C and D-F depict the detection of a sub-population of stem cells exhibiting nuclear expression of the stem cell transcription factor Nanog. Nanog positive cells (plates A and D) were negative for the widely expressed neuro-progenitor marker Nestin (plates B and E). Note that Nanog positive cells exhibited larger nuclei compared to surrounding cells (see overlays with hoescht in C and F). Scale bars=100µm.

Varied Structures And Expression Of Multiple Markers Of Differentiation In Sections Of A Tumour Derived From Human ES Cells NCL-1



structures. Positive expression occurred in small patches of cells in a similar pattern to that previously observed in RA treated TERA2.sp12 cells *in vitro*.

In summary, extensive regions of neural differentiation were observed in all tumours, displaying a range of differentiation stages and both neuronal and glial marker expression. Epithelial cells within the teratomas were made up of both endoderm and ectoderm as detected by specific markers, and formed the histologically distinctive structures observed in haematoxylin and eosin stained sections. No definitive evidence of differentiation into mesoderm derivatives was detected; very little Brachyury expression was observed, and although positive staining was detected with the smooth muscle marker α smooth muscle actin, this observation was treated with caution because of the accompanying high levels of non-specific staining. In addition, (as previously discussed) smooth muscle may be either neural crest (ectoderm) or mesoderm derived, so markers of smooth muscle do not necessarily represent a mesoderm derivative.

The relative composition of different markers of germ layers was grossly consistent in all of the tumours tested, although one tumour contained organised tubular structures of a greater size and complexity compared to other tumours, and also exhibited higher levels of HNF3 β expression. This was observed in the tumour isolated 17 weeks after injection, compared to the other tumours which were isolated after 7-14 weeks. It is also important to note that only a sample of sections from regions through-out the tumours were observed, and in a relatively small sample number of tumours, so it is possible that some phenotypes were not detected in this study.

Discriminating between mouse and human cells within the tumours

As substantial tumours are extensively vascularised by the host, the tumours examined were likely to contain –at the least– vascular structures derived from mouse tissue. Indeed, structures that resembled blood vessels were observed within all tumour sections. These were lined with cells containing distinctive flattened nuclei (endothelium-like) and these structures also frequently exhibited high levels of non-specific staining. In addition, TERA2.sp12 derived tumours were typically surrounded in a layer of fat-like tissue and structures resembling hair follicles were frequently observed in this outer layer and sometimes deeper within the tumour tissue. These regions demonstrated relatively high levels of background staining even with extensive blocking treatments, and cells lining the hair follicle-like structures stained brightly with epithelial keratin.

The aim of the following experiment was to discern between structures formed from TERA2.sp12 cells or the mouse host and determine whether the range of phenotypes previously described could truly be attributed to TERA2.sp12 cell differentiation. To

discriminate between mouse and human cells within the tumour explants, serial sections were immuno-stained with mouse anti-Human nuclear antigen (Chemicon) used at a dilution of 1:50. As this antibody was raised in mouse, sections were pre-treated with a Fab fragment of anti-mouse IgG as previously described. Unfortunately, as this human specific antibody was only available as an antibody raised in mouse, non-specific staining was observed in all tumours, despite extensive blocking treatments. However the background staining was low enough in all tumours for a difference in staining specificity and intensity to be detected, as the antibody only brightly stained the nuclei of human cells, compared to lower intensity non-localised staining in mouse cells (see Figure 5.6).

This enabled me to observe that the blood vessel-like structures, the outer layer of fatty tissue and hair follicles were all consistently negative for human nuclear antigen. The remaining cell types and structures all exhibited positive nuclear immuno-staining for human nuclear antigen, demonstrating that the majority of the tumour was derived from TERA2.sp12 cells.

Detection of stem cell populations within tumour explants

To assess whether these tumours contained a sub-population of cells that retained their stem cell phenotype or determine whether the tumours increased in size solely as a result of the proliferation of progenitor cells, tumour sections were screened with an antibody to the stem cell transcription factor Nanog. Serial sections through the tumours were stained with an antibody to Nanog (raised in goat) following the protocol outlined in Section 2.5, with an initial blocking step in donkey serum and primary incubation overnight at 4°C.

Although relatively high background was observed in all tumour sections except those from the tumour that was perfusion fixed, a distinctive Nanog immuno-positive population was observed in all tumours. In the 17 week perfusion fixed tumour, a substantial population of Nanog positive cells was observed and these cells were negative for the neural progenitor marker Nestin (Figure 5.7). In addition, Nanog positive cells were typically observed to be closely associated with structures resembling vascular tissue. This was consistent with the character of TERA2.sp12 cells as EC cells whereby a stem cell population is retained in the tumour.

Comparison with human ES cell derived tumour

To compare the differentiation within TERA2.sp12 derived tumours with that of a human ES cell line, sections of tumour explants derived from hES-NCL1 cells injected beneath the capsule of the kidney of adult male severe combined immune deficient (SCID) mice were either stained with haematoxylin and eosin following the procedure in Section 2.6 or immuno-labelled following the protocol in Section 2.5.3 with antibodies to germ layer markers listed in

Table 5.2. The tumour sections were obtained from a collaboration project between Stefan Przyborski (University of Durham) and Miodrag Stojkovic (University of Newcastle), (Stojkovic 2004). The cells were prepared in Newcastle and the tumours processed by Rebecca Stewart, a fellow lab member. To summarise, 3000 hES-NCL1 cells grown on mouse embryonic fibroblasts were injected beneath the kidney capsule. After a palpable tumour had formed, the mice were sacrificed and the tissues dissected, fixed in PFA, embedded in wax, processed and sectioned following the protocol described in Section 2.5.

Sections exhibited much greater variation and complexity in structure formation under gross examination, although some structures were very similar to those found in tumours derived from TERA2.sp12 cells. Unfortunately only a sub-set of antibodies worked successfully on the tumour sections, because the tumour was embedded in wax, so a detailed immuno-logical comparison with TERA2.sp12 derived tumours was not possible. Figure 5.8 depicts representative images from sections of the human embryonic stem cell derived tumour after both histological and immuno-staining. These initial results are consistent with the hypothesis that TERA2.sp12 cells differentiate in vivo into a more restricted range of cell types than hES-NCL1 cells, although further experiments are required to support this hypothesis.

Varied Structures And Expression Of Multiple Markers Of Differentiation In Sections Of A Tumour Derived From Human ES Cells NCL-1

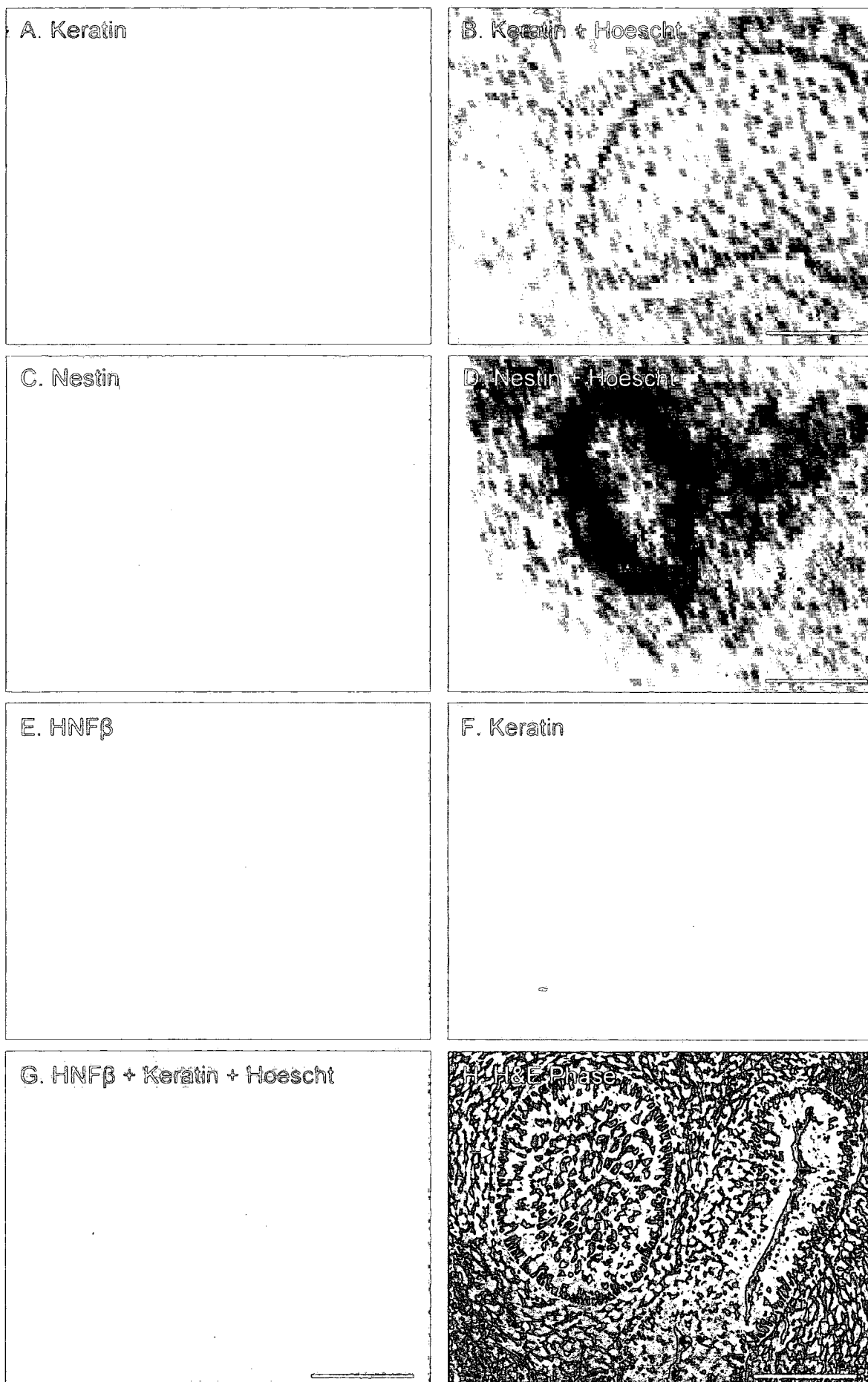


Figure 5.8. Varied structures and expression of multiple markers of differentiation in sections of a tumour derived from human ES cells NCL-1. Cells were injected beneath the left kidney capsule of a SCID mouse, and upon generation of a palpable tumour the tumour was removed, fixed in 4% PFA and embedded in wax. 10 μ m sections were either immuno-stained with pan epithelial keratin (red, A with overlay in B) or Nestin (green, C with overlay in D) or double immuno-labelled with epithelial keratin (red) and HNF3 β (green), (E-F with overlay in G) and the nuclei counter stained blue with hoescht. Tumour sections were also stained with haematoxylin and eosin and viewed under phase contrast (H). Discrete patches of tissue were immuno-positive for the neural progenitor marker Nestin (C and overlay with hoescht D), and organised, morphologically distinct multicellular structures exhibiting epithelial keratin staining were observed (A-B). Plate H depicts a similar structure under phase contrast (arrow). In contrast, relatively simple arrangements of epithelial keratin and HNF3 β positive cells around a central cavity were also observed in sections (E, F and overlay with hoescht in G). Scale bars = 100 μ m

5.3 DISCUSSION

5.3.1 Summary

As in the previous chapters it was demonstrated that TERA2.sp12 cells could form non-ectodermal derivatives *in vitro*, in this chapter the differentiation of TERA2.sp12 cells *in vivo* was characterised by both histological and immuno-staining analyses, in order to more fully explore the differentiation potential of these cells. Sub-cutaneous injection of TERA2.sp12 cells into immune-deficient mice generated readily palpable tumours after 11-17 weeks that contained a range of differentiated phenotypes of predominantly TERA2.sp12 origin. Neural differentiation was dominant in all the tumours generated, but a substantial population of epithelial structures indicative of simple non-neural epithelial ectoderm was also observed. In addition, a stem cell population was detected in all tumours, consistent with the properties of TERA2.sp12 cells as an embryonal carcinoma cell line. Histological comparison with tumours derived from human stem cells demonstrated a markedly restricted range of structures and phenotypes in TERA2.sp12 cell derived tumours compared to human ES cell derived tumours, supporting the hypothesis for TERA2.sp12 cell differentiation being restricted to primarily an ectodermal phenotype. However, immuno-staining analyses identified some selected expression of endoderm markers, indicating that TERA2.sp12 cells still retain the capacity to differentiate into non-ectodermal lineages *in vivo*. In this study the ability of TERA2.sp12 cells to form mesoderm derivatives was not demonstrated, so they may still display a restricted differentiation potential.

5.3.2 Histological analysis of TERA2.sp12 derived tumours

Haematoxylin and eosin staining of tumour sections identified a range of phenotypes within the TERA2.sp12 derived tumours, including neural rosettes and organised epithelial structures consisting of layers of cells surrounding a central cavity. However the majority of the tissue was disorganised, and compared to the extensive range of phenotypes and more complex structures observed in sections of a tumour derived from the human ES cell line NCL-1, TERA2.sp12 derived tumours exhibited a markedly reduced range of differentiation phenotypes. As only one tumour was examined from the human ES cell line NCL-1, and more importantly this tumour was derived from a different site of injection, only limited comparisons can be made. However multiple reports documenting the formation of tumours from ES cells injected into different sites in immune-deficient mice all demonstrate the formation of multiple tissue types representing all three germ layers. In these reports they present histological evidence of readily identifiable tissues such as smooth muscle, striated

muscle, bone, cartilage, fetal glomeruli, gut, respiratory epithelium, keratinizing squamous epithelium, hair, neural epithelium, ganglia and highly organised structures such as teeth, hair follicles and gut (Heins 2004, Reubinoff 2000, Stojkovic *et al.* 2004b, Thomson 1998) for review (Oderico 2001).

In comparison, the markedly restricted range of phenotypes and simple epithelial structures observed in TERA2.sp12 cells supports the theory that TERA2.sp12 cells are more restricted in their capacity to differentiate to that of ectoderm derivatives (as we have previously reported (Przyborski 2004, Stewart 2005). A limited differentiation capacity has also been previously observed in teratomas formed from NTERA2.cl.D1 cells and other clonal lines derived from TERA2 by Andrews *et al.* (Andrews *et al.* 1984). They reported that injection into immuno-compromised mice generated teratocarcinomas containing neural elements and simple tubular structures resembling primitive gut.

However, experiments described in previous chapters demonstrate the ability of TERA2.sp12 cells to form derivatives expressing markers of endoderm (Gata4), mesoderm (Brachyury, α -smooth muscle actin) and trophoectoderm (HCG) *in vitro*, questioning the hypothesis that TERA2.sp12 differentiation is restricted to ectoderm derivatives. To more closely examine the differentiation phenotypes within TERA2.sp12 derived tumours, cells were immuno-stained with a range of markers expressed in derivatives of all three germ layers.

5.3.3 Immuno-staining analysis of TERA2.sp12 derived tumours

Characterising differentiated populations within TERA2.sp12 derived tumours

Immuno-staining of tumour sections demonstrated that TERA2.sp12 cells had embarked on extensive differentiation into ectodermal derivatives, with extensive neural marker expression in discrete regions of tissue and also substantial epithelial keratin expression (Stewart 2005). Organised arrangements of layers of cells surrounding a cavity stained positive for pan-epithelial keratin and simple epithelial keratins 8 and 18, and a proportion of these structures also expressed the epidermal stem cell antigen Tp63. It was interesting to note that Tp63 expression was restricted to the outer surrounding layer of epithelial keratin positive cells, similar to the pattern of normal epidermal development where basally localised epithelial stem cells generate overlying progenitors by asymmetric division.

Importantly, selected regions within tumours derived from TERA2.sp12 cells also immuno-stained positive for markers of other germ layers. The endoderm transcription factor Gata4 was detected in regions of epithelial keratin positive tissue, as was the endoderm transcription factor HNF3 β – a member of the fork head family of proteins initially identified in drosophila mutants with limited gut development (Lai *et al.* 1993). HNF3 β is expressed in

visceral and definitive endoderm during early mouse embryogenesis (Ang *et al.* 1993, Lai 1993, Sasaki 1993) and is required for normal primitive streak morphogenesis (Dufort *et al.* 1998). Gata4 is also expressed in primitive and definitive endoderm, and later in development in the liver and gut epithelium (Arceci *et al.* 1993, Laverriere *et al.* 1994). Taken together this indicates that the Gata4 and HNF3 β immuno-staining observed in regions of less organised tissue represents primitive or definitive endoderm. HNF3 β expression was also detected in organised epithelial keratin positive structures that were frequently branched and structurally resembled primitive gut epithelia. This corresponds with observations by Andrews *et al.* (1984b) of gut-like structures in tumours derived from NTERA2.cl.d1. Through immuno-staining analyses Gertow *et al.* (2004) also demonstrated that the epithelial components of tumours derived from HS181 human embryonic stem cells consisted of endoderm and ectoderm derivatives. They observed similar simple epithelial structures and gut like structures staining positive for simple epithelial keratins as well as the presence of more complex duct-like structures not observed in TERA2.sp12 derived tumours.

Gata4 is also expressed in mesoderm derived developing and mature cardiomyocytes (Arceci 1993, Heikinheimo *et al.* 1994, Laverriere 1994). However, there was no morphological evidence of differentiation along this lineage, and the similar pattern of immuno-staining to HNF3 β suggests that Gata4 expression was linked to endoderm lineages in these tumours. No evidence of the primitive endoderm marker AFP was detected in these tumours, implying that the differentiation observed was primarily definitive endoderm. However technical problems with using the antibody on wax sections may have hindered observations of AFP expression, and thus primitive endoderm differentiation cannot be excluded. To further characterise these endoderm derivatives including the primitive gut-like structures and also effectively discriminate between visceral or primitive endoderm, more extensive immuno-staining analyses including co-staining with a greater range of markers are necessary.

The early mesoderm transcription factor Brachyury was also detected in tumour sections, but not in association with organised tissue and in relatively few cells, following a similar pattern to that observed in adherent cultures of RA treated cells. Together with the report that the embryonal carcinoma cell line TERA2 expresses Brachyury with no obvious mesoderm differentiation (Gokhale *et al.* 2000), this does not conclusively provide evidence of mesoderm differentiation *in vivo*. Expression of the general marker of smooth muscle, α smooth muscle actin, was detected within these tumours, which could provide supporting evidence for mesoderm differentiation. However, as previously discussed in chapter 4, smooth muscle can also be derived from neural crest cells of the ectoderm. Together with the absence of histological evidence of mesoderm derivatives such as bone or striated muscle, this

indicates that mesoderm differentiation was limited or absent in these tumours. Staining with further markers of mesoderm (for example with desmin) would clarify whether mesoderm derivatives are present.

In summary, immuno-staining evidence of predominantly ectoderm differentiation, (both neural and non-neural simple epithelial) and some endoderm differentiation, (putative simple gut epithelia) was detected in TERA2.sp12 cell derived tumours, whereas no solid evidence of mesoderm differentiation was detected. This is in contrast to reports by Heins *et al.* (2004) and Gertow *et al.* (2004) demonstrating the expression of markers of all three germ layers in immuno-staining analyses of tumours generated from human ES cell lines.

Identification of a stem cell population within TERA2.sp12 derived tumours – the EC phenotype

A sub-population of cells expressing the stem cell transcription factor Nanog was identified in all of the TERA2.sp12 derived tumours surveyed, in contrast to a report by Gertow *et al.* (2004) in which they demonstrated the absence of a stem cell population in tumours derived from human ES cells, attributing tumour growth solely to progenitor proliferation. This is consistent with the inherent tumourigenic nature of embryonal carcinoma cells, as embryonal carcinoma cell lines were originally derived from the undifferentiated stem cell component (embryonal carcinoma) of teratomas. Correspondingly, embryonal carcinoma cells are typically easily maintained to exhibit a stem cell phenotype *in vitro*, in contrast to embryonic stem cells, which spontaneously differentiate unless they are cultured on feeder layers or together with a host of growth factors. This may reflect the transient nature of their counterparts in development, as a pluripotent stem cell population only exists in the developing blastocyst prior to gastrulation (for review Andrews 2002).

It is also interesting to note that in a recent report, the human embryonic stem cell sub-line H7-s6, which after prolonged culture had accumulating karyotypic abnormalities and displayed enhanced growth characteristics, was demonstrated to form a tumour containing a stem cell-like sub-population upon injection into immune-deficient mice, and that this stem cell population could be explanted and sub-cultured *in vitro* (Andrews *et al.* 2005). This demonstrates a link between malignant EC cells and their non-malignant ES cell counterparts. Together with the observation that the karyotypic abnormalities accumulated in ES cultures are typical of those found in EC cell lines (Draper *et al.* 2004, Liu *et al.* 1997, Skotheim *et al.* 2002), this also suggests that 'culture adaptation' of ES cells mirrors to some extent the progress towards malignancy in the formation of germ cell tumours and their EC components.

It was also interesting to note that the stem cell population in TERA2.sp12 derived tumours was frequently observed to be closely associated with host blood vessels. This may

reflect the influence of growth factors and more optimal growth conditions on stem cell maintenance but is also likely to be a result of the angiogenic properties of embryonal carcinoma stem cells as tumour cells.

Contribution of host cells versus implanted TERA2.sp12 cells in tumour formation

Immuno-staining analyses clearly demonstrated that over 90% of each tumour consisted of TERA2.sp12 cells, whilst surrounding outer layers containing fatty tissue and hair follicles were host derived, as well as blood vessel-like structures through-out the tumours. Similar results were generated by Gertow *et al.* (2004) who analysed human ES derived tumours using FISH screening to detect human DNA, and reported that the contribution of mouse cells was largely restricted to endothelial cells of the nutriating vessels. However, they also reported evidence of some interactions between human and mouse cells, such as observations of blood vessel linings consisting of both mouse and human derived endothelial cells. They confirmed these observations with immuno-staining for anti-human endothelial markers CD31 and CD34, indicating that some co-oporation in tumour vascularisation between the host and transplanted cells was occurring.

The data generated in my study using an antibody to anti-human nuclei alone indicates that blood vessels in TERA2.sp12 cells were solely host derived. However in the light of the report by Gertow *et al.* this requires more detailed examination. Additional immuno-staining with an antibody to human endothelial markers CD31 and CD34 may serve to more definitively determine whether or not blood vessels were entirely host derived. However, the absence of evidence for mesoderm differentiation in tumours from this study is in marked contrast to the presence of extensive mesoderm derivatives in the ES derived tumours analysed by Gertow *et al.*, which increases the likely-hood that the vasculature in TERA2.sp12 derived tumours may be indeed entirely host-derived.

5.3.4 A restricted differentiation potential for TERA2.sp12 cells?

Support for a restricted differentiation potential for TERA2.sp12 cells

The immuno-staining and histological results from this study indicate that although tumours do contain endoderm derivatives and are therefore not restricted to ectoderm in their differentiation, no solid evidence was obtained for mesoderm differentiation and extensive differentiation similar to embryonic stem cells was not displayed. Theoretically, TERA2.sp12 cells could easily exhibit a restricted differentiation potential compared to ES cells as a result of their tumourogenic nature. EC cells including TERA2.sp12 are highly aneuploid and have generally adapted over many generations to growth either in culture or as a tumour in a host mouse. As their differentiated derivatives typically have a limited lifespan and are non-malignant, the accumulation of mutations that inhibit differentiation could provide EC cells

with a selective advantage. As previously described, the cell line from which TERA2.sp12 cells were cloned was itself derived from embryonal carcinoma cells isolated from a teratoma generated from injection of TERA cells into immuno-compromised mice, and of course the TERA cell line was initially established from the embryonal carcinoma cells of a teratoma. So as well as being subjected to selection pressure under pro-longed culture conditions, TERA2.sp12 cells and its parent cells were also subjected to *in vivo* selection through multiple steps of tumour formation and isolation of undifferentiated cells. Thus there are multiple windows of opportunity in which TERA2.sp12 cells could have acquired a reduced differentiation potential. Along these lines, it would be interesting to directly compare the differentiation of tumours derived from the parent cell lines TERA and TERA2 with TERA2.sp12 to determine whether an increasingly restricted differentiation phenotype is observed in tumour explants from these cells respectively.

If for example TERA2.sp12 cells have lost the ability to differentiate along mesodermal lineages, this would in part explain the reduced complexity of structures observed within these tumours compared to ES cells. Complex epithelial-mesenchymal interactions control the formation of organized tissue structures during normal embryogenesis, and the absence of mesoderm lineages would prevent these interactions from occurring and therefore the establishment of organised tissue structures.

An alternative explanation for the restricted differentiation observed in TERA2.sp12 derived tumours

The presence of a stem cell population and the reduced variety of differentiation and complexity in TERA2.sp12 derived tumours compared to ES derived tumours is likely to be inherently linked. This suggests an alternative means of explanation for the more restricted range of phenotypes manifested in TERA2.sp12 derived tumours than the hypothesis that TERA2.sp12 cells possess a restricted differentiation potential. In this alternate hypothesis, I suggest that TERA2.sp12 cells injected into immuno-compromised mice are slower to embark on differentiation compared to ES cells, with the majority of cells initially proliferating as stem cells, such that by the time the tumour has become large enough to be palpable and is removed, only early forms of differentiation, (such as ectoderm and some simple endoderm) are observed. In essence this hypothesis proposes that embryonal carcinoma cells do not form such a range of differentiated structures and germ layer derivatives compared to embryonal stem cells because they are in less advanced phases of differentiation. To investigate this hypothesis, very young tumours derived from TERA2.sp12 cells and ES cells could be examined. If such TERA2.sp12 derived tumours consisted mainly of stem cells and early ES derived tumours demonstrated a more restricted range of phenotypes (due to less time for differentiation to occur), this would provide supporting evidence for this hypothesis.

Alternately, older tumours from TERA2.sp12 cells could be examined to investigate whether the increased time allowed for more advanced differentiation and the generation of derivatives of all three germ layers. However, this is not practically possible when the welfare of the host is taken into consideration, because the tumour would become very large and consequently place unacceptable levels of stress on the host.

As previously discussed, a recent report documents the ability of a 'culture adapted' human embryonic stem cell sub-line to form a tumour containing a small stem cell component after injection into immune-deficient mice. No accompanying restriction in differentiation was reported. However, this may reflect the low incidence of stem cells generated within these tumours (only one population in one tumour was detected), in the tumours generated from transplantation

5.3.5 Conclusion

Although initial histological examination indicated TERA2.sp12 cells exhibited restricted differentiation to ectodermal derivatives during tumour formation *in vivo*, subsequent immuno-staining analyses identified derivatives of endoderm as well as ectoderm. No solid evidence for mesoderm differentiation was generated, suggesting that TERA2.sp12 cells may still show a more restricted differentiation potential compared to human ES cells in their ability to form all three germ layers. A sub-population of stem cells was identified in every tumour examined, reflecting the malignant nature of TERA2.sp12 cells. Two alternative hypotheses for the restricted differentiation of TERA2.sp12 cells were presented: The first hypothesis proposes that the accumulation of mutations in embryonal carcinoma cells under selection pressure to maintain a malignant phenotype has led to a loss of functional genes that confer the ability to differentiate along certain lineages, and this is reflected in the restricted differentiation phenotypes observed *in vivo*. The second hypothesis proposes that the reduced complexity and range of differentiation observed in TERA2.sp12 derived tumours is a result of a delay in differentiation of injected TERA2.sp12 cells, such that only early stages of *in vivo* differentiation are observed in harvested tumours. This hypothesis implies that TERA2.sp12 cells are not restricted in their developmental potential and under suitable conditions are able to differentiate into all three germ layers. Although the observations of predominantly ectodermal differentiation *in vitro* previously reported in studies of TERA2.sp12 cells support the first hypothesis, this may reflect the limited range of culture conditions and growth factors used. In the previous experimental chapters I have demonstrated that TERA2.sp12 cells have the ability to differentiate into endoderm and trophoectoderm and express early markers of mesoderm differentiation. It is also likely that TERA2.sp12 cells exhibit a restricted differentiated phenotype *in vivo* as a result of the combined effects described in both hypotheses, or that TERA2.sp12 cells are not restricted to ectodermal

differentiation but exhibit an increased preference for differentiation along this lineage due to the accumulation of mutations in a few key genes.

In summary, taken together with experiments in earlier chapters this data suggests that TERA2.sp12 cells are not restricted to ectodermal differentiation *in vitro* or *in vivo*. The tumours derived from TERA2.sp12 injection into immune-deficient mice do not demonstrate the formation of all three germ layers, although the strong malignant component within these tumours may have limited the extent of time for differentiation resulting in less advanced phenotypes. In future experiments I suggest a more comprehensive survey *in vitro* using a range of growth factors to more exhaustively determine the differentiation potential of TERA2.sp12 cells. Most importantly future experiments should specifically test whether TERA2.sp12 cells can form mesoderm derivatives by treating cultures with previously described mesoderm inducers (such as the TGF β ligand Activin). Determining whether TERA2.sp12 cells can form complex mesodermal derivatives after prolonged incubation *in vivo* would be difficult as previously discussed. One suggestion to overcome this problem is to subject TERA2.sp12 cells to mesoderm inducing treatment prior to transplantation into immuno-compromised mice. If TERA2.sp12 cells are subsequently demonstrated not to retain the ability to differentiate into mesodermal derivatives, it would be interesting to test whether co-transplantation with mesoderm progenitors would allow for the formation of more complex structures similar to the those observed in ES cell derived tumours.

CHAPTER 6
GENERAL DISCUSSION

6.1 SUMMARY OF THE FINDINGS FROM THIS STUDY

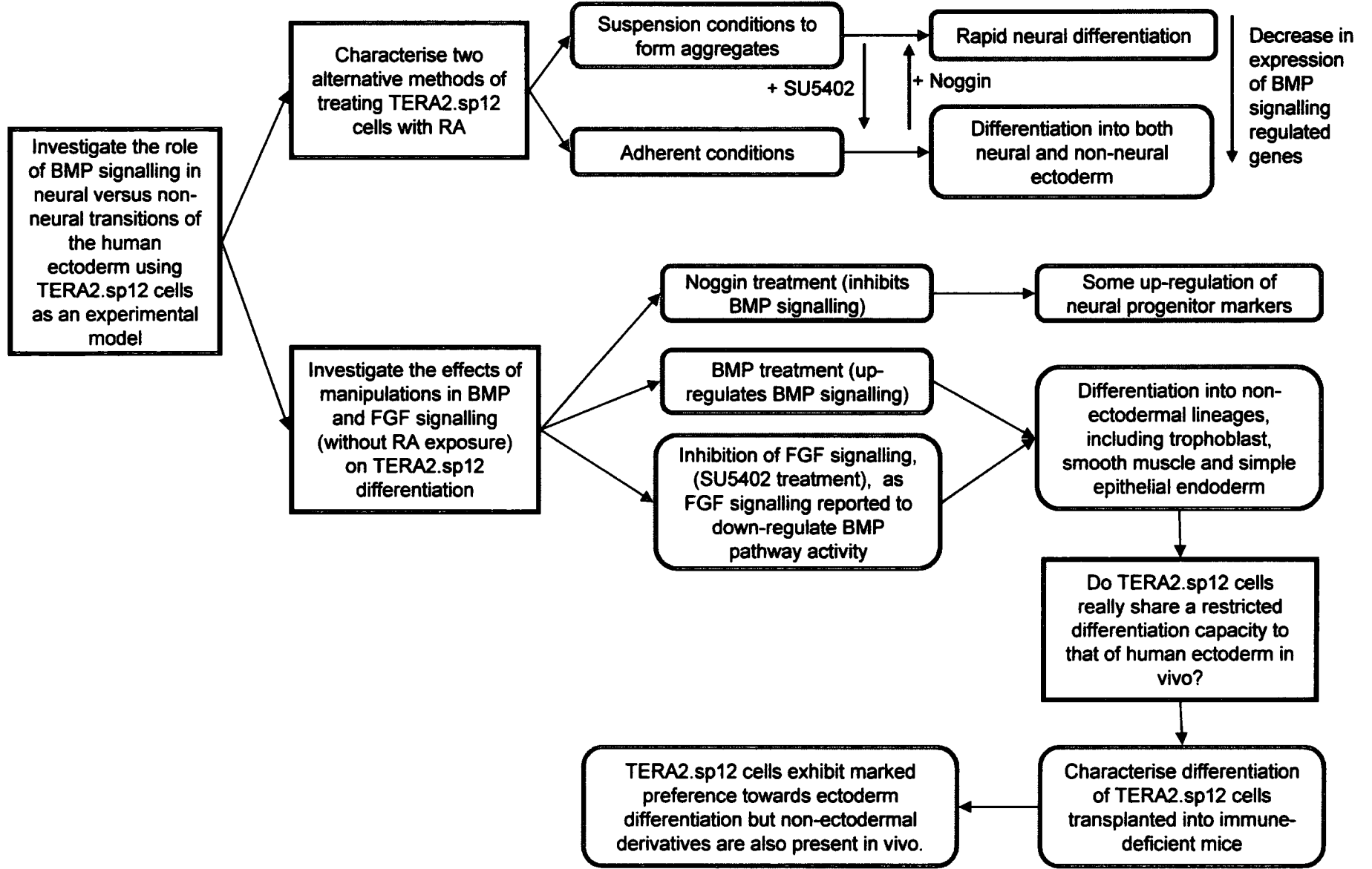
The central aim of this study was to investigate the mechanisms governing the transition of developing human ectoderm into neural or non-neural progenitors using TERA2.sp12 cells as an experimental model, with focus on determining whether the role of BMP signalling in this event is conserved in human development. A flow chart in Figure 6.1 summarises the main routes of investigation in this study.

To begin with, the RA induced differentiation of TERA2.sp12 cells under two alternative culture conditions was characterised, in which the level of neural differentiation was markedly different. TERA2.sp12 cells treated with RA under suspension culture conditions to form aggregates demonstrated more rapid and extensive differentiation into neural derivatives compared to adherent cultures. In contrast to cultures treated under suspension conditions, RA treated adherent cultures also displayed both neural and non-neural differentiation, (which was characterised as early non-neural ectoderm in this study). The dissociation and culture of TERA2.sp12 cells under suspension conditions draws parallels with dissociation experiments in *Xenopus* ectoderm explants (Grunz & Tacke 1989, Hemmati-Brivanlou & Melton 1997) and changes in BMP signalling as a result of dissociation and culture in suspension were hypothesised to be influencing neural versus non-neural ectoderm fate decisions in TERA2.sp12 cultures.

Molecular analysis of components reported to be transcriptionally up-regulated by BMP signalling (Hollnagel *et al.* 1999, Suzuki *et al.* 1997) supported the hypothesis that BMP signalling is up-regulated in TERA2.sp12 cells cultured under suspension conditions compared to adherent cultures, and the manipulation of the BMP signalling pathway was demonstrated to markedly influence the level of neural differentiation in TERA2.sp12 cells. This indicates that a key role for BMP signalling in inhibiting neural induction in the early ectoderm is conserved in human development. However, under the conditions used in this study, the manipulation of BMP signalling in the absence of the differentiation inducer RA was not sufficient to induce differentiation into either non-neural ectoderm or committed neural progenitors in TERA2.sp12 cells. In response to BMP treatment TERA2.sp12 cells formed non-ectodermal derivatives, (trophoblast, endoderm and smooth muscle lineages), similar to the reported differentiation of human ES cells into either endoderm or trophoblast derivatives in response to BMP treatment (Pera *et al.* 2004, Xu *et al.* 2002). In mouse ES cells, BMP treatment has been demonstrated to induce differentiation into non-neural ectoderm, however only under certain conditions, such as in cells that have been previously been treated with the neural differentiation inducing factor SCID (Kawasaki *et al.* 2000). The

Figure 6.1

Flow Chart Summarising The Main Investigations In This Study



presence of growth factors in the serum may have influenced the effects of BMP treatment on the differentiation of these cells, and further investigations with manipulations of the BMP pathway and culture conditions are necessary to determine whether, under conducive conditions, BMP pathway up-regulation is able to induce the differentiation of TERA2.sp12 cells towards a non-neural ectoderm progenitor phenotype, or whether other signalling cues are also required.

Correspondingly, treatment of TERA2.sp12 cells with the BMP inhibitor Noggin was not sufficient to differentiate the population of TERA2.sp12 cells towards a committed neural progenitor phenotype, although some neural progenitor markers such as Nestin were up-regulated in a proportion of the culture. This may reflect an insufficient level of BMP signalling inhibition, or alternatively suggests that other signalling cues are required before TERA2.sp12 cells are competent to respond to an inhibition of BMP signalling by differentiating into neural precursors. Pera *et al.* (2004) had also reported that treatment of human ES cells with Noggin generated cells which under subsequent neural progenitor inducing conditions, were able to generate greater numbers of neural derivatives than untreated cells. However the generation of neural progenitors from human ES or EC cells from inhibition of BMP signalling alone has not yet been demonstrated.

Treatment of TERA2.sp12 cells with the FGF receptor 1 inhibitor SU5402 was demonstrated to induce a markedly similar response to BMP treatment. As FGF mediated signalling has been reported to inhibit BMP pathway activity through inhibitory phosphorylation of Smad1 (Pera *et al.* 2003), this mechanism may explain the similar response of TERA2.sp12 cells to either BMP pathway stimulation or FGF pathway inhibition. Further experiments are required to determine whether this is indeed the mechanism by which FGF mediated signalling inhibits the BMP pathway in TERA2.sp12 cells, and also to fully characterise the differentiation of these cells in response to changes in BMP pathway activity.

Culture of TERA2.sp12 cells under suspension conditions in the absence of RA was also demonstrated to induce differentiation, with a proportion of cells expressing markers of endoderm and mesoderm progenitors. The aggregates formed shared similarities with early embryoid bodies derived from ES cells, further indicating that TERA2.sp12 cells are more similar to human ES cells than previously thought. However, TERA2.sp12 cells cultured to form aggregates differentiated even though they were subjected to the same media conditions which are routinely used to maintain self-renewal. In contrast embryoid bodies are formed from ES cells cultured under suspension conditions and in the absence of factors necessary for stem cell maintenance, (such as fibroblast feeders or growth factors). The results from this study identify the potent effects of aggregation alone on differentiation, which warrants further investigation as this may more closely reflect the signalling environment of the early

embryo and also of cells within teratomas.

As TERA2.sp12 cells had demonstrated their capability of differentiating into non-ectodermal lineages during these investigations *in vitro*, in the last chapter of this study the differentiation potential of TERA2.sp12 cells was more fully explored by further characterising the derivatives formed in tumours derived from transplanting TERA2.sp12 cells into immune-deficient mice. This investigation demonstrated that TERA2.sp12 cells have a greater differentiation potential than previously thought, and are capable of forming non-ectodermal derivatives *in vivo*, although the range and complexity of differentiation is still markedly reduced compared to tumours derived from human ES cells. Further characterisation of these derivatives with a range of lineage markers is necessary to determine the full range of differentiation in these tumours.

6.2 DISCUSSION

In characterising the effect of BMP signalling on neural differentiation of TERA2.sp12 cells, this investigation has touched on key aspects that relate to both human development and the field of embryonic stem cell research, which are reviewed in the following discussion.

6.2.1 The effects of dissociation and aggregate formation

In this study, the remarkable effects of dissociation and culture under suspension conditions on both up-regulated neural differentiation (RA treated cultures) and also differentiation into early derivatives of all three germ layers (untreated cultures) has been characterised in TERA2.sp12 cells. As previously discussed, temporary dissociation and culture under suspension conditions has been used in many systems to induce differentiation. However, the key signalling events that underpin these changes in behaviour have not yet been addressed. As previously suggested, one such event may be a down-regulation in BMP pathway activity mediated by an up-regulation in MAPK activity associated with dissociation (Kuroda *et al.* 2005). However the cells are then exposed to more complex and 3-dimensional interactions with increased cell-cell contacts as they form aggregates in suspension, mediating more localised signalling pathways. This may more closely mimic the environment of cells in a developing embryo, and that also that of cells within teratomas, in which more extensive differentiation is observed (as shown in Chapter 5).

Evidence of a community effect in the down-regulation of stem cell marker expression was observed in TERA2.sp12 cells grown to form aggregates under suspension conditions without RA, suggesting that localised signalling to down-regulate stem cell marker expression was occurring: Characterisation of these signalling events would be pertinent to several aspects of development-particularly in the down-regulation of stem cell markers in the

developing blastocyst.

The results of this study do not clearly distinguish whether the changes in behaviour of TERA2.sp12 cells treated with RA under suspension culture conditions (as opposed to adherent cultures) are from the initial state of dissociated single cells in suspension or the effects of growth in a more three dimensional context as aggregates form, although it is likely that the results of this study were generated from a combination of these effects. However, preliminary experiments where suspension cultures were treated with RA immediately after dissociation and culture in suspension, (compared to 24 hours later) exhibited a less marked up-regulation of neural differentiation markers, indicating that this initial stage is key to promoting neural differentiation. This correlates with the proposed mechanism by which MAPK mediated BMP pathway down-regulation occurs as a direct result of dissociation. Careful analysis of MAPK and BMP pathway activity is needed to confirm this.

6.2.2 Investigating the default model of neural induction in humans

Mimicking *Xenopus* ectoderm explant dissociation experiments in mammalian cells

Early in this study, parallels were drawn between aggregate formation and the dissociation experiments in *Xenopus* naïve ectoderm explants which induced neural differentiation. Although there were several similarities, namely the initial dissociation step and its effects on neural differentiation, there were also inherent differences between these experiments (such as the presence of serum and/or RA in TERA2.p12 cultures) and it would be interesting to more closely model the early *Xenopus* explant dissociation experiments with TERA2.sp12 cells: For example, one could investigate whether culture of dissociated TERA2.sp12 cells under suspension conditions (without RA) in either very low serum conditions or with no serum generates entirely neural differentiation, and whether this effect can be rescued by the addition of either FGFs or BMPs. However, difficulties may be encountered in maintaining cell survival under low serum conditions. Shulz *et al.* (2004) report that transfer of colonies of human embryonic stem cells into suspension conditions and culture in media with serum replacer promotes neural differentiation, including into dopaminergic neurons, and that this is blocked by early exposure to BMP4. This indicates that simply removing serum and culturing in suspension (without the dissociation step) encourages neural differentiation, corresponding somewhat with the default theory in that a reduced signalling environment is conducive to increased neural induction.

Other approaches to investigate the default model of neural induction

In the course of this investigation adherent cultures of TERA2.sp12 cells were treated with Noggin to actively inhibit BMP signalling. This induced an up-regulation of some neural

progenitor markers such as Nestin, but only in a proportion of the population, and was not sufficient to induce neural differentiation. This may reflect the requirement for a greater inhibition or complete abrogation of BMP pathway activity, which could be generated by using a cocktail of BMP inhibitors at high concentrations (or using BMP receptor inhibitors). Alternatively, this may reflect a requirement for other instructive signals for neural induction to occur in TERA.sp12 cells (contrary to the default model of neural induction). Again the use of serum in the culture media provides an undefined source of a host of growth factors that could be influencing differentiation in TERA2.sp12, so ideally experiments should be carried out using either low serum conditions or with no serum to minimise this effect.

Another aspect that hasn't been addressed is the effect of the initial low seeding density on the results of these experiments: Seeding at a low density may encourage TERA2.sp12 cells to differentiate and also influence differentiation fates. Within the first few days of seeding at a low density a proportion of the population was observed to down-regulate the stem cell marker SSEA3, and Andrews *et al.* (1984b) reported an increased expression of Fibronectin and changes in morphology in TERA2 derived clonal lines cultured under low densities. Changes in seeding density have also been demonstrated to modify the differentiation fate of human embryonic stem cells in response to BMP signalling (Xu 2002). It would therefore be highly relevant to investigate whether similar results are generated in experiments where TERA2.sp12 cells are treated under maintenance density conditions, or even as confluent cultures, although the subsequent passaging required would introduce another variable to these experiments. This would determine whether signalling cues generated or eliminated as a result of seeding at low density have modulated the response of TERA2.sp12 cells to these treatments. Conversely, one could determine whether maintaining cells at very low densities (by constant reseeded), results in robust differentiation along a characterised lineage.

A very recent study by Smukler *et al.* (2006) has markedly demonstrated the effects of seeding density and the absence of serum on differentiation behaviour in mouse embryonic stem cells, and also generated evidence strongly supporting the default model of neural induction: They reported that mouse embryonic stem cells differentiate into primitive neural stem cells in the absence of any extrinsic cues (although a large proportion of cells do not survive) and proposed that neural differentiation may indeed be the default state of cells that are not exposed to extrinsic cues. In their investigation, single mouse embryonic stem cells seeded at very low density (to abrogate intercellular signalling) in the absence of serum or feeder layers (to remove extrinsic factors) displayed neural stem cell marker expression, although subsequently the majority of cells died. Furthermore, they demonstrated that treatments to increase viability – such as the addition of exogenous survival factors, activation

of the endogenous cAMP pathway, or genetic interference with apoptosis – generated increased numbers of primitive neural stem cells, without any non-neural differentiation being detected. They also demonstrated that FGFs were important for the proliferation and subsequent survival but not the generation of the default pathway derived primitive neural stem cells. It is interesting to note that ES cells seeded under these conditions in minimal media and treated with SU5402 at the same concentrations used in this study, were not inhibited from differentiating into primitive neural stem cells, and in marked contrast to TERA2.sp12 cells exhibited no evidence of trophoblast or endoderm differentiation. This suggests that the presence of other factors in the serum of cultures in which TERA2.sp12 cells were treated, or generated as a result of the higher seeding density in which they were cultured, may have influenced the effect of SU5402 on TERA2.sp12 cell fate. It would be highly valuable to mimic these experiments in a human model such as TERA2.sp12 cells, and determine if they exhibit a similar ‘neural induction’ response.

6.2.3 Maintenance of the stem cell phenotype

A thorough understanding of the pathways regulating stem cell maintenance and self-renewal in stem cells is necessary not only for efficient propagation of human embryonic stem cells in the absence of animal derived products, but is also important in understanding and minimising tumour formation in transplanted grafts. From a developmental perspective, stem cell regulation is inherently linked with differentiation and cell fate – as many of the pathways involved maintain the stem cell phenotype at least in part by inhibiting specific routes of differentiation, such as the inhibition of pro-neural transcription factors by BMP pathway components such as Id hlh factors (as described in Ying *et al.* 1993). Accordingly, gaining an understanding of the mechanisms controlling stem cell maintenance will have direct relevance to elucidating the mechanisms controlling early induction events and cell fate in the embryo.

Xu *et al.* (2005) recently demonstrated that human embryonic stem cells could be maintained undifferentiated in culture without serum, feeder layers or conditioned medium when exposed to both high levels of FGF and the BMP inhibitor Noggin. Thus they proposed that through antagonising the BMP signalling pathway the stem cell phenotype was maintained in these cells. However, human embryonic stem cells could not be clonally propagated using this method, suggesting that other signalling pathways are also necessary to maintain the stem cell phenotype. In support of this observation, TERA2.sp12 cells seeded at low densities and then treated with Noggin did not maintain the expression of stem cell markers, indicating that inhibition of BMP signalling alone was not sufficient to maintain undifferentiated TERA2.sp12 cells.

In the last two years several studies have rapidly advanced our understanding of the

pathways involved in maintenance of human stem cells, and a key point that emerges is the marked differences in the pathways involved compared to mouse embryonic stem cells. In summary, activation of the Wnt signalling pathway has also been demonstrated to maintain the stem cell phenotype in human embryonic stem cells cultured without feeder layers or conditioned medium (Sato *et al.* 2004), and two recent reports by Vallier *et al.* (2005) and James *et al.* (2005) have demonstrated that activation of another branch of the TGF β signalling pathway through Nodal/Activin signalling is necessary for stem cell maintenance in human ES cultures. They also demonstrate that both FGF and Wnt mediated maintenance of stem cell pluripotency requires an active Nodal/Activin signalling pathway, and Vallier *et al.* report that human embryonic stem cells can be maintained long-term in unconditioned, serum free media without serum replacer or growth on feeder layers, when cultured in the presence of FGF2 and either Nodal or Activin. However, in all cases these cells were cultured on matrigel and maintained in colonies or at high densities, so more factors may be necessary to maintain human embryonic stem cells as single cells in the absence of matrigel or feeder layers. Figure 6.2 outlines the pathways reportedly involved in maintenance of the pluripotent stem cell phenotype in human embryonic stem cells.

It could prove highly constructive to directly compare the expression of these signalling pathway components between human embryonic stem cells and a feeder independent embryonal carcinoma cell line such as TERA2.sp12, as it is likely that these pathways also play key roles in maintaining the stem cell phenotype of TERA2.sp12 cells. A key step is to establish whether TERA2.sp12 cells can also be maintained in serum free media with the addition of Activin/Nodal and FGF, and whether these components are necessary, or whether TERA2.sp12 cells have acquired activating mutations in these pathways or exhibit strong autonomous signalling loops.

The signalling conditions required to maintain human embryonic stem cells as single cells or at very low seeding densities in the absence of a feeder layer or matrigel have not yet been elucidated: As seeding at low density also induces TERA2.sp12 cells to down-regulate stem cell markers, TERA2.sp12 cells could provide a simpler experimental system in which to investigate the roles of a range of factors in maintaining the stem cell phenotype in single cells. For example, one could investigate the ability of exogenous addition of a range or combination of signalling ligands to maintain levels of SSEA3 expression in TERA2.sp12 cells seeded at a very low density.

6.2.4 Manipulation of the BMP and FGF mediated signalling pathways

Considering temporal regulation of BMP signalling

A key issue that emerged from the study of BMP signalling in TERA2.sp12 cells (see Chapter

Schematic Outline Of Key Signalling Pathways That Are Thought To Regulate Stem Cell Maintenance In Human ES Cells

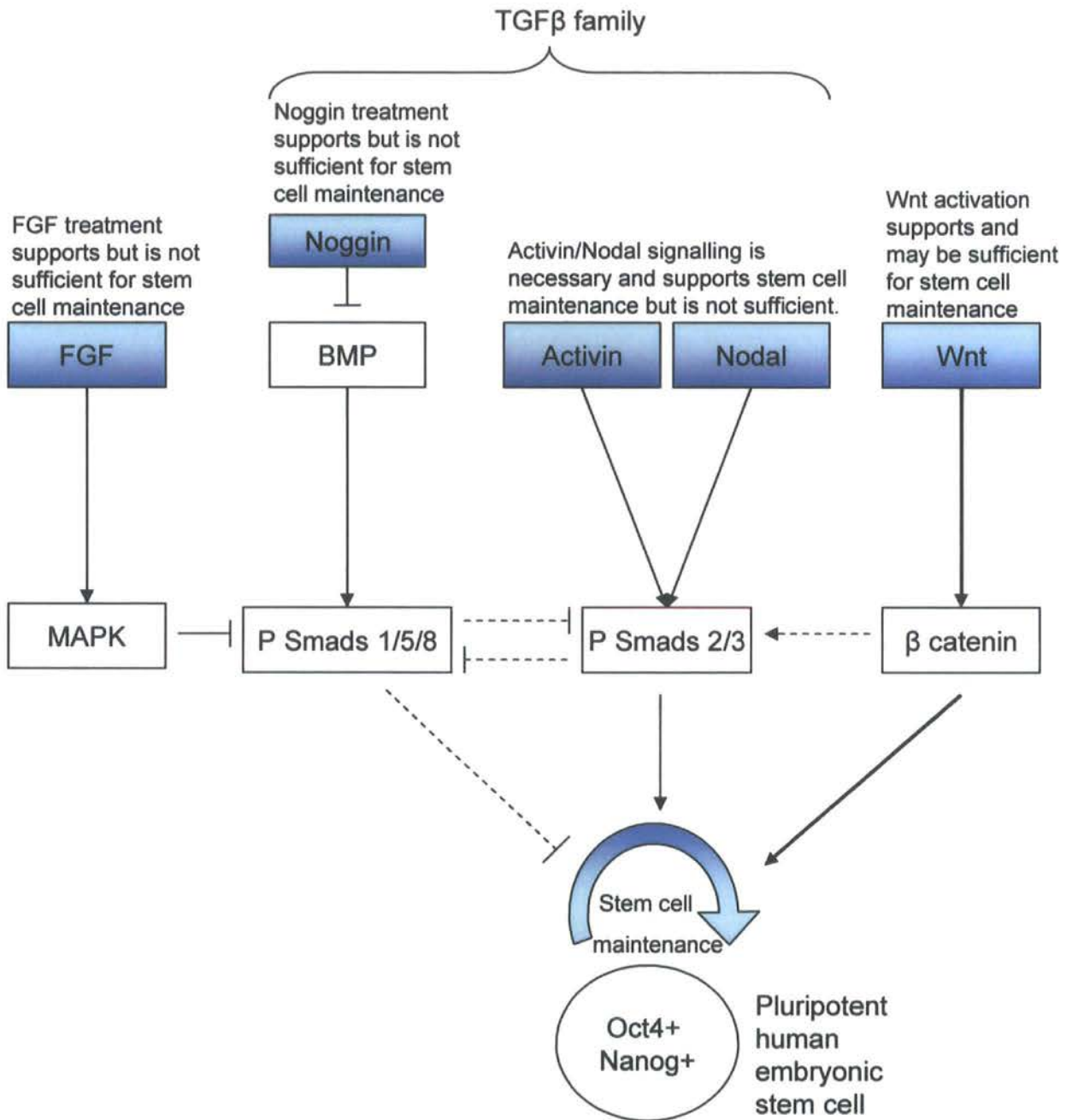


Figure 6.2. An outline of the signalling pathways thought to be important in maintaining the stem cell phenotype in human embryonic stem cells. Xu et al. (2005) reported that FGF and Noggin treatment can maintain undifferentiated colonies of human embryonic stem cells in the absence of feeder conditioned medium when cultured on matrigel in the presence of serum replacer. Sato et al. (2004) reported that activation of the Wnt signalling pathway was sufficient to maintain human ES cells in the absence of feeder conditioned medium or serum, although cells were cultured as colonies on matrigel. James et al. (2005) and Vallier et al. (2005) demonstrated that Activin/Nodal signalling was necessary to maintain human stem cell maintenance, and Wnt mediated stem cell maintenance required an active Activin/Nodal signalling pathway. Vallier et al. also report that FGF mediated stem cell maintenance requires an active Nodal/Activin signalling pathway, and that culture with FGF2 and either Activin or Nodal is sufficient to maintain human embryonic stem cells in the absence of feeder layers, conditioned medium, serum or serum replacer. No reports have yet demonstrated the sustained maintenance of human embryonic stem cells as single cells and in the absence of matrigel and serum replacer.

4) was the wide-ranging effects that manipulations in BMP signalling can generate, such that in TERA2.sp12 cells induced to differentiate down ectodermal lineages by RA, BMP treatment markedly inhibited neural differentiation and promoted non-neural ectoderm-like differentiation, whereas BMP treatment of TERA2.sp12 cells alone induced differentiation into derivatives that were previously uncharacterised in this cell line – such as trophoblast, endoderm and smooth muscle. It is also important to consider embryonic stem cells in the context of their ability to form all the tissues of the body, and note that many of these derivatives are temporally and differentially modulated by BMP signalling. Consequently ES cells provide the opportunity to explore the effects of modulating BMP signalling in different embryonic cell types. For example, stem cells of the central nervous system (CNS) respond to BMPs by differentiating into a wide variety of dorsal CNS and neural crest cell types: BMP 2, 4 and 7 are reported to inhibit neurogenesis and induce astrocytogenesis of mouse fetal neuroepithelial cells, and reduce oligodendrocyte differentiation (Gomes *et al.* 2003, Yanagisawa *et al.* 2001). It is interesting to note that BMP4 mediated glial differentiation has been reported to occur via the FRAP/STAT pathway, (Rajan *et al.* 2003), for review see (Hall & Miller 2004). In addition, BMP signalling has been reported to induce dendritic growth in cultured sympathetic neurons (Lein *et al.* 2002), induce differentiation of mesencephalic precursors into dopaminergic neurons (Brederlau *et al.* 2002) and modulate the differentiation of post-natal cells of the rat cerebellum (Anglely *et al.* 2003). Thus the effects of BMP modulation on the differentiation and specification of these types could also be investigated in human TERA2.sp12 cells that have been previously differentiated to form neural sub-types.

Modulating BMP pathway activity in the context of other signalling cues

Another promising avenue of investigation is to determine whether various concentrations or combinations of BMPs induce differentiation of TERA2.sp12 cells towards different lineages, in order to more fully elucidate the graded effects of BMP signalling on differentiation: BMP treatment has been demonstrated to induce both trophoectoderm and primitive endoderm differentiation in human ES cells (Pera 2004, Xu 2002) and it is plausible that low levels of BMP signalling may instead promote differentiation into non-neural ectoderm. In addition, it would be highly constructive to investigate the effect of BMP treatment in embryonic cells subjected to different signalling cues – such as in cells cultured with or without serum and/or at very low densities, in the presence of Nodal/Activin signalling or Wnt signalling, and in the presence/absence of FGF mediated signalling. Evidently control experiments that characterise the individual effects of these signalling cues on TERA2.sp12 cells would be essential. This would generate a better understanding of both the ability of other signalling pathways to block BMP mediated behaviour (such as inhibiting differentiation), and also their ability to perhaps modify the effects of BMP pathway activity on differentiation (for example in the presence of

high FGF signalling BMP treatment may induce trophoectoderm as opposed to endoderm differentiation (Pera 2004, Xu 2002).

Results from this study indicate that in TERA2.sp12 cells, a principle function of FGF signalling is to antagonise BMP pathway activity, as treatment with the FGF receptor inhibitor SU5402 results in differentiation analogous to treatment with BMP2 or BMP4. As discussed previously, in the developing *Xenopus* embryo FGF mediated inhibition of BMP pathway activity through Smad 1 phosphorylation has been proposed to play a key role in promoting neural induction (for review see Pera 2003). This hypothesis remains to be substantiated in TERA2.sp12 cells – and future investigations should determine whether high FGF signalling can block BMP mediated pathway activity and whether this is indeed mediated through ERK1/2 phosphorylation of Smad 1.

However, other signalling cues may function independently of their activity on the BMP pathway, such as by changing the competence of the cell to respond to BMP signalling, or changing the state of the cell such that it will respond differently to BMP pathway activity. If future studies demonstrate that TERA2.sp12 cells dramatically modify their differentiation response to BMP treatment when subjected to different signalling cues, by comparing the results with data demonstrating the effects of various concentrations of recombinant BMPs on differentiation fate, you could also gauge whether these effects are generated simply through attenuating BMP pathway activity or whether the resulting differentiation is unique to that induced by changes in BMP signalling alone. The results from these experiments would have direct relevance to embryonic development, increasing our understanding of how different combinations of signalling cues act on identical cells from the blastocyst to generate the complex and wide ranging derivatives required in a developing embryo.

6.2.5 Working with TERA2.sp12 cells – a useful experimental tool in which to study human development?

With regard to the rapid advances in the last few years in the characterisation and establishment of multiple human embryonic stem cell lines, together with recent technological advances that have greatly reduced their extensive culture requirements, the question naturally arises as to whether TERA2.sp12 cells – as an EC cell line – are no longer a compelling system in which to study human development. As previously discussed, TERA2.sp12 cells provide a robust and simple culture system for manipulation experiments. However, as cancer cells they are malignant and display genetic instability, and like all human EC cell lines typically have undergone extensive culture *in vitro*, an opportunity in which to accumulate further mutations, some of which may reflect a reduced capacity for differentiation. In fact recent reports also document the accumulation of mutations and the formation of aneuploid

karotypes in human embryonic stem cell lines that have undergone prolonged culture, (specifically the recurrent gain of chromosomes 12 and 17q) demonstrating that culture conditions are selective for mutations (Draper *et al.* 2004). It is interesting to note that the accumulated chromosomal abnormalities in human ES cells are typically similar to those observed in human EC cells, and are associated with sites of stem cell maintenance. Significantly, these mutations have not been reported to reduce the pluripotency of these embryonic stem cell lines *in vitro* or *in vivo* (Andrews *et al.* 2005, Draper 2004, Liu *et al.* 1997).

As malignant cells, EC cell lines are unlikely to be used preferentially for transplantation therapies. However, in one sense their greater tumorigenic tendencies can be exploited: If human EC cell derivatives are manipulated such that they demonstrate integration and differentiation upon transplantation into animal hosts without tumour formation, this provides a highly rigorous test of the ability of a technique to prevent tumour formation upon transplantation, which could then be safely applied to human ES cells. The current approach for transplantation therapies is to differentiate embryonic stem cells extensively *in vitro* prior to transplantation, although this also can have a negative impact on survival after transplantation. The potential of extensive *in vitro* differentiation prior to transplantation to prevent tumour formation has been successfully demonstrated with a subclone of the NTERA2 embryonal carcinoma line. Differentiated neurons derived from RA treated NT2D1 cells have been reported to generate significant behavioural recovery upon injection into ischemic rats in the absence of tumour formation, and in current clinical trials with patients suffering from stroke or cerebral (basal ganglionic) infarction, abnormal tissue masses or tumour formation from NT2D1 neuron cell grafts have not been detected. These transplantation studies and the current progress of clinical trials are extensively reviewed by Newman *et al.* (2005).

Despite their malignant character, as demonstrated in this study, in many aspects embryonal carcinoma cell lines may exhibit very similar differentiation behaviour to human ES cells and as a simple culture tool, provide a means in which to investigate key questions in human embryonic development that could subsequently be confirmed in human stem cell lines. In order to truly validate observations of human development derived from studies of human TERA2.sp12 cells, it would be necessary to test these results in not one but multiple other human embryonic stem cell lines, to demonstrate that the results generated are not due to the variant behaviour of an individual cell line. With the increasing availability of embryonic stem cell lines, this is becoming easier.

In summary, although the emphasis of human stem cell research has undoubtedly shifted to human embryonic stem cell lines, robust and well characterised embryonal

carcinoma cell lines such as TERA2.sp12 cells remain a useful tool if they are studied – not in isolation – but together with embryonic stem cells, as a simple and robust culture model in which to carry out investigations.

6.2.6 The future of embryonic stem cell research

Models in which to study human embryogenesis

Embryonic stem cells provide a highly accessible model in which to study events such as germ layer induction and lineage specification. However, this requires the development of defined protocols for lineage specification which do not require serum or other animal derived products, and the development of further markers of specific lineages. Additionally, the functions of specific genes in early development could be explored using gene knock out techniques, such as RNAi mediated inhibition of specific genes.

As with all culture models, it is also important to take into consideration the limitations of *in vitro* experiments, and try to test any results in an *in vivo* system. In the case of humans this is not possible, but other mammalian developmental models are well characterised, and human ES cell derived neural progenitors transplanted into the ventricles of newborn mice and adult rats exhibit migration and neural differentiation (Reubinoff *et al.* 2001, Tabar *et al.* 2005, Zhang *et al.* 2001). In addition, chimeric embryos have recently been successfully generated from human embryonic stem cells injected into either a developing chick or mouse embryo (Goldstein *et al.* 2002, Muotri *et al.* 2005).

These reports of successful differentiation and integration of human ES cells and their derivatives with host murine and chick embryonic tissues have important implications. The study of chimeric embryos provides a means of exploring the differentiation potential of human embryonic stem cells in the context of early development *in vivo* and importantly, suggests that fundamental developmental pathways are indeed conserved between these species. Future studies utilising the manipulation of embryonic stem cells both *in vitro* and *in vivo* will allow researchers to unravel the mechanisms of human embryonic development and also possibly reconcile differing developmental theories between species – such as the conflicting theories for the mechanism of neural induction.

Clinical/Therapeutic applications

As discussed, significant advances have been made in characterising human embryonic stem cells and unravelling various aspects of human development. In addition, significant steps have been taken towards the use of embryonic stem cells in medical applications. These include providing differentiated derivatives for drug testing and for transplantation therapies in diseases generated by the loss of specific cell types, such as diabetes, heart disease, liver

disease and Parkinsons disease. Since Parkinsons disease is caused by loss of a specific neural sub-type (dopaminergic neurons), extensive research has been invested towards using embryonic stem cell derived dopaminergic neurons in transplantation therapies and subsequently will be discussed here as a specific example. The key requirements for the clinical application of stem cells include the ability to direct cells along a specific lineage in defined conditions and isolate large numbers of these cells for transplantation, ensuring that the transplant is not contaminated with other differentiated phenotypes or with undifferentiated (potentially tumour forming) cells.

Using a rat model for Parkinson's disease, Kim *et al.* (2002) demonstrated that mouse ES cell-derived dopamine neurons survived, developed functional synapses and displayed electrophysiological properties characteristic of midbrain neurons following transplantation into these animals. In addition, the animals showed some recovery, suggesting that the transplanted cells were functional. In 2004, Perrier *et al.* reported the directed differentiation of human embryonic stem cell lines (using co-culture techniques) to generate high-yields of dopaminergic neurons (Perrier *et al.* 2004). In addition, recent transplantation experiments with neural progenitors and dopaminergic neurons derived from primate ES cells into the rat model for Parkinsons disease have also demonstrated that a small proportion of dopaminergic neurons survive in the host, but significant functional recovery as a result of improved dopaminergic neuron function has not been generated (Ben-Hur *et al.* 2004, Sanchez-Pernaute *et al.* 2005). Thus to meet the requirements for a pre-clinical trial, researchers need to generate grafts which exhibit extensive survival and integration of the transplanted cells, retain the required dopaminergic phenotype, and furthermore are able to mediate functional recovery. A final obstacle to be overcome is donor/recipient compatibility and graft rejection, although a recent report indicates that human embryonic stem cells are less susceptible to immune rejection than adult cells (Drukker *et al.* 2006).

Another important avenue of investigation with enormous potential is the generation and characterisation of human ES cell lines with genotypes characteristic of different diseases. Human ES cell lines from patients suffering from a variety of different diseases could be generated through the use of somatic cell nuclear transfer (Hochedlinger & Jaenisch 2003). These lines could then be used as *in vitro* models in which to study and further characterise these diseases, and as a model for testing new approaches to treat diseases (see review Keller 2005). To summarise, in order to safely utilise embryonic stem cells and their derivatives in transplantation therapies we need to further characterise the mechanisms governing stem cell maintenance and the specification and differentiation of specific lineages. Currently our understanding is restricted. We have yet to unleash and – more importantly– control the full differentiation potential of human ES cells.

6.2.7 Conclusions

Research using human embryonic stem cells has potent implications for both clinical applications and for the study of human embryogenesis and development. The results of this study have contributed to the elucidation of the role of the BMP pathway in neural versus non-neural transitions of the developing ectoderm in humans. An increased understanding of human embryogenesis and the basic molecular mechanisms that control the specification of lineages will be in turn highly applicable to the manipulation of stem cells towards specific cell lineages for medical applications such as drug development and transplantation therapies.

APPENDIX

7.1 RECIPES FOR CELL CULTURE

ACID WASHED GLASS BEADS

Cover beads with concentrated HCL leave o/night in fume-hood. Pour off HCL. Wash beads three times in distilled water. Drain, autoclave. Leave to dry in vacuum oven. Aliquot 20 beads per test tube. Re-autoclave beads in tubes.

7.2 RECIPES FOR WESTERN BLOT ANALYSIS

LYSIS BUFFER

In 10 mls:

100µl Igepal, (detergent)

500µl 1M TrisCL (ph 8)

500µl 3M NaCl

10µl 1M MgCl

8960µl mqH₂O

Add proteinase inhibitors at a ratio of 1:7

PROTEINASE INHIBITORS

Complete MINI protease inhibitor cocktail tablets (Roche Biosciences)

(Dissolve 1 tablet in 1.5ml MQH₂O.

5X SDS REDUCING BUFFER (PROTEIN SAMPLE BUFFER)

2.5ml 1M Tris pH 6.8

5ml Glycerol

1g SDS

0.05g bromophenol blue

2.5ml 2 beta-mercaptoethanol

10X TRIS-BUFFERED SALINE

6.1g Tris Base

43.8g NaCl

500mls H₂O, adjust pH to 7.5

10% GEL (RESOLVING GEL)

4.9mls dH₂O

2.5mls Tris HCL (pH 8.8)

100ul 10% SDS stock

2.5mls 40% Acrylamide Bis Phosphate, (37.5:1)

Just before pouring into mould add the following setting agents

50µl 10% Ammonium persulfate

5µl TEMED

4% GEL (STACKING GEL)

6.4mls dH₂O

2.5mls Tris HCL (pH 8.8)

100ul 10% SDS stock

1ml 40% Acrylamide Bis Phosphate, (37.5:1)

Just before pouring into mould over the set resolving gel add the following setting agents

50µl 10% Ammonium persulfate

10µl TEMED

10x RUNNING BUFFER

9g Tris Base

43.2g Glycine

3g SDS

dH₂O to 600mls.

2x TRANSFER BUFFER

3g Tris Base

28.8g Glycine

400mls Methanol

600mls dH₂O

PONCEAU STAIN

0.2% Ponceau, 3% trichloro-acetic acid in dH₂O

PONCEAU DESTAIN

5% Acetic acid in dH₂O

BLOCKING BUFFER

5% milk powder, (Marvel)

0.2% Tween 20 (Sigma) in TBS.

DEVELOPING SOLUTION 1

100ul Luminol (Sigma), (250mM in DMSO)

44ul P Coumaric Acid, (Sigma), (90mM in DMSO)

1ml 1M TrisCl ph 8.5

9ml H2O

DEVELOPING SOLUTION 2

6.4µl H2O2

1ml 1M TrisCl ph 8.5

9ml H2O

7.3 RECIPES FOR IMMUNO-STAINING**4% PFA**

Add 4g of PFA powder (Sigma) to 100mls of PBS. Add a few drops of 0.5M NaOH and agitate at 40°C until solution clears, then filter through filter paper.

7.4 RECIPES FOR PCR ANALYSIS**5X TBE BUFFER**

27g Tris Base

13.75g Boric Acid

10ml 0.5M EDTA (pH8), (or 1.861g)

Make up to 500ml with H2O

RUNNING BUFFER

25mls 5x TBE

225mls H2O

AGAROSE GEL

5 ml 5X TBE

45 ml H₂O

1% (0.5g) agarose

Microwave in plastic beaker for 1min (High)

Cool beaker under running water (1-2mins).

Add 2.5ul ethidium bromide, (TOXIC)

Pour into gel cast and leave to set. (Takes 20-40mins, turns opaque when set).

After gel is set, remove casting ends and well casts, place gel in electrophoresis tank. Add running buffer until surface of gel is just covered.

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