Differentiation of Pluripotential Murine Embryonic Stem Cells

A thesis submitted to the University of Adelaide

for the Degree of Doctor of Philosophy

by

Julie-anne Lake, B.Sc. (Hons)

Department of Biochemistry

University of Adelaide

Adelaide, South Australia

January 1996

Summary ix

Statement xii

Acknowledgements xiii

CHAPTER ONE: INTRODUCTION 1

1.1 The Study of Development 2

1.2 Why Study Mammalian Embryogenesis? 3

1.2.1 The Mouse as a Model System for Mammalian Embryogenesis 4

1.3 Murine Embryogenesis 4

1.3.1 Preimplantation Development 4

1.3.2 Postimplantation Development 6

1.3.3 Gastrulation 7

1.3.4 The Origin of Germ Cells 9

1.4 Pluripotential Cell Maintenance During Development 10

1.5 Pluripotential Murine Embryonic Stem Cells *In Vitro* 11

1.5.1 The Isolation of Murine Embryonal Carcinomal (EC) Cells 11

1.5.2 The Isolation of Murine Embryonic Stem (ES) Cells 12

1.5.3 Cytokines which Prevent the Differentiation of ES Cells *In Vitro* 12

1.5.4 ES Cell Differentiation *In Vitro* 14

1.5.5 ES Cells and Gene Targeting 15

1.5.6 The Identification of Mammalian Developmental Control Genes 16

1.5.7 ES Cells and the Identification of Mammalian Developmental Control Genes 17

1.6 ES Cells and ICM Cells 18

1.7 ES Cells as an *In Vitro* Model for Murine Development 19

1.8 Background to the Project 20

1.8.1 The Factors Responsible for X Cell Formation and Maintenance 21

1.8.2 The Gene Expression of X Cells 21

1.8.3 The Significance of the ES to X Cell Transition 22

1.9 Aims and Approach 23

CHAPTER TWO: MATERIALS AND METHODS 25

2.1 Abbreviations 26

2.2 Tissue Culture Materials 27

2.2.1 Chemicals 27

2.2.2 Tissue Culture Plasticware 28

2.2.3 Miscellaneous 28

2.2.4 Cell Staining Kits 29

2.2.5 Cytokines 29

2.2.6 Antibodies 29

2.2.7 Radiochemicals 30

2.2.8 Solutions 30

2.2.9 Media 31

2.2.10 Tissue Culture Cell Lines 32

2.3 Tissue Culture Methods 32

2.3.1 Gelatinised Petri Dishes 33

2.3.2 Determination of Cell Number 33

2.3.3 Preparation of Stem Cell Lines for Storage 33

2.3.4 Thawing Frozen Stem Cell Lines 33

2.3.5 Maintenance of COS-1 Cells 33

2.3.6 Preparation of COS-1 Cell Conditioned Medium Containing Murine LIF 34

2.3.7 Preparation of MED2 Conditioned Medium 35

2.3.8 Routine Maintenance of ES Cells 35

2.3.9 Routine Formation and Maintenance of X Cells 36

2.3.10 Routine Formation of ES Cells from X Cells (X Cell Reversion) 36

2.3.11 Routine Maintenance of XB Cells 37

2.3.12 Harvesting Cells for RNA Preparation 37

2.3.13 ES Cell Assays at Clonal Cell Density 37

2.3.14 X Cell Assays at Clonal Cell Density 39

2.3.15 Spontaneous Differentiation of ES Cells 40

2.3.16 Preparation of Embryoid Bodies 40

2.3.17 Scoring Beating Muscle in Embryoid Bodies 41

2.3.18 Scoring Neurons in Seeded Embryoid Bodies 42

2.3.19 Harvesting Embryoid Bodies for RNA Preparation 42

2.3.20 Differentiation of Cells with Retinoic Acid 42

2.3.21 Cytokine Assays 43

2.3.22 Maintenance of Swiss 3T3 Cells 44

2.3.23 Swiss 3T3 Growth Factor Assay 44

2.3.24 Alkaline Phosphatase Staining 45
2.3.25 Detection of Neuronal Antigens by Indirect Immunofluorescence 45
2.3.26 Detection of SSEA-1 by Indirect Immunofluorescence 46
2.3.27 Generation of Chimeric Mice 46
2.4 Molecular Materials 47
2.4.1 Chemicals and Reagents 47
2.4.2 Radionucleotides 48
2.4.3 Kits 48
2.4.4 Enzymes 49
2.4.5 Buffers 49
2.4.6 DIG Riboprobe Dot Blot Buffers 50
2.4.7 Cloning and Expression Vectors 51
2.4.8 Cloned DNA Sequences 51
2.4.9 Oligonucleotides 52
2.4.10 Bacterial Strains 53
2.4.11 Bacterial Growth Media 53
2.4.12 DNA Markers 54
2.4.13 Miscellaneous Materials 54
2.5 Molecular Methods 54
2.5.1 Restriction Endonuclease Digestion of DNA 54
2.5.2 Gel Electrophoresis 54
2.5.3 Purification of Linear DNA Fragments 55
2.5.4 DNA Ligation Reactions 55
2.5.5 Preparation of Competent Cells 55
2.5.6 Transformation of Competent Cells 56
2.5.7 Rapid Small Scale Preparation of DNA (Mini-prep) 56
2.5.8 Double Stranded Sequencing of Plasmid DNA 56
2.5.9 Large Scale Plasmid Preparation 57
2.5.10 Isolation of Cytoplasmic RNA from Cultured Cells 58
2.5.11 Isolation of RNA from Embryoid Bodies and RA Treated Aggregates 58
2.5.12 Northern Blot Analysis 59
2.5.13 Ribonuclease Protection 60
2.5.14 Synthesis of Radioactive DNA Probes 60
2.5.15 Synthesis of Radioactive RNA Probes 61
2.5.16 Hybridization of Radioactive Probes to Nylon Filters 62
2.5.17 Synthesis of Digoxygenin (DIG) Labelled RNA Probes 62
2.5.18 Dot Blot Assay for DIG Probe Reactivity 63

2.5.19 *In Situ* Hybridisation of Cell Monolayers 63
2.5.20 Phosphorimager Analysis and Photographic Scanning 64
2.5.21 Containment Facilities 65

CHAPTER THREE: DEFINITION OF CULTURE CONDITIONS FOR X CELL FORMATION AND MAINTENANCE 66
3.1 Introduction 67
3.2 MED2 Induces the Differentiation of ES Cells into X Cells in the Presence of LIF at Clonal Cell Density 68
3.3 The Stability of X Cells Cultured in 50% MED2 and LIF 69
3.4 50% MED2 is the Optimal Condition for X Cell Maintenance at Clonal Cell Density 71
3.5 LIF is Required for the Maintenance of X Cells at Clonal Cell Density 71
3.6 Addition of Murine LIF is not Required for X Cell Formation at High Cell Density but Improves X Cell Maintenance 72
3.7 X Cells Revert to ES Cells when Cultured in the Presence of LIF and the Absence of MED2 75
3.8 MED2 Contains Biologically Active Human LIF 76
3.9 Discussion 78

CHAPTER FOUR: GENE EXPRESSION IN X CELLS 81
4.1 Introduction 82
4.2 Expression of Marker Genes that Discriminate Between the ICM and Primitive Ectoderm in X Cells Passaged with and without Added LIF 84
4.2.1 Northern Blot and RNAase Protection Analysis of Pluripotential Cell Marker Gene Expression 84
4.2.2 *In Situ* Hybridisation Analysis of *FGF-5*, *Rex-1* and *Gbx-2* Expression 87
4.3 Comparison of X Cell Cultures and Spontaneously Differentiated ES Cell Cultures 88
4.3.1 The Morphology of Spontaneously Differentiated ES Cell Cultures 88
4.3.2 The Gene Expression of Spontaneously Differentiated ES Cell Cultures 90
4.4 Discussion 91
4.4.1 The Isolation of XB Cells from Embryoid Bodies 92

CHAPTER FIVE: X CELL GENE EXPRESSION DEFINES THREE DISTINCT PLURIPOTENTIAL CELL STATES 94

5.1 Introduction 95

5.2 Gene Expression in Reverted X Cells 96

5.3 The Cyclic Nature of X Cell Gene Expression During Long Term Culture 98

5.4 The Behaviour of X Cell Cultures at Clonal Cell Density 99

5.5 Discussion 101

5.5.1 A Model for X Cell Formation, Maintenance and Differentiation 101

5.5.2 Implications for Pluripotential Cell Isolation from Mammalian Embryos 103

CHAPTER SIX: THE DIFFERENTIATION POTENTIAL OF X CELLS 105

6.1 Introduction 106

6.2 X Cells can Differentiate as Embryoid Bodies. 106

6.3 Comparison of ES, Xp0+ and Xp0- Cell Differentiation as Free Floating Embryoid Bodies 108

6.3.1 The Formation of Cardiac Muscle in ES, Xp0+ and Xp0- Embryoid Bodies 109

6.4 Comparison of ES, Xp0+ and Xp0- Cell Differentiation as Seeded Embryoid Bodies 111

6.4.1 The Formation of Beating Muscle and Neuronal Networks in Seeded Embryoid Bodies 112

6.5 Reverted X Cells and Embryoid Body Formation 113

6.6 X Cells form Neuronal Networks at High Frequency when Treated with Retinoic Acid 114

6.7 Gene Expression During Embryoid Body Differentiation 117

6.7.1 Formation of Primitive Ectoderm in Embryoid Bodies 117

6.7.2 Formation of Mesoderm in Embryoid Bodies 118

6.7.3 Formation of Primitive Endodermal Derivatives in Embryoid Bodies 119

6.8 Discussion 122

6.8.1 The Differentiation Potential of X Cells Compared to ES Cells 122

6.8.2 Gene Expression Correlates with Differentiation Potential 124

6.8.3 The Formation of Primitive Endoderm *In Vitro* and *In Vivo* 125

6.8.4 The Differentiation Potential of X Cells in Relation to the Two State Model of X Cell Formation and Maintenance 126

CHAPTER SEVEN: DIFFERENTIATION OF X CELLS WITH MESODERM INDUCING CYTOKINES 128

7.1 Introduction 129

7.2 Basic FGF Induces Specific Differentiation of X Cells but not ES Cells 130

7.2.1 The Effect of bFGF on ES Cells and X Cells 131

7.2.2 Basic FGF Induces X Cell Differentiation 132

7.3 The Effect of Heparin on bFGF X Cell Differentiation 134

7.4 The Effect of Other Members of the FGF Family on the Differentiation of X Cells 135

7.5 Activin A Induces the Differentiation of X Cells 136

7.5.1 Activin A Induces the Differentiation of X Cells but not ES Cells 137

7.5.2 Comparison of bFGF-Induced and Activin A-Induced X Cell Differentiation 138

7.6 The Gene Expression Associated with X Cell Differentiation in Response to Mesoderm Inducing Cytokines 138

7.6.1 Gene Expression in bFGF-Treated X Cell Cultures 139

7.6.2 Gene Expression in Activin A-Treated X Cell Cultures 142

7.7 Discussion 143

CHAPTER EIGHT: REINTRODUCTION OF X CELLS INTO THE EARLY MURINE EMBRYO 147

8.1 Introduction 148

8.2 Contribution of X Cells and Reverted X Cells to Murine Embryos 148

8.3 Contribution of Reverted XB17 Cells to Murine Embryos 151

8.4 Discussion 152

CHAPTER NINE: FINAL DISCUSSION 154

9.1 The Embryological Equivalent of X cells 155

9.2 X Cell Gene Expression is Consistent with the Early Primitive Ectoderm 155

9.3 X Cell Differentiation Capacity is Consistent with the Early Primitive Ectoderm 156

9.3.1 Differentiation of X Cells in Response to Retinoic Acid 156

9.3.2 Differentiation of X Cells as Embryoid Bodies 156

9.3.3 Differentiation of X Cells in Response to Mesoderm Inducing Cytokines 157

9.4 X Cells and *In Vivo* Differentiation Events 158

9.5 X Cell Reversion 158

9.6 The Relationship Between X Cell Gene Expression and Differentiation Capacity 159
9.7 The Primitive Ectoderm as an Alternative Target for Pluripotential Cell Isolation 160
9.8 X Cells and the Primitive Ectoderm 161
9.9 Future Work 162

REFERENCES 165

THESIS SUMMARY

Murine embryonic stem (ES) cells are derived from the inner cell mass of the early murine embryo. ES cells which have been cultured *in vitro* can be reintroduced into the early murine blastocyst where they contribute differentiated progeny to all tissues, including the germ line. This demonstrates that ES cells can both generate and respond to all the environmental signals that regulate normal mouse development. The availability of homogeneous populations of undifferentiated stem cells which can be manipulated and differentiated *in vitro* provides an attractive system for the identification of factors which regulate embryogenesis. This system has already been successfully employed for the identification of the cytokine Differentiating Inhibiting Activity (DIA)/Leukaemia Inhibitory Factor (LIF) which prevents the differentiation of ES cells *in vitro*. Biological molecules that can direct the specific differentiation of ES cells into defined lineages had not been characterised at the outset of this work.

Conditioned medium (MED2) derived from a human hepatocarcinoma cell line was found to induce the uniform and specific differentiation of ES cells to a morphologically distinct, novel cell population, termed X cells. The conversion of ES cells to X cells occurred in MED2 in the presence or absence of LIF. X cells could be maintained indefinitely by culture and passage in the presence of MED2. If MED2 and LIF were removed, X cells spontaneously differentiated into a multitude of cell types. This suggested that X cells, like ES cells, retained differentiation capability. Significantly, X cells cultured in the presence of LIF but in the absence of MED2 reverted to an ES cell phenotype. Preliminary analysis of the properties of X cells suggested that these cells may represent a pluripotential embryonic cell type, possibly primitive ectoderm. The overall aim of this thesis was therefore to examine the biological and biochemical properties associated with X cells to allow comparison with ES cells and definition of a potential embryonic equivalent.

Extensive analysis of X cell gene expression with a variety of marker genes revealed that X cell gene expression is consistent with cells of the early primitive ectoderm.

X cells, like ES cells, were capable of embryoid body formation and differentiation. Consistent with gene expression analysis, during embryoid body differentiation X cells

exhibited a higher capacity to form late stage primitive ectoderm and mesodermal derivatives than ES cells. Differentiation *via* aggregation in the presence of retinoic acid revealed that X cells had a lower potential to form primitive endodermal derivatives and a higher capacity to form mesodermal derivatives than ES cells. The differentiation potential associated with X cells is consistent with their proposed relationship with the early primitive ectoderm.

Mesoderm induction studies in *Xenopus* have implicated the involvement of members of the FGF and TGFβ growth factor families. The primitive ectoderm is the substrate for mesoderm induction during gastrulation in the murine embryo. Members of the FGF family, including basic FGF, acidic FGF and FGF-4 and activin A (a TGFβ) were found to induce specific, alternative differentiation of X cells to mesodermal cell types *in vitro*. Consistent with mesoderm induction experiments carried out in *Xenopus*, *Brachyury* but not *goosecoid* was expressed in X cell cultures treated with bFGF, while activin A-treated cultures expressed both *Brachyury* and *goosecoid*. Furthermore the patterns of *goosecoid* and *Brachyury* expression observed within differentiated X cell cultures were consistent with the expression pattern associated with these genes during gastrulation in the mouse. The ability of X cells to differentiate in response to bFGF and activin A into early mesoderm is consistent with their proposed relationship with the primitive ectoderm, and suggests that X cells may constitute an *in vitro* system for the analysis of factors involved in gastrulation.

X cells which had been reverted to ES cells by culture in the presence of LIF and the absence of MED2, were found to possess a similar gene expression and differentiation potential to ES cells. Significantly, reverted X cells, but not X cells, were able to contribute to murine tissues when injected into early murine blastocysts. The relationship between X cells and the primitive ectoderm suggests that embryonic primitive ectoderm may have a similar capacity for reversion and embryonic contribution when isolated *in vitro*. Thus the primitive ectoderm may represent an alternative target tissue for the isolation of pluripotential stem cells *in vitro*.

The evidence accumulated and described in this thesis suggests that X cells constitute an *in vitro* analogue of embryonic primitive ectoderm, and establish X cells as an *in vitro* system for the analysis of further developmental events occurring during murine embryogenesis.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED ..... **DATE**.....5/1/96.....

ACKNOWLEDGMENTS

I would like to thank Dr. G. E. Rogers for giving me the opportunity to undertake a Ph.D. in the Department of Biochemistry. I would also like to thank all the academic staff members for their support, particularly Dr. Barry Egan.

I would like to formally thank Dr. Peter Rathjen for his excellent propensity for supervision, enormous support, understanding throughout the years and for being absolutely consistent while critically reading this thesis. Also thankyou for your intellectual prowess shown in all areas, other than washing machines and April Fools Day jokes. To the members of the lab, past and present, thankyou for the cohesiveness and immense support you have shown throughout the years. Thankyou to Joy Rathjen for her critical reading of this thesis, discovering X cells, tissue culture prowess and friendship. Thankyou to every one in the lab for their friendship and extreme funniness. In particular, Linda Whyatt for her little collection, hijinx and my favourite phrase " The eagle has landed", Bryan " the train" Haines for dancing with pot plants, for taking three panadols on regular occasions and "Her name was Lola", Roger " Walnut" Voyle for being from New Zealand, Tricia Pelton for building block jokes, Jenny "MED2" Washington for tissue culture conversations, Mikey " wasn't me" B, for having a nickname Bettess the Lettuce, Gavin " Fritz" Chapman for that unforgettable evening of RNAase protections. Tom Shultz for keeping the ship afloat in times of rough weather, Paul Q. Thomas for the limbo and being a scientific rock, Brett Johnson for balcony company, Jackie for taking my bench, Shiwani for finding a damn marker and a reluctant thankyou to Joe Wrin for giving me a "Lake locater" (I think it worked).

A special thanks to the support staff, particularly, Jan, Jackie, Judy, Jenny 1 and Jenny 2 and Sheila for media and solution preparation and ordering things really quickly.

Thanks also to Reedy for leaving, Lones for being a Lones, Michael C., Jilly and John, Reebs, Sads, Kym, Dora, Gayathri (woops), Tim, Lizal, Pete, Rick and Racheal (Where have you all gone??). Thankyou to Ms Emma Parkinson for her close friendship, really excellent parties and for cleaning her choppers. Thankyou to my crazy family for always having interesting and such poignant problems. Thankyou Mum for all your help, thankyou Dad, my best friends and life will get better.

To Tim, thankyou, thankyou, thankyou for your patience, love, support, friendship, help (were do I stop) and company at such late hours in what have been very trying times. I am really looking forward to some normality in our lives. And last but by no means least, to my little girl Caitlin, thankyou for being so cute and putting up with being in creche, more than you are with me.

CHAPTER ONE:

INTRODUCTION

CHAPTER ONE: INTRODUCTION

1.1 The Study of Development

Understanding the mechanism by which a single cell generates the diversity of cell types and complexity of pattern required for the formation of an independent living organism has been a fundamental aim of developmental biology for over a century. The study of development encompasses relatively disparate fields such as genetics, physiology, embryology, evolutionary, molecular and cellular biology and hence represents one of the most fascinating and interesting fields of investigation.

Two important concepts of molecular developmental biology have arisen from investigation of development in lower vertebrate species, in particular *Xenopus laevis* and in the metazoan, *Drosophila melanogaster*. The first of these concepts was that differential gene expression dictates cell specification and pattern formation during development. Although in retrospect this appears obvious, it was not until extensive analysis of the genes responsible for developmental mutations in *Drosophila* that this concept was fully realised (reviewed by St Johnson and Nusslein-Volhard 1992). These investigations led to the discovery of developmental transcriptional control genes. Of these genes perhaps the best known are the homeobox genes (reviewed in Scott *et al.* 1989). Since their initial discovery in *Drosophila* the importance of these genes in the development of vertebrate species has been recognised repeatedly (reviewed in Scott *et al.* 1989, McGinnis and Krumlauf 1992, Lobe 1992).

Early *Drosophila* development involves nuclear divisions prior to cellularisation (St Johnson and Nusslein-Volhard 1992). Therefore specification of cell types can be dictated by the differential expression of transcription factors. Vertebrate development is cellular in nature and therefore requires different mechanisms to account for this additional complexity. The analysis of development in the lower vertebrate, *Xenopus*, has been particularly informative in this regard.

Extensive analysis of molecular development in *Xenopus* has given rise to a second important concept which indicates that growth factors regulate cell specification and pattern formation during development. Early development in *Xenopus* is characterised by cellular subdivision of the one cell embryo. At the 32 cell stage the *Xenopus* embryo

contains two specified cell types, cells which give rise to ectoderm (animal pole) and cells which give rise to endoderm (vegetal pole). Vegetal cells induce overlying animal cells in the equatorial region to form mesoderm (reviewed in Sive 1993). One of the major advances in developmental biology was the discovery that growth factors play a role in this specification event (reviewed in Slack 1990, Ruiz i Altaba and Melton 1990, Cooke 1991, Sive 1993). When animal cells which are normally fated to form atypical epidermis are cultured with members of the fibroblast growth factor (FGF) or transforming growth factor (TGF) β family, mesoderm is induced. The array of tissues induced depends on the factor and the concentration used in the experiment.

The concepts of differential gene expression and growth factor induction merged when it was shown that growth factors induce the expression of a range of developmentally regulated transcription factors which in turn specify cell identity (Ruiz i Altaba and Melton 1989a, 1989b, Sive 1993).

1.2 Why Study Mammalian Embryogenesis?

The human thirst for self knowledge and preservation makes the study of mammalian development intrinsically interesting. Not only does an intimate knowledge of mammalian development give an insight into how the human form is generated and has evolved, it provides us with an opportunity to understand the mechanism of human disease and ultimately to develop corresponding therapies. While parallels can be drawn between the development of lower organisms and mammals, many aspects of early development are intrinsically different. Thus it follows that while investigation of the development of lower species can be informative, mammalian development is best characterised using a mammalian system. Unfortunately the use of mammalian systems for molecular developmental analyses is complicated by their relatively large genome, long generation time, small litter size and inherent ethical considerations. Furthermore, the mammalian embryo develops in the uterine environment within the mother and therefore is relatively inaccessible to experimentation.

1.2.1 The Mouse as a Model System for Mammalian Embryogenesis

The mouse constitutes the primary mammalian experimental system for the analysis of development. More than 1000 genes, many affecting murine development, have been genetically mapped and identified (Copeland *et al.* 1993, Dietrich *et al.* 1994). In addition the morphological alterations associated with murine embryogenesis have been extensively characterised by histological examination (Kaufman 1992). Finally, the ability to isolate and culture pluripotential murine embryonic stem (ES) cells *in vitro* (Martin 1981, Evans and Kaufman 1981) has been of unprecedented technical and conceptual importance. These cells can be genetically manipulated *in vitro* and reintroduced into murine blastocysts where they contribute to all murine tissues, including the germ line (Bradley *et al.* 1984, Cappechi 1989). This allows the generation of mice with specific genetic alterations and thus allows the developmental function of genes to be determined *in vivo*.

1.3 Murine Embryogenesis

In the following sections the differentiation events occurring during early murine embryogenesis are described (for reviews see Rossant and Pedersen 1988, Kaufman, 1992, Hogan *et al.* 1994). A summary of the cell lineages formed during murine embryogenesis which are described in this section are depicted in Figure 1.1.

A basic theme of murine embryogenesis is the formation of a population of equipotent pluripotential stem cells. This population is maintained in various forms during early murine embryogenesis and culminates in the formation of totipotent primordial germ cells. Specification of cell types occurs relatively late during mammalian development and appears to be dependent on zygotic transcription (Kidder 1992). This contrasts with development in other species, such as *Xenopus* and *Drosophila*, where specification of cell types occurs much earlier and appears to be dependent on localised deposition of positional maternal information in the oocyte (Melton 1991).

1.3.1 Preimplantation Development

The developmental events occurring during preimplantation development from fertilisation through to blastocyst formation are depicted in Figure 1.2. Fertilisation

FIGURE 1.1 Summary of the cell lineages arising during murine development.

Darkly shaded areas designate tissues which will give rise to the embryo proper and extraembryonic tissues. Unshaded areas represent tissues of the embryo proper, while lightly shaded areas indicate extraembryonic tissues.

Adapted from Hogan *et al.* 1994.

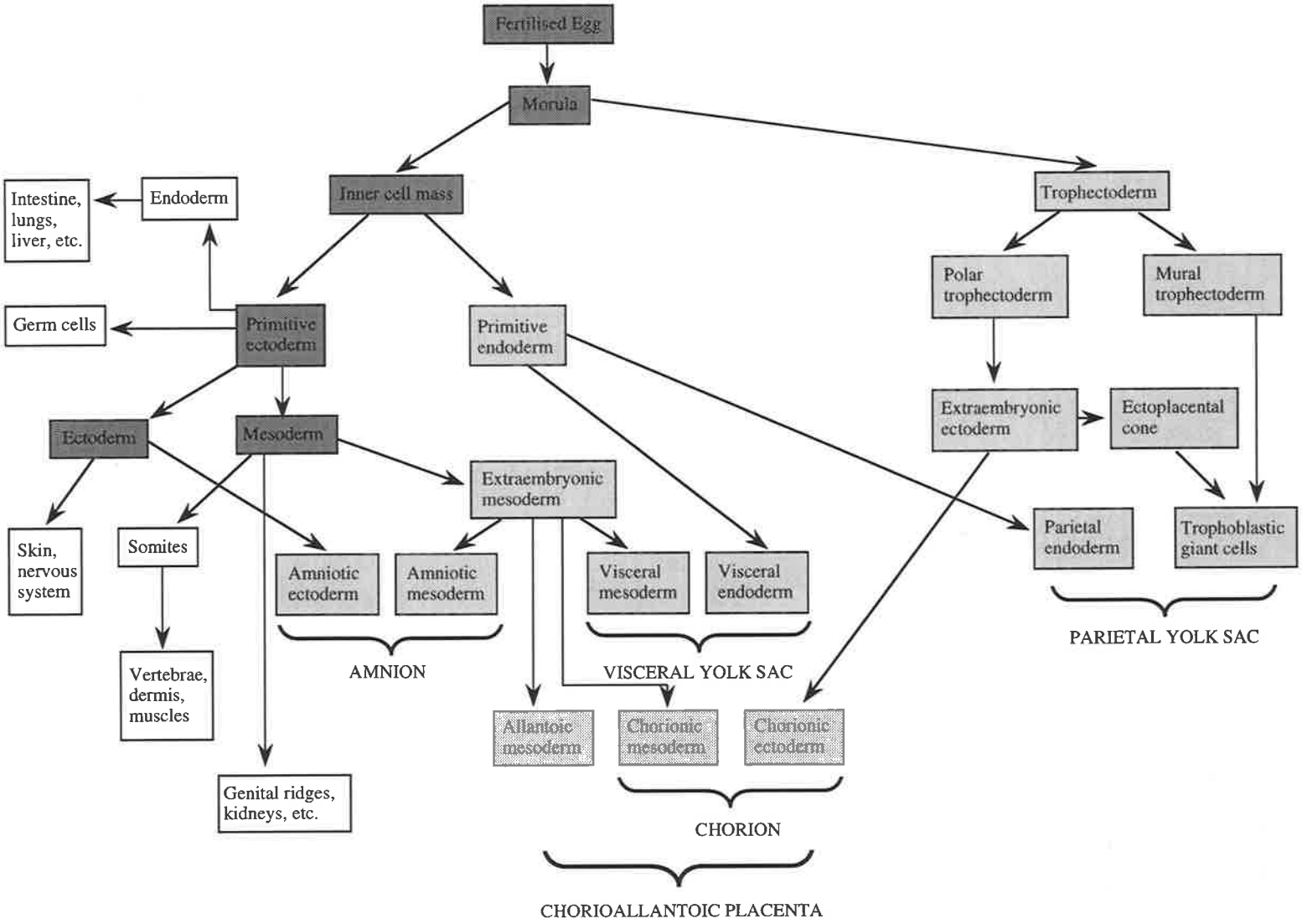
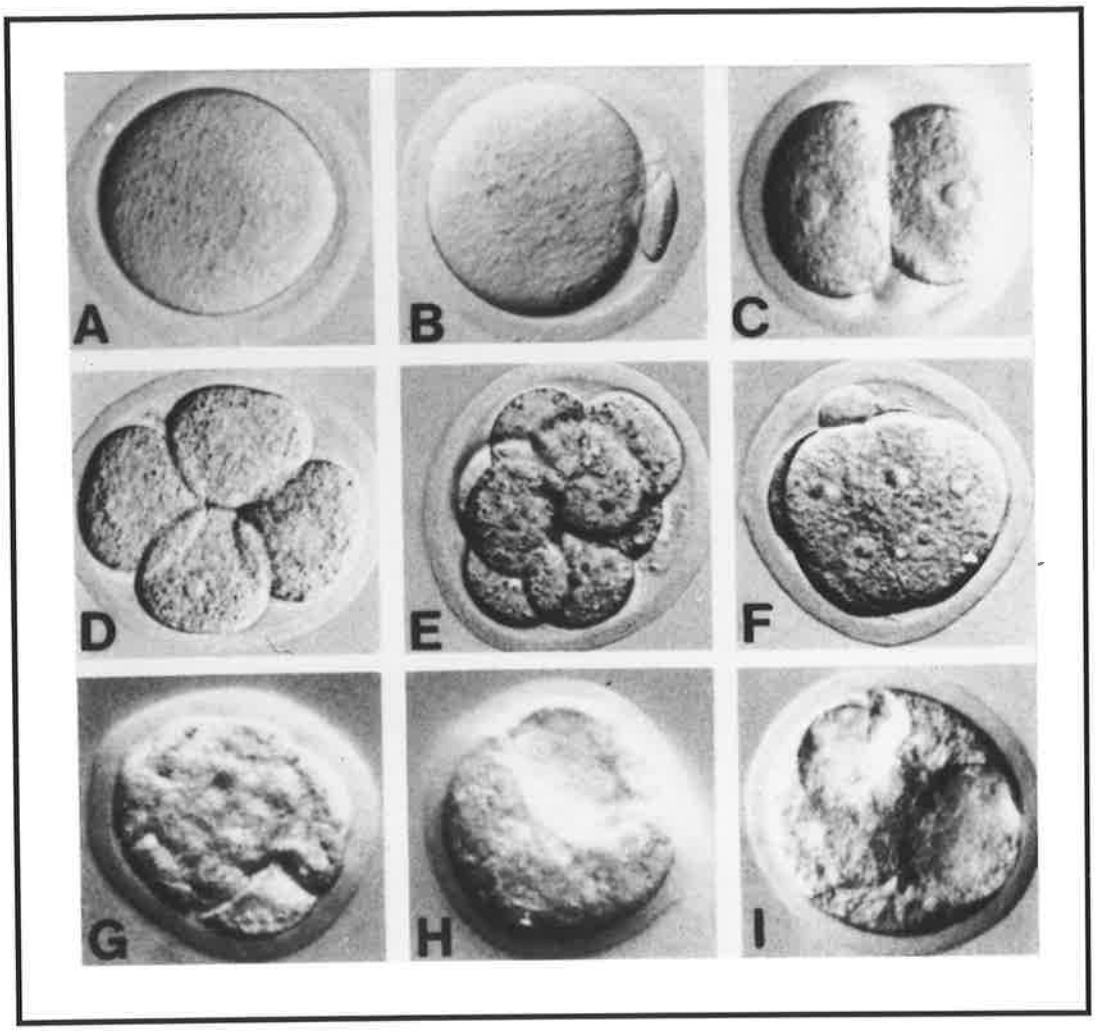

Fertilised Egg
Morula
Inner cell mass
Trophectoderm
Endoderm
Intestine, lungs, liver, etc.
Polar trophectoderm
Mural trophectoderm
Germ cells
Primitive ectoderm
Primitive endoderm
Extraembryonic ectoderm
Ectoplacental cone
Ectoderm
Mesoderm
Extraembryonic mesoderm
Parietal endoderm
Trophoblastic giant cells
Skin, nervous system
Somites
Amniotic ectoderm
Amniotic mesoderm
Visceral mesoderm
Visceral endoderm
PARIETAL YOLK SAC
Vertebrae, dermis, muscles
AMNION
VISCERAL YOLK SAC
Allantoic mesoderm
Chorionic mesoderm
Chorionic ectoderm
Genital ridges, kidneys, etc.
CHORION
CHORIOALLANTOIC PLACENTA

FIGURE 1.2 Development of the preimplantation murine embryo.

(A) An oocyte arrested at metaphase II of meiosis. (B) The fertilised egg, showing extrusion of the second polar body. (C) The embryo at the two cell stage. (D) The four cell stage embryo. (E) The eight cell stage embryo prior to compaction. (F) The eight cell stage embryo after compaction. (G) The 16 cell stage embryo. (H) The 32 cell stage embryo. (I) The 32 cell stage embryo at the onset of cavitation.

Adapted from Maro *et al.* 1991.


A
B
C
D
E
F
G
H
I

marks the beginning of murine development. The sperm penetrates the outer layers (cumulus mass and zona pellucida) of the oocyte and enters the cytoplasm. Fertilisation triggers completion of meiosis and extrusion of the second polar body (Figure 1.2 B), such that the oocyte contains a haploid complement of maternal chromosomes. Nuclear membranes form around the maternal and paternal chromosomes and the resulting pronuclei move toward the centre of the egg. During this migration DNA replication takes place. The nuclear membranes of the pronuclei break down and the chromosomes align in preparation for the first cleavage event (Figure 1.2 C).

These embryonic cells or blastomeres divide every 20 hours giving rise to the eight cell embryo (Figure 1.2 E). Until the mid-two cell stage the embryo relies on maternal protein and RNA synthesised during oogenesis. At this stage maternal mRNA appears to be degraded and zygotic transcription commences (Latham *et al.* 1991, Kidder 1992). This contrasts with development in other species, including *Drosophila* and *Xenopus* where maternal constituents are required until more advanced stages of embryogenesis (Melton 1991). Until the eight cell stage (morula), individual blastomeres are equipotent. Cell specification has not taken place and each cell has the capacity to generate an entire and normal organism (Pederson 1988). After this stage there is a gradual restriction in developmental potential.

After the formation of the eight cell embryo, compaction occurs (Figure 1.2 F). Individual blastomeres flatten thereby increasing cell-cell contact. Gap junctions between cells are established and the cells become polarised both cytocortically and cytoplasmically. Cell polarisation is thought to be the basis for the first differentiation event which occurs during murine embryogenesis (Johnson and Maro 1988). As the embryo divides, cells positioned in or near the centre of the embryo form the Inner Cell Mass (ICM). These pluripotential cells contribute to all embryonic and extraembryonic tissues except the trophoblast and trophoblast-derived lineages. Cells which are positioned on the outside of the embryo form an epithelial layer known as the trophectoderm. The trophectoderm ultimately gives rise to extraembryonic tissues of the murine embryo including the chorion and the placenta. Concomitant with this differentiation is the formation of the blastocoelic cavity (Figure 1.2 I). Trophectoderm

which surrounds the inner cell mass is termed polar trophectoderm while that which surrounds the blastocoelic cavity is known as mural trophectoderm.

At approximately four days of development a second differentiation event occurs shortly before implantation. Cells covering the blastocoelic surface of the inner cell mass form an epithelial layer known as the primitive endoderm (Figure 1.3). Progeny of the primitive endoderm are restricted to the formation of visceral and parietal yolk sac endoderm which constitute part of the extraembryonic tissues surrounding the murine embryo. As the embryo expands the zona pellucida surrounding the embryo becomes thinner and eventually breaks, releasing the blastocyst into the uterine lumen.

1.3.2 Postimplantation Development

The development of the murine embryo from the blastocyst stage at 4.5 days until the egg cylinder stage at day 6.0 is schematically represented in Figure 1.4. At 4.5 days of development (Figure 1.4 A) the blastocyst adheres to the uterine wall *via* mural trophectoderm located furthest from the ICM (the abembryonic pole). This attachment induces surrounding uterine stroma to form a spongy mass of cells, known as decidual tissue. With time the decidual cells degenerate and the trophoblastic cells invade the surrounding tissue. The cells of the primitive endoderm begin to differentiate, migrate and eventually cover the blastocoelic surface of the mural trophectoderm. These cells are termed the parietal endoderm, while primitive endodermal cells in direct apposition to the cells of the inner cell mass/primitive ectoderm are termed visceral endoderm. Parietal endoderm cells secrete components which compose Reichert's membrane, a basement membrane which lies between the trophectoderm and parietal endoderm cells of the murine embryo. This membrane constitutes the major barrier between maternal and embryonic environments.

At day 4.5 the inner cell mass consists of approximately 20-25 cells. These cells proliferate rapidly and constitute a pluripotential cell population of approximately 250 cells by day 6 of development (Snow 1977). The substantial increase in the number of cells constituting the inner cell mass results in their movement towards the abembryonic pole (Figure 1.4 B). The proaminotic cavity forms and cells of the ICM are arranged into an epithelial layer termed the primitive ectoderm or epiblast (Figure 1.4 C). Cells of the

FIGURE 1.3 The murine blastocyst at 4.5 days of development.

A histological cross section of the murine blastocyst at day 4.5 of development, showing the inner cell mass (Epiblast) and an underlying layer of primitive endoderm (PrEnd), the blastocoelic cavity (Blastocoel) and surrounding trophectoderm (TE).

Reproduced from Hogan *et al.* 1986.

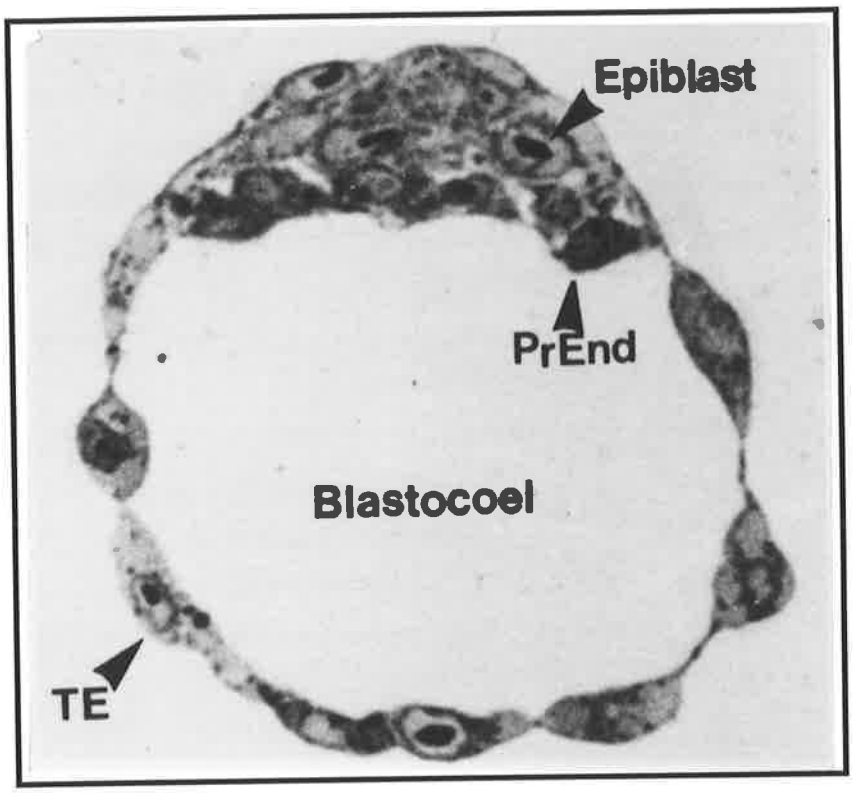

Epiblast
PrEnd
Blastocoel
TE

FIGURE 1.4 Postimplantation murine development from day 4.5 to day 6.0.

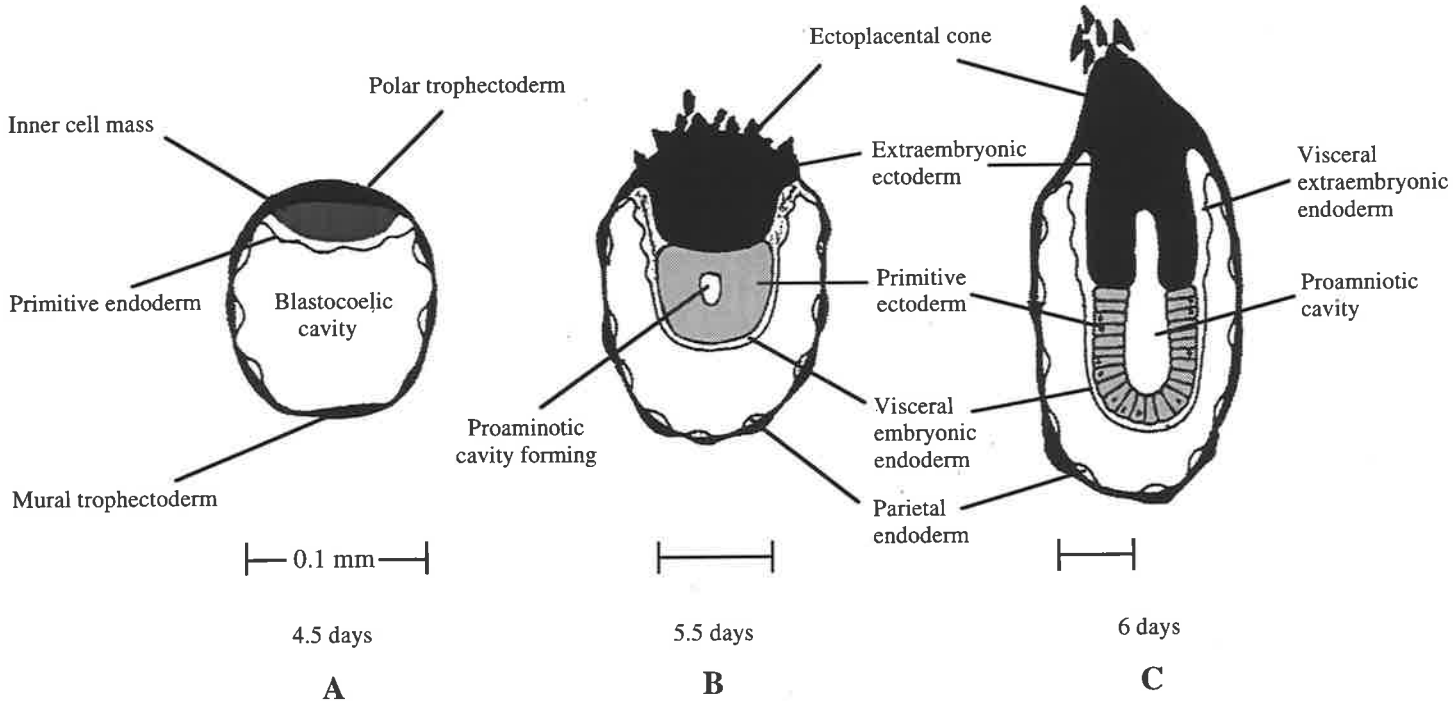
Schematic representation of postimplantation development.

(A) At 4.5 days of development the embryo consists of three distinct cell lineages, the inner cell mass, primitive endoderm and trophectoderm.

(B) At 5.5 days the proaminotic cavity begins to form within the pluripotential cells of the murine embryo which are now termed primitive ectoderm. The primitive endoderm has generated two distinct endodermal cell types, the parietal endoderm lines the inner surface of mural trophectoderm, while the visceral endoderm surrounds the primitive ectoderm.

(C) At 6.0 days (the egg cylinder stage) of development the cells of the primitive ectoderm flatten and expand forming an epithelial layer surrounding the proamniotic cavity.

Adapted from Hogan *et al.* 1986.


Inner cell mass
Polar trophectoderm
Primitive endoderm
Blastocoelic cavity
Mural trophectoderm
0.1 mm
4.5 days
A
Ectoplacental cone
Extraembryonic ectoderm
Primitive ectoderm
Proaminotic cavity forming
Visceral embryonic endoderm
Parietal endoderm
5.5 days
B
Visceral extraembryonic endoderm
Proamniotic cavity
6 days
C

polar trophectoderm proliferate and extend into the proaminotic cavity forming the extraembryonic ectoderm and invade the uterine decidua forming the ectoplacental cone.

1.3.3 Gastrulation

The development of the murine embryo during gastrulation from day 6.5 to day 8.5 is represented schematically in Figure 1.5. At 6.5 days of development the primitive ectoderm consists of approximately 660 cells (Snow 1977) and, through the process of gastrulation, gives rise to all of the embryonic tissues including the germ cells and the remaining extraembryonic tissues (Tam and Beddington 1987, Tam 1989, Lawson *et al.* 1991, Tam and Beddington 1992, Quinlan *et al.* 1995). Gastrulation commences at 6.5 days with the formation of the primitive streak (Figure 1.5 A). The primitive streak is first apparent as a cellular thickening in the proximal region of the primitive ectoderm adjacent to the extraembryonic/embryonic ectoderm boundary, and demarcates the posterior aspect of the embryonic anteroposterior axis. Cells of the primitive ectoderm move toward and accumulate in the streak region which extends distally during gastrulation. Cells which pass through the streak emerge as a layer of mesoderm between the primitive ectoderm and visceral endoderm. Newly formed mesoderm moves in two directions. A proportion of these cells migrate proximally, crossing the boundary between the extraembryonic and embryonic ectoderm and form extraembryonic mesoderm. The remainder move laterally toward the anterior end of the embryo between the primitive ectoderm and the visceral endoderm, giving rise to embryonic mesoderm. Mesodermal cells emerging from different regions of the streak have different developmental fates. Axial, paraxial, lateral and extraembryonic mesoderm arise from successively more posterior regions of the streak (Tam and Beddington 1987, Lawson *et al.* 1991).

At 7.5 days (Figure 1.5 B), cells which will give rise to definitive gut endoderm emerge from the distal region of the streak (the node) and move anteriorly, incorporating into and replacing the outer layer of visceral endoderm (Tam and Beddington 1992). Definitive gut endoderm later gives rise to a number of derivatives including the lungs, liver and pancreas. Other cells emerging from the node form axial mesoderm including the head process and notochord. Primitive ectoderm cells which do not pass through the

FIGURE 1.5 The gastrulation phase of murine embryogenesis.

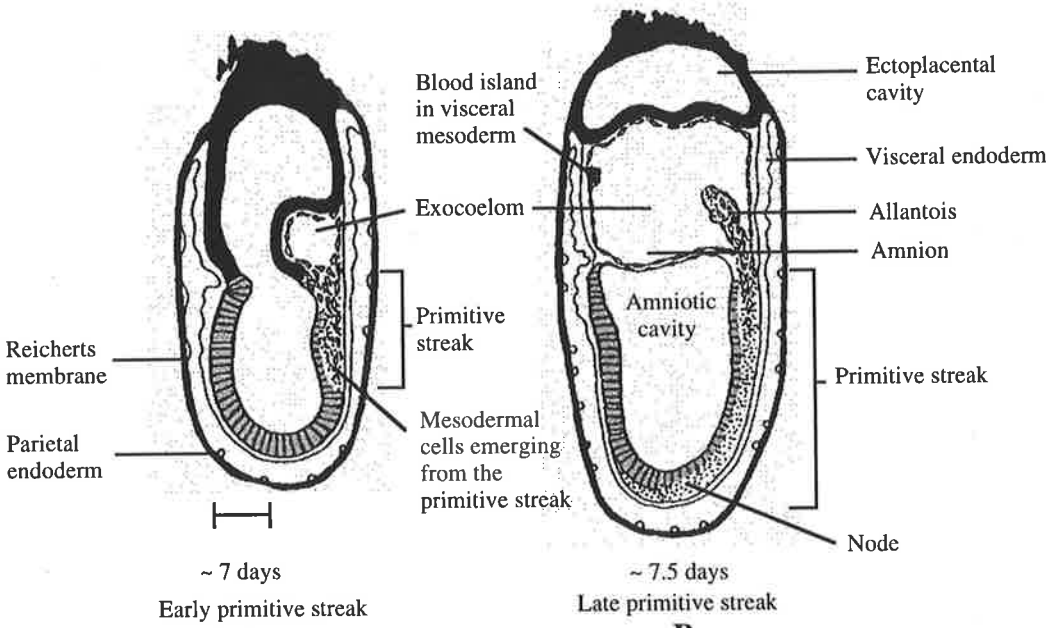
Schematic representation of murine development from day 7.0 to day 8.5.

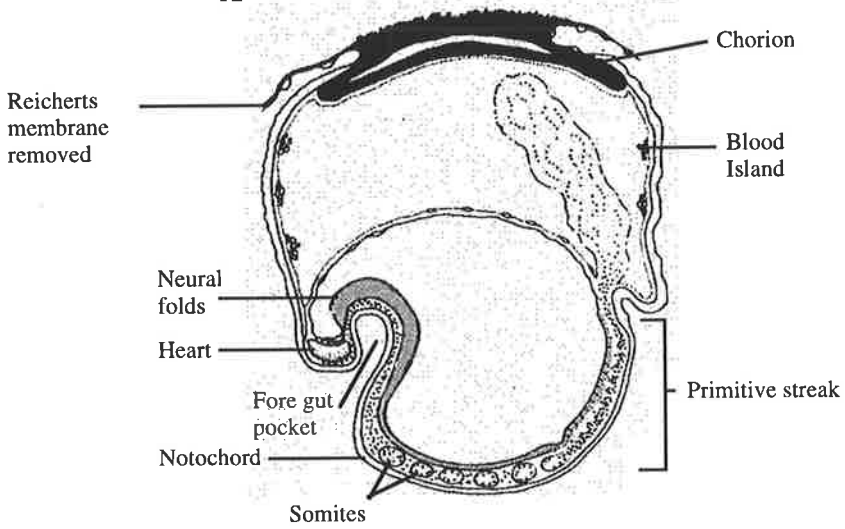
(A) At 6.5-7.0 days the onset of gastrulation is marked by the formation of the primitive streak, in the proximal region of the primitive ectoderm.

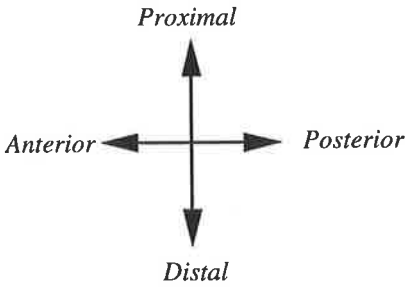
(B) Newly formed mesoderm moves proximally, anteriorly and laterally generating a distinct mesodermal layer between the primitive ectoderm and visceral endoderm boundary.

(C) By 8.5 days of development a variety of tissues can be distinguished along the anterior posterior axis of the embryo, including neural tissue, notochord, somites, as well as precursors of the definitive gut endoderm and heart.

Adapted from Hogan *et al.* 1986.


Reicherts membrane
Parietal endoderm
Primitive streak
Mesodermal cells emerging from the primitive streak
Exocoelom
Blood island in visceral mesoderm
Ectoplacental cavity
Visceral endoderm
Allantois
Amnion
Amniotic cavity
Primitive streak
Node
~ 7 days
Early primitive streak
A
~ 7.5 days
Late primitive streak
B


Reicherts membrane removed
Chorion
Blood Island
Neural folds
Heart
Fore gut pocket
Notochord
Somites
Primitive streak
8.5 days
C


Proximal
Anterior
Posterior
Distal

primitive streak are the precursors of the neuroectodermal (nervous system) and epidermal (skin) cell lineages (Tam 1989, Quinlan *et al.* 1995). Newly formed extraembryonic mesoderm gives rise to a mesoderm lined cavity termed the exocoelum and a structure known as the allantois which forms a major component of the chorioallantoic placenta.

By 8.5 days, newly formed embryonic mesoderm can be subdivided into cranial, cardiac, presomitic, somitic, lateral and intermediate mesoderm based on morphology and anatomy. Presomitic mesoderm (paraxial mesoderm) condenses into paired blocks or somites along the anterior-posterior axis of the embryo (Figure 1.5 C). Somites later give rise to the vertebra, ribs and muscle of the embryo. Lateral mesoderm will give rise to the viscera and connective tissues of the limbs, the mesothelial lining of the body cavity and the mesenteries, while intermediate mesoderm will form the kidneys and genital ridges of the embryo.

Gastrulation is a pivotal process in establishing the anterior/posterior body axis of the embryo and in specification of the embryonic germ layers. In a matter of days the relatively uniform primitive ectodermal cell population is transformed into an embryo containing multiple differentiated tissues and organ precursors in the correct position and orientation.

Investigation of early murine development has largely been restricted to the gastrulation stage onwards for a number of reasons. Firstly, gastrulation represents one of the most interesting developmental periods, since this is the stage where all the tissues which will ultimately form the embryo are laid down. Secondly, developmental similarities between species are more easily discernible at this stage than at earlier stages where the developmental strategy of mammals diverges significantly. Consequently developmental control genes identified in simpler systems during post-gastrulation development are more likely to be conserved between species. Genes found to have a developmentally restricted expression pattern during gastrulation in one species, particularly *Xenopus*, can be used for the identification of murine homologues which have a similar expression pattern during murine gastrulation. Finally, the murine embryo itself is considerably larger, more accessible and less fragile at this stage of development than at earlier stages and therefore more suitable for experimental analysis and manipulation. Therefore while the molecular mechanisms which underlie the earliest

stages of murine embryogenesis remain largely undefined, a multitude of genes expressed during gastrulation and organogenesis have been identified.

An array of genes have been shown to be differentially expressed within the primitive streak during gastrulation (reviewed in Hogan *et al.* 1994) including the transcription factors *goosecoid* (Blum *et al.* 1992), *Brachyury* (Wilkinson *et al.* 1990, Herrmann 1991) *HNF-3β* (Monaghen *et al.* 1993, Sasaki and Hogan 1993) and *evx-1* (Bastian and Gruss 1990), and growth factors such as *nodal* (Zhou *et al.* 1993) and various members of the FGF family (Crossley and Martin 1995). Differential gene expression within this region is thought to underlie the initial specification of mesodermal and endodermal cell types within the murine embryo. While functional roles consistent with gene expression have been shown for *Brachyury* (Herrmann 1990), *HNF-3β* (Ang and Rossant 1994) and *nodal* (Conlon *et al.* 1991, Zhou *et al.* 1993, Conlon *et al.* 1994), the specific functional roles of other genes expressed in this region have not been defined.

Many of the genes expressed during early murine gastrulation exhibit similar expression patterns at equivalent stages during *Xenopus* embryogenesis (Beddington and Smith 1993, DeRobertis *et al.* 1994). Members of the TGFβ and FGF family have been implicated in the process of mesoderm induction in *Xenopus* embryogenesis (reviewed in Sive 1993). It is therefore thought that members of these growth factor families will play similar roles during murine gastrulation. While the expression patterns associated with these factors during gastrulation are consistent with roles in mesoderm induction (Crossley and Martin 1995, Manova *et al.* 1995), the precise functions of these factors remain unclear.

1.3.4 The Origin of Germ Cells

Primordial germ cells arise from the proximal and posterior region of the primitive ectoderm and are first detected at day 7.0 of development in the extraembryonic mesoderm at the base of the allantois (Ginsberg *et al.* 1990). PGCs express high levels of tissue non-specific alkaline phosphatase and have a large round cellular morphology. At 8.5 days of development the PGC population consists of approximately 75 cells. As development proceeds these cells continue to proliferate and migrate from the base of the allantois to the genital ridges. By 13 days of development approximately 25,000 PGCs

have colonised each prospective ovary/gonad (Tam and Snow 1981). Female germ cells cease proliferation and at day 12.5 enter meiosis, arresting at prophase of the first meiotic division, while male germ cells enter mitotic arrest at day 14 and do not undergo meiosis until much later in development (Monk and McLaren 1981, Shultz *et al.* 1988).

1.4 Pluripotential Cell Maintenance During Development

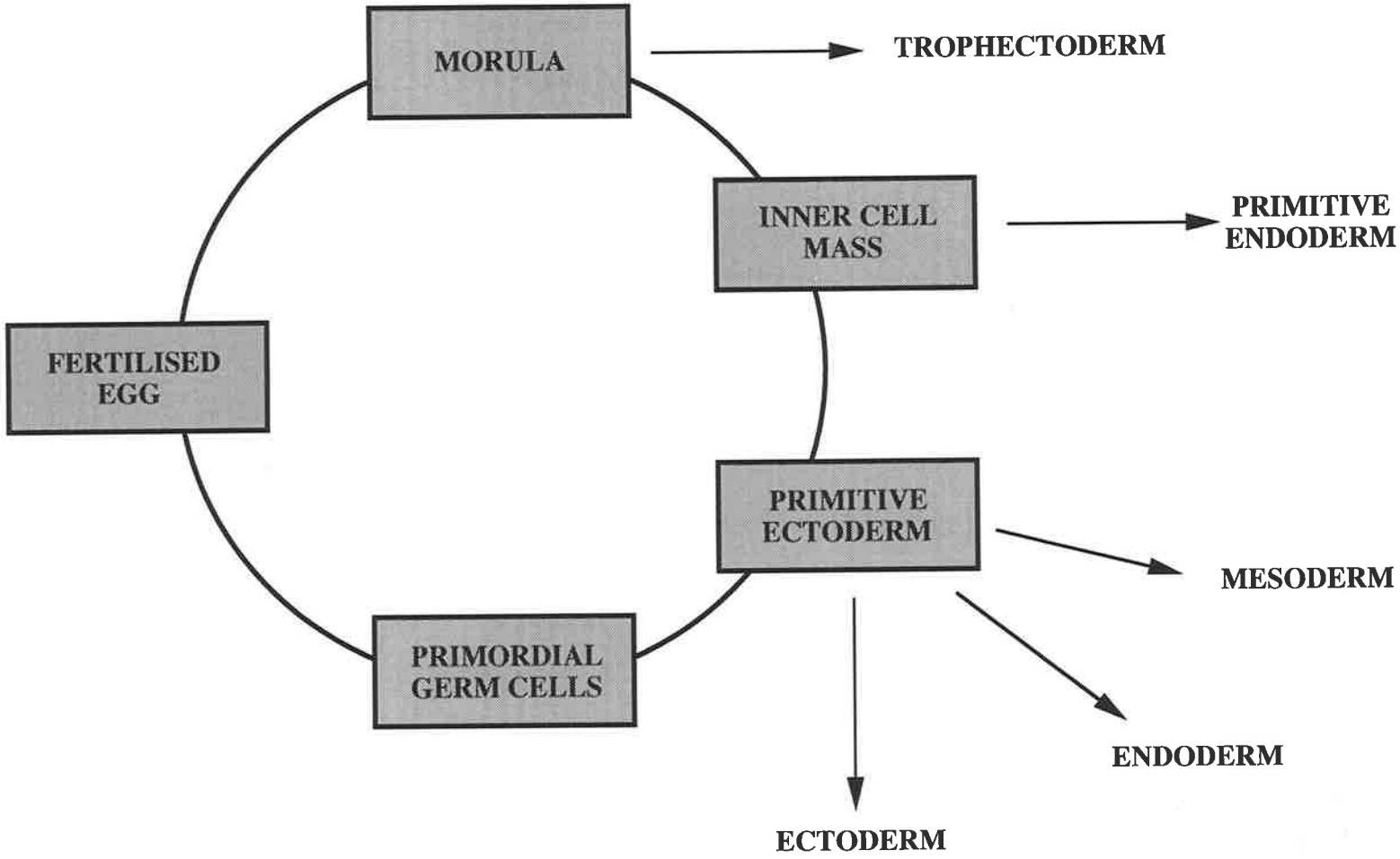
The mechanism of early mammalian development involves the maintenance and coordinated differentiation of a population of pluripotential cells (Figure 1.6). The fertilised egg divides giving rise to the morula which contains equipotent pluripotential blastomeres. As the morula gives rise to the blastocyst, pluripotential stem cells are maintained in the ICM and subsequently the primitive ectoderm. With the onset of gastrulation, the pluripotential cell population is maintained in the form of primordial germ cells which give rise to further generations, allowing continuation of the species. These cell populations can be traced throughout development by expression of the homeobox gene *Oct-4* (Rosner *et al.* 1990, Scholer *et al.* 1990, Yeom *et al.* 1991) which is confined to pluripotential cell types. A relationship between these cell populations is implied by the ability of PGC cells to revert to an ES cell phenotype *in vitro* (Matsui *et al.* 1992, Resnick *et al.* 1992, Rossant 1993).

The flexibility associated with pluripotential stem cell differentiation and maintenance is considered to underlie the considerable developmental lability exhibited by the murine embryo (Smith 1992). Aggregation of several preimplantation embryos will result in the formation of a normal sized embryo while, destruction of 80% of the cells in the early murine embryo prior to organogenesis still allows formation of a normal embryo (Snow and Tam 1979). This implies that the pluripotential stem cells of the embryo have the capacity to reprogram their development.

Murine embryogenesis is therefore reliant on the formation, maintenance and coordinated differentiation of pluripotential stem cells. Access to these cells *in vitro* enables the analysis of signalling events which control these developmental decisions.

FIGURE 1.6 The maintenance and differentiation of pluripotential cell populations during murine development.

Murine development begins with the fertilised egg. Subsequent cleavage gives rise to the morula which contains pluripotent blastomeres. The pluripotential cell lineage is subsequently maintained in the inner cell mass and primitive ectoderm, culminating in the formation of germ cells. The differentiated tissues arising from each pluripotent cell lineage during this progression are also depicted.


MORULA
TROPHECTODERM
INNER CELL MASS
PRIMITIVE ENDODERM
FERTILISED EGG
PRIMITIVE ECTODERM
MESODERM
PRIMORDIAL GERM CELLS
ENDODERM
ECTODERM

1.5 Pluripotential Murine Embryonic Stem Cells *In Vitro*

The finding that it is possible to isolate and culture pluripotential stem cells of the early murine embryo *in vitro* (Evans and Kaufman 1981, Martin 1981), which can give rise to all tissues of the embryo when reinjected *in vivo* (Bradley *et al.* 1984), has revolutionised the study of murine embryogenesis. Not only have embryonic stem cells allowed the development of highly efficient gene targeting strategies for the investigation of gene function *in vivo*, they also constitute an *in vitro* system in which factors and genes controlling pluripotential stem cell differentiation can be identified.

1.5.1 The Isolation of Murine Embryonal Carcinomal (EC) Cells

Teratocarcinomas are gonadal tumours which contain a multitude of differentiated cell types derived through differentiation of a population of pluripotential stem cells which maintain the tumour. The first pluripotential stem cells to be isolated and cultured *in vitro* were the stem cells of teratocarcinomas (Rosenthal *et al.* 1970, Evans 1972), termed embryonal carcinoma (EC) cells. EC cells possess antigenic determinants, biochemical properties and cell ultrastructure similar to cells of the inner cell mass and primitive ectoderm of the early murine embryo. In addition, EC cells differentiate *in vitro* as embryoid bodies and in response to chemical inducers, generating a multitude of differentiated cell types (Martin and Evans 1975a, 1975b, Strickland and Mahdavi 1978). The most significant discovery was that a proportion of EC cell lines were able to incorporate into the ICM of host blastocysts and participate in normal embryogenesis, contributing to range of tissue in chimeric offspring (Papaioannou *et al.* 1975).

The use of EC cells as an *in vitro* system for the analysis of differentiation, and as a vehicle for the generation of transgenic animals, was compromised by their inability to colonise the germ line of murine embryos. This could be explained by the fact that many EC cell lines are aneuploid and may not undergo meiosis (Smith 1992). However, through the isolation and characterisation of EC cells the methods and approaches which were required for the isolation of normal stem cells from the early embryo itself were established.

1.5.2 The Isolation of Murine Embryonic Stem (ES) Cells

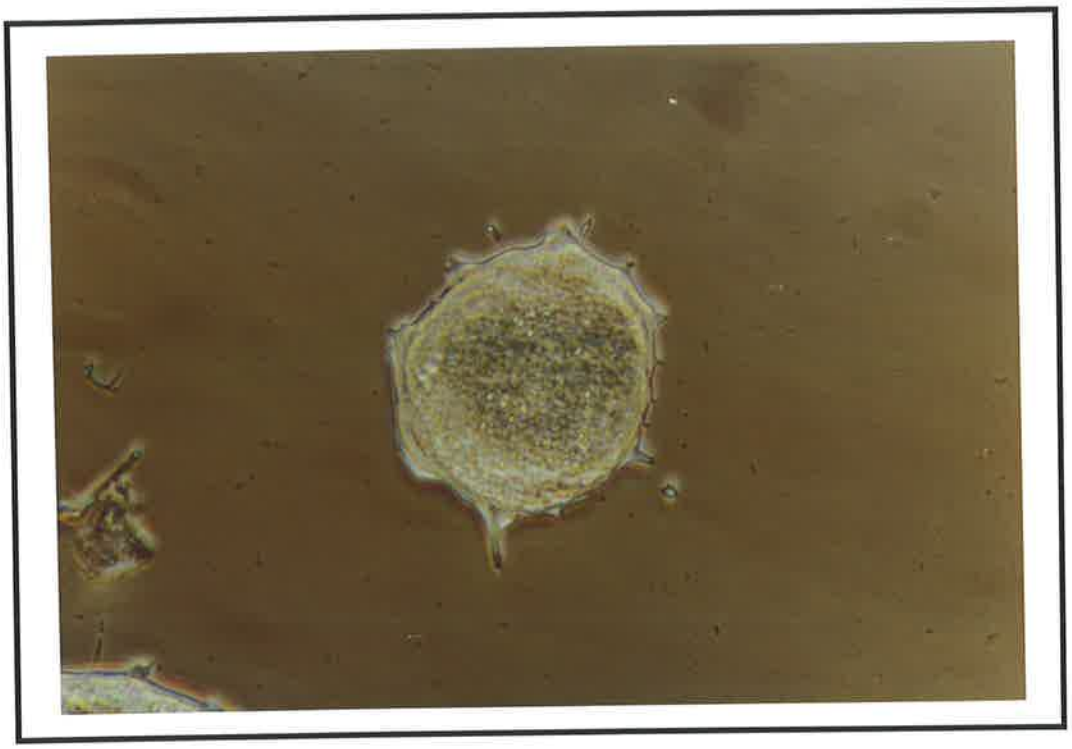
Embryonic stem cell lines were derived directly from the ICM of murine blastocysts in 1981. These cell lines were established from whole blastocysts (Evans and Kaufman 1981) and immunosurgically isolated ICM cells (Martin 1981). In both methods cells were maintained in the undifferentiated state by culture *in vitro* on feeder layers of mitotically inactivated mouse embryonic fibroblasts. An important property of ES cells is that upon reintroduction into the murine blastocyst they contribute at a high frequency to all tissues which constitute the murine embryo including the germ line (Bradley *et al.* 1984, Robertson 1987). This demonstrated that ES cells, in contrast to EC cells, retain full biological pluripotency after culture *in vitro*. This property has enabled the development of ES cells as an *in vitro* system for the analysis of cell differentiation, the generation of transgenic animals and the identification of developmental control genes.

1.5.3 Cytokines which Prevent the Differentiation of ES Cells *In Vitro*

ES cells were originally isolated and maintained by co-culture on mitotically inactivated fibroblasts. On feeder layers these cells grow as compact colonies, consisting of small cells with relatively large nuclei and minimal cytoplasm. If ES cells were cultured in the absence of these feeder layers they underwent extensive differentiation. This suggested that the feeder cells produced an activity which actively suppressed ES cell differentiation and promoted self renewal. This was supported by the observation that various cell lines secrete a soluble factor which inhibits stem cell differentiation This factor, termed Differentiation Inhibiting Activity (DIA) was originally purified from Buffalo Rat liver (BRL) epithelial cells (Smith and Hooper 1987). DIA is a single chain glycoprotein which was found to be identical to a previously described regulator of myeloid differentiation, Leukemia Inhibitory Factor (LIF) (Gearing *et al.* 1987, Hilton 1988, Williams *et al.* 1988). Addition of DIA/LIF to ES cell cultures *in vitro* was sufficient to maintain ES cells in the undifferentiated state and allow their continuous propagation in the absence of feeder layers (Smith *et al.* 1988, Williams *et al.* 1988). ES cells cultured in the presence of LIF grow as a relatively uniform population of cells which possess a dome-shaped, compact colony morphology, similar to the morphology exhibited on feeders (Figure 1.7). ES cells cultured in the presence of LIF retain full

FIGURE 1.7 The morphology of embryonic stem (ES) cells.

ES cells cultured in the presence of LIF grow as compact dome-shaped colonies. ES cells were cultured for three days (2.3.8) and photographed using phase contrast optics at a magnification of 50x.



biological potency, both *in vitro* and *in vivo* (Hooper *et al.* 1987, Smith and Hooper 1987, Smith *et al.* 1988). Furthermore, LIF can also be used as an alternative to feeder layers in the isolation of ES cells from murine embryos (Pease *et al.* 1990). The ability to culture ES cells in the absence of feeder layers has simplified and facilitated their *in vitro* manipulation and allows these cells to be used as an *in vitro* assay system for the identification of factors and genes controlling differentiation.

LIF, together with Steel factor also plays an intrinsic role in the maintenance of primordial germ cells *in vitro* where it inhibits apoptosis and promotes proliferation (Matsui *et al.* 1991, Pesce *et al.* 1993, Cheng *et al.* 1994). When bFGF is added to PGC cultures these cells revert to ES-like cells, termed EG cells (Matsui *et al.* 1992, Resnick *et al.* 1992). EG cells display differentiation properties including germ line contribution, similar to ES cells derived from the ICM (Stewart *et al.* 1994).

Since the discovery of LIF as a cytokine which can maintain ES cells *in vitro*, a number of cytokines exhibiting similar properties have been discovered. These include Oncostatin M (OSM), Ciliary neurotrophic factor (CNTF) and Cardiotrophin 1 (CT-1). Interleukin (IL)-6 in conjunction with a soluble form of the IL-6 receptor is also able to maintain ES cells *in vitro* (Yoshida *et al.* 1994). These factors effect the maintenance of ES cells *via* a common signalling receptor subunit, gp130 (Gearing and Bruce, 1992, Conover *et al.* 1993, Pennica *et al.* 1995).

LIF is expressed by many cell lines *in vitro* and upon spontaneous differentiation of ES cells in culture, is produced by differentiated cells. The action of this LIF on remaining pluripotential cells in the culture results in the maintenance of a pluripotential stem cell pool *via* a feedback mechanism (Rathjen *et al.* 1990). This together with the ability of LIF to maintain ES and EG cells *in vitro* has led to the proposal that LIF might act *via* a similar mechanism to maintain pluripotential stem cells of the embryo during embryogenesis. LIF is known to be expressed at low levels in the murine embryo during early development (Rathjen *et al.* 1990) but this expression does not appear to be necessary for embryological development (Stewart *et al.* 1992). However, mice homozygous for a mutation of the LIF locus are sterile, as blastocysts fail to implant. While LIF expression within the embryo itself is not vital, maternal LIF expression may play a role in embryogenesis. Functional interpretation of LIF action in early

embryogenesis by gene targeting is complicated by the possibility that factors with similar functions, such as OSM, CNTF, CT-1, IL-6 and IL-11 may be present within the embryo and compensate for the loss of LIF. Resolution of this problem requires the generation of mutant mice carrying multiple mutations in each of these genes.

1.5.4 ES Cell Differentiation *In Vitro*

In the absence of LIF, ES cells differentiate spontaneously forming a multitude of differentiated cell types (Smith *et al.* 1988, Williams *et al.* 1988). ES cells can also be induced to differentiate *in vitro* by culture in the presence of chemical inducers such as retinoic acid (RA), 3-methoxybenzamide (MBA) and dimethylsulfoxide (DMSO) (Smith 1991). However, the cell types produced by these differentiation protocols have not been defined unequivocally. While these differentiation protocols can be useful in the identification of developmentally regulated genes (1.5.7), their significance with respect to *in vivo* differentiation events is questionable.

Perhaps more relevant to the study of *in vivo* differentiation events is the study of ES cell differentiation during embryoid body formation. When ES cells are aggregated by culture in suspension in the absence of LIF, they form structures known as embryoid bodies. ES cell aggregates cultured under these conditions differentiate in a sequential manner giving rise to tissues which are present in early murine embryos. Thus the development of embryoid bodies begins with the formation of an outer layer of endoderm. Subsequently, cavitation and the formation of an inner layer of ectoderm-like cells occurs. Based on morphology and gene expression these events are thought to represent respectively, the formation of the primitive endoderm, proamniotic cavity and primitive ectoderm *in vivo* (Doetschman *et al.* 1985, Shen and Leder 1992). When embryoid bodies differentiate further they form a variety of tissues including muscle, cartilage, neurons, blood and pigmented epithelium (Evans and Kaufman 1981, Martin *et al.* 1981, Doetschman *et al.* 1985, Robertson 1987). ES cell derived embryoid bodies have been used by a number of investigators, in the study of many aspects of murine development (reviewed in Pederson 1994). These investigations have included analysis of gene expression during differentiation (Lindenbaum *et al.* 1990, Poirier *et al.* 1991, Yamada *et al.* 1994), the effect of growth factors on differentiation (Shen and Leader

1992, Johansson and Wiles 1995), myogenesis (Rohwedel *et al.* 1994, haemopoiesis (Schmitt *et al.* 1991, Keller *et al.* 1993), cardiac development (Wobus *et al.* 1991, Andrews 1994, Lyons *et al.* 1995) and vascular development (Wang *et al.* 1992). While the *in vitro* development of embryoid bodies is considered to constitute a more accessible system for the analysis of development than the embryo, specific developmental events can not be examined in isolation due to the heterogeneity of the differentiation system.

1.5.5 ES Cells and Gene Targeting

The isolation and culture of pluripotential embryonic stem cells *in vitro*, together with the development of homologous recombination technology has enabled the development of efficient gene targeting systems for the analysis of gene function *in vivo* (Cappechi 1989, Schwartzburg *et al.* 1989). This enables any mutation to be introduced into any gene of interest within the murine embryo. In general the methodology involves introduction of a targeting vector into ES cells cultured *in vitro*. The targeting vector contains the gene of interest carrying the desired mutation and a selectable marker gene. Stably transfected ES cells are selected, expanded and screened for homologous recombination as opposed to random integration *via* Southern analysis. ES cells containing the desired mutation in the specific gene of interest are then reintroduced into early murine blastocysts by microinjection, generating chimeric mice. The use of appropriate coat colour mutations in ES cells and host blastocysts allows easy identification of chimeric animals. Embryos heterozygous for the mutated allele can be generated by backcrossing chimeric animals. Subsequently mice homozygous for the mutant allele can be derived through heterozygous intercrosses.

This technique represents a powerful method for the functional analysis of developmental control genes and has also allowed the development of murine models of human disease. Hence many investigators have employed ES cells as a tool for *in vivo* analysis of gene function (Brandon *et al.* 1995a, 1995b). The advantages of ES cells for the elucidation of differentiation and isolation of potential regulators of development have, by contrast, only been recently exploited.

1.5.6 The Identification of Mammalian Developmental Control Genes

Application of molecular and genetic techniques to murine embryogenesis has been hampered by the small size, fragility and inaccessibly of the murine embryo. A successful approach to the identification of developmental control genes in mammals has involved identification of murine homologues of genes found to be important in experimentally manipulative systems, such as *Drosophila* and *Xenopus*. The similarity between diverse vertebrate species at mid-gestation predicts that the underlying mechanisms of gastrulation will be conserved between species (Gould 1977, DeRobertis *et al.* 1994). Indeed the homologues of many of the genes expressed during gastrulation in *Xenopus* have murine homologues which are expressed at related stages and cell types in the mouse (Blum *et al.* 1992, Beddington and Smith 1993). More surprising is the finding that many of the developmental control genes expressed during *Xenopus* and murine development were isolated by virtue of homology to *Drosophila* developmental genes, identified by classical genetic approaches (Scott *et al.* 1989). While this approach has proved extremely successful, developmental control genes involved in aspects of development peculiar to mammals are unlikely to be identified *via* homology screens.

Genes specific for murine development can also be identified by classical approaches using spontaneous and induced murine developmental mutations (Herrmann 1991). However this technique is dependent on an observable mutant phenotype and mutations affecting early development, which are commonly aborted *in utero*, are difficult to detect. Furthermore the large genome size of mammals hampers the isolation of genes responsible for such mutations.

Another approach has involved the isolation of genes based on their expression patterns during development. Despite the inherent difficulty in accumulating sufficient murine embryonic material, cDNA libraries specific for regions of the mid-gastrulation murine embryo have been constructed. Using subtractive hybridisation techniques, genes with restricted expression in particular tissues of the murine embryo have been identified (Harrison *et al.* 1995). This approach has the advantage of allowing identification of novel genes important specifically during murine development. However, the expertise required for the isolation and dissection of embryonic tissues limits the use of this

technique for a majority of researchers. Furthermore, this approach has not been directed towards the isolation of developmental control genes.

1.5.7 ES Cells and the Identification of Mammalian Developmental Control Genes

The identification of novel developmental genes in mice can be approached by the manipulation of murine embryonic stem cells *in vitro* followed by reconstitution of murine blastocysts by microinjection (Robertson 1991). Insertional mutagenesis in ES cells was first used in the identification of novel genes in 1986 (Robertson *et al.* 1986). This method involved infection of ES cells with a defective retrovirus carrying the Neo^R gene and relies on the insertion of the retrovirus into a developmentally important gene such that the gene is inactivated. Cells in which the retrovirus had inserted were selected in culture and these cells were reintroduced into murine blastocysts. Lines of mice from which homozygous mutants could be bred were generated. One of these lines, deficient in mesoderm formation, was later found to have an insertion in *nodal*, a gene encoding a TGFβ related protein which is expressed around the node during gastrulation (Conlon *et al.* 1991, Zhou *et al.* 1993).

This method has been refined in a number of ways and has led to the development of gene trap vectors which are used in a similar manner to the retroviruses described above (Gossler *et al.* 1989, Friedrich and Soriano 1991, Joyner 1991, Skarnes *et al.* 1992). Gene trap vectors contain a *lac Z* reporter gene and are designed such that *β-galactosidase* (*β-gal*) is expressed only if the vector is integrated into a host gene. The expression pattern of *β-gal* in chimeric animals can be visualised directly by histochemical staining, and developmentally restricted and/or interesting patterns of expression selected. *Lac Z* then acts as a marker which facilitates the cloning of the host gene. In addition the expression of *lac Z* in stably transfected ES cells can be followed during *in vitro* differentiation of ES cells. This enables potentially important insertions to be identified prior to blastocyst injection. The recent development of vectors which identify specific classes of genes represents a significant advance for the identification of novel developmentally regulated genes (Skarnes *et al.* 1995).

Novel genes with restricted patterns of gene expression during murine development have been identified *via* PCR screening of undifferentiated ES cell cDNA (Thomas 1994, Chapman 1994). The ability of ES cells to differentiate *in vitro* (1.5.4) by either spontaneous differentiation in the absence of LIF, in response to chemical inducers or as embryoid bodies has enabled potential regulators of development to be identified based on restricted expression pattern in ES cells and/or differentiated derivatives. While differential display PCR and differential screening of cDNA libraries (T. Shultz, unpublished observations, Poirier *et al.* 1991) have been used to identify genes expressed in this manner, the isolation of developmental control genes is not ensured. The recent identification of an autonomously replicating extrachromosomal vector (episome) (Gassman *et al.* 1995) which can be maintained in ES cells should allow identification of novel control genes *via* functional assays on the basis of molecular complementation *in vitro*. This technique, coupled with the identification of factors which induce the lineage specific and homogeneous differentiation of ES cells *in vitro*, should potentially constitute a powerful and accessible system for the identification of development control genes.

1.6 ES Cells and ICM Cells

For various reasons it has been argued that ES cells are more closely related to the cells of the primitive ectoderm than the inner cell mass (Smith 1992). The nature of developmental restrictions occurring during murine embryogenesis appear to be progressive and at this stage of development are poorly understood. Primitive ectoderm is a term ascribed to cells of the inner cell mass which have not become restricted to primitive endoderm (Rossant 1988). Several observations suggest that ES cells are the *in vitro* equivalent of the inner cell mass rather than primitive ectoderm. Firstly, ES cells are routinely isolated from the inner cell mass of murine blastocysts at approximately day 4 (Hogan *et al.* 1994). They have the capacity to contribute to all tissues in the murine embryo, including the primitive endoderm and its derivatives when reintroduced into early murine embryos (Beddington and Robertson 1989). Secondly, ES cells undergo a program of development and gene expression *in vitro* very similar to that undertaken by cells of the inner cell mass itself, when cultured as embryoid bodies (Doestchman *et al.* 1985, Shen and Leder 1992). This program entails the development of an outer layer of

primitive endoderm followed by the formation of an inner layer of primitive ectoderm. Finally, genes expressed in by the inner cell mass *in vivo* are also expressed by ES cells *in vitro* (Rogers *et al.* 1991, G. Chapman, T. Shultz unpublished observations).

ES cells are isolated from the inner cell mass and exhibit a gene expression and differentiation potential consistent with the cells of the inner cell mass. ES cells can therefore be considered as the *in vitro* equivalent of cells of the inner cell mass.

1.7 ES Cells as an *In Vitro* Model for Murine Development

The molecular events occurring during early murine development prior to gastrulation remain poorly defined. This is largely due to experimental limitations associated with early mammalian embryogenesis such as small size, fragility and inaccessibility of murine embryos at early stages. In addition, while similarities between species are found during and after gastrulation, earlier events of mammalian embryogenesis are not shared with experimentally more manipulative animals such as *Drosophila* and *Xenopus*.

In vitro approaches have proved invaluable in the identification of molecules which regulate haemopoiesis (Metcalf 1989). The isolation and purification of factors responsible for haemopoietic stem cell commitment have consequently been used successfully in the treatment of a range of human diseases (Metcalf 1991). Early mammalian development also relies on the coordinated differentiation, proliferation and maintenance of pluripotential stem cells. Pluripotential cells of the murine ICM (ES cells) are accessible *in vitro*. The ability of these cells to respond to all developmental signals *in vivo* and differentiate *in vitro* raises the possibility that similar functional assays might prove instructive for the identification of factors which control early mammalian development.

This system has been successfully employed in the identification of biological factors which prevent the differentiation of ES cells (1.5.3). Biological factors able to induce the specific differentiation of ES cells had not been characterised at the outset of this work. A variety of factors known to be active in a range of developmental processes such as gastrulation, including members of the FGF and TGFβ families, have no effect on the differentiation of ES cells *in vitro* (P. Rathjen, personal communication).

Molecules which override the LIF signal and induce the specific differentiation of pluripotential stem cells are likely to be of pivotal importance in developmental events taking place during early murine embryogenesis. It should be possible to identify such factors using the specific differentiation of ES cells *in vitro* as an assay system.

Identification of biological factors which regulate stem cell developmental decisions such as differentiation and proliferation *in vitro* is of primary importance to our understanding of early mammalian embryogenesis *in vivo*. The ability to induce specific differentiation of ES cells *in vitro* is potentially a very powerful system for the analysis of events which occur during early embryogenesis. The specific differentiation of ES cells *in vitro* may ultimately allow the formation and isolation of somatic stem cells which would be of considerable importance for successful application of gene therapy to genetic disease.

1.8 Background to the Project

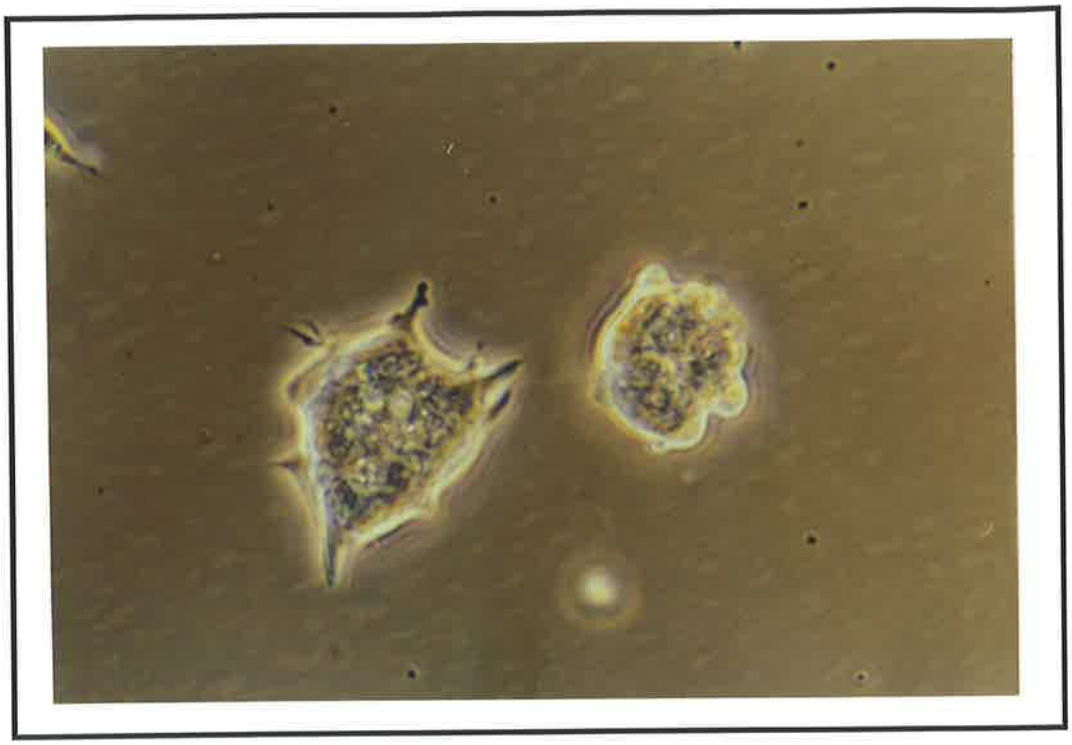
Dr. Joy Rathjen screened a variety of conditioned media for an observable effect on ES cell differentiation in the presence of LIF. One such medium, conditioned by exposure to a human hepatocarcinoma cell line, contained such an inductive activity, and was termed MED2. Culture of ES cells in 50% MED2 in the presence of exogenous LIF resulted in the formation of a morphologically unrelated cell type which were termed X cells. The formation of X cells from ES cells could be seen within 24 hours of culture and appeared to be a highly uniform differentiation event. In contrast to the compact dome-shaped morphology of ES cells, X cells colonies grew as monolayer colonies in which individual cells, containing nuclei with one or more prominent dark nucleoli structures, could be distinguished (Figure 1.8). The morphology of X cells was similar to that exhibited by P19 EC cells which possess properties similar to the embryonic primitive ectoderm (Rudnicki and McBurney 1987, Rogers *et al.* 1991).

The conversion of ES cells to X cells occurred in MED2 in the presence or absence of LIF. X cells could be maintained indefinitely by culture and passage in the presence of MED2. The formation of a highly uniform and maintainable cell population from ES cells contrasts with the multitude of differentiated cell types produced when ES cells are spontaneously differentiated by culture in the absence of LIF. Cell types arising from

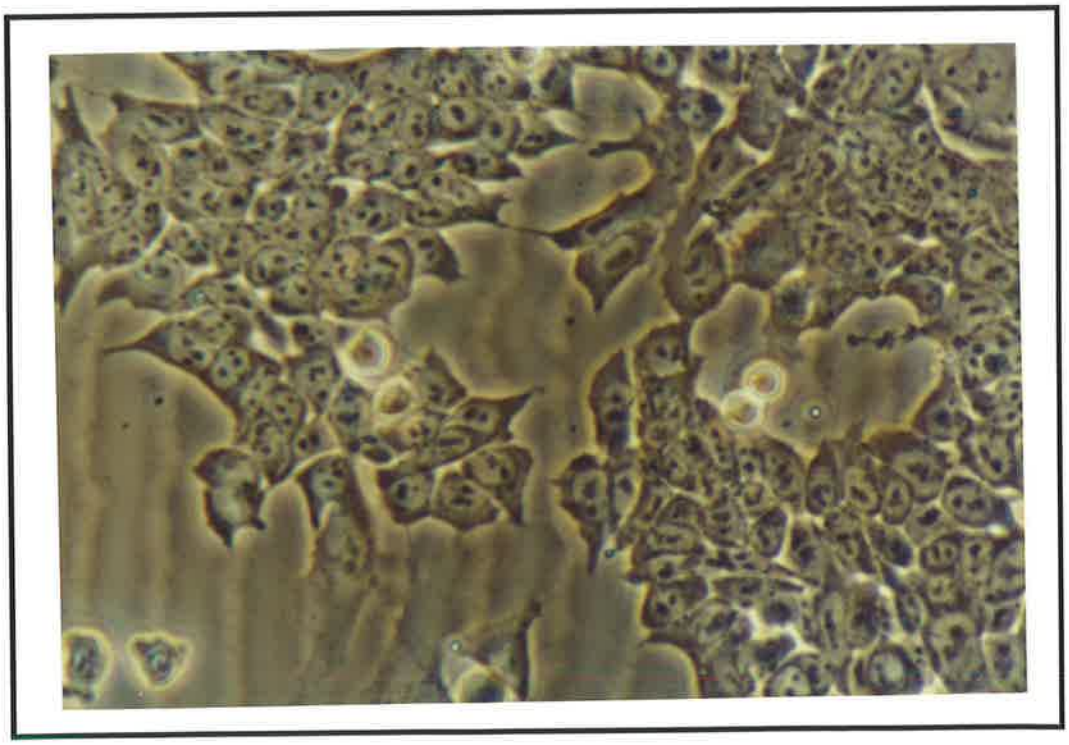
FIGURE 1.8 The morphology of ES cells and X cells.

ES cell colonies are shown in A and X cell colonies are shown in B. Photographs were taken at a magnification of 100x using phase contrast optics.

A



B



such differentiation can not be maintained indefinitely in culture and are morphologically distinct from X cells. When both MED2 and LIF were removed X cells, like ES cells, spontaneously differentiated forming a variety of differentiated cell types. This suggested that X cells, like ES cells, retained differentiation capability. Of particular interest was the observation that when X cells were cultured in the presence of LIF, and absence of MED2, X cells appeared to revert at a high frequency to an ES cell phenotype (J. Rathjen, unpublished observations).

1.8.1 The Factors Responsible for X Cell Formation and Maintenance

The induction and maintenance of X cells is absolutely dependent on the continued presence of MED2. Size exclusion and ion exchange chromatography indicates a requirement for at least two factors, a protein of > 30kD and an uncharacterised factor of approximately 0.7kD (J. Rathjen, J. Washington and M. Bettess, unpublished observations). Purification of the active components of MED2 is in progress and did not form part of this project.

1.8.2 The Gene Expression of X Cells

X cells were found to express the stem cell specific marker alkaline phosphatase by enzymatic based histochemical staining (J. Rathjen, unpublished observations). Immunofluoresence antibody staining revealed that X cells also express the stem cell specific cell surface antigen SSEA-1 (J. Rathjen, unpublished observations). Furthermore, investigation of X cell gene expression suggested that X cells express *Oct-4* (Thomas 1994). Alkaline phosphatase, SSEA-1 and *Oct-4* are expressed in pluripotential cells *in vitro* and *in vivo* (Solter and Knowles 1978, Smith *et al.* 1988, Richa and Solter 1988, Pease *et al.* 1990, Rosner *et al.* 1990, Scholer *et al.* 1990, Yeom *et al.* 1991). These observations were consistent with the spontaneous differentiation of X cells upon withdrawal of MED2 and suggested that X cells retained pluripotential differentiation capability.

Immunofluorescent antibody staining revealed that X cells, in contrast to ES cells, express low levels of the intermediate filament cytokeratin, Endo A (J. Rathjen, unpublished observations). Endo A is expressed in the trophectoderm, primitive

endoderm derivatives, primitive ectoderm and early mesoderm in murine embryos (Franke *et al.* 1982, Brulet *et al.* 1980, Adamson 1988) but is not expressed in the cells of the ICM. This suggested that X cells were different from pluripotential cells of the ICM.

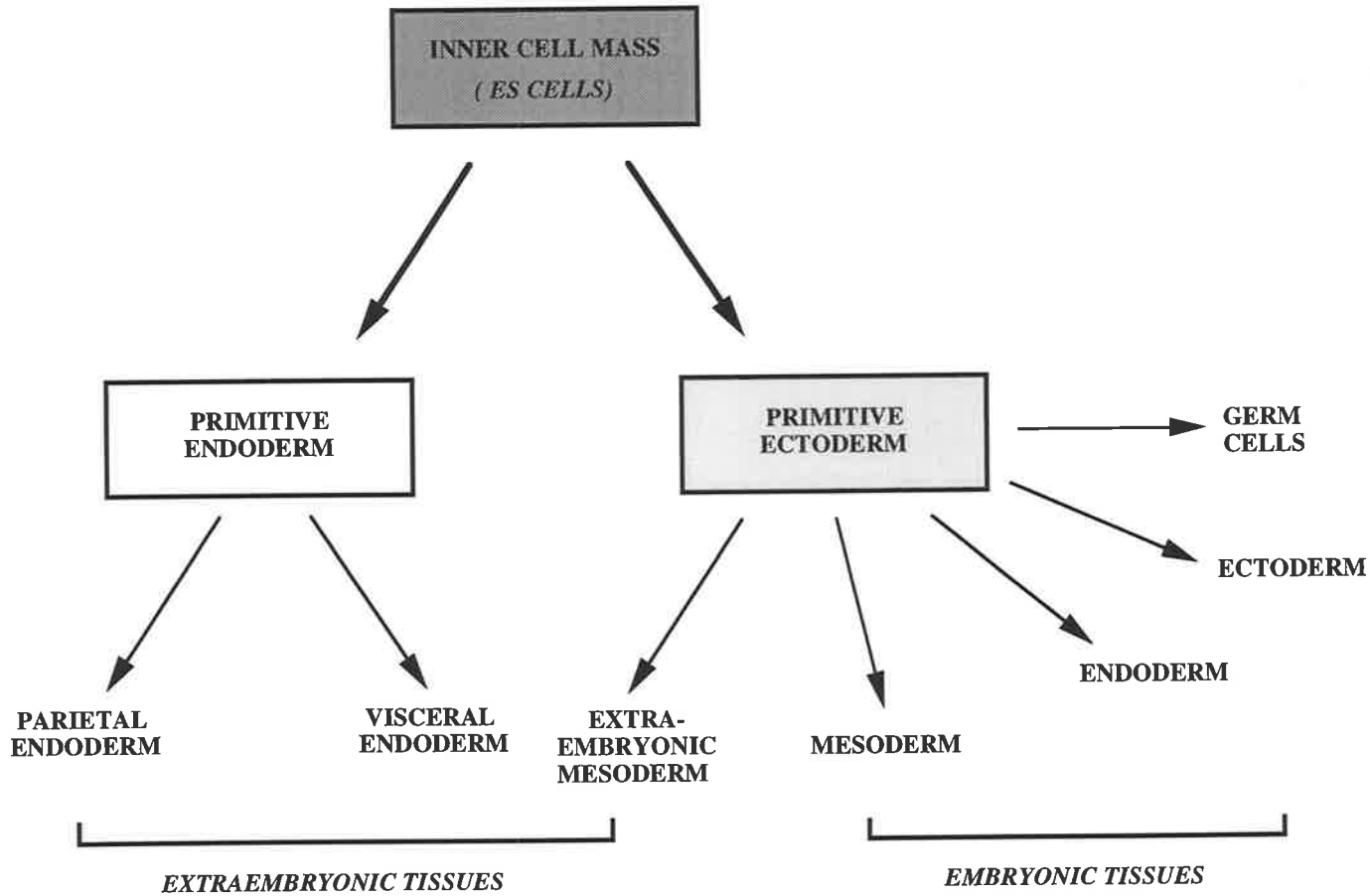
1.8.3 The Significance of the ES to X Cell Transition

The identification of haemopoietic regulatory factors *via in vitro* culture systems demonstrates that differentiation *in vitro* in response to biologically-derived molecules results in the formation of biologically relevant cell types. The differentiation of ES cells into X cells may therefore represent a developmental event occurring during early murine embryogenesis. ES cells are analogous to cells of the inner cell mass (1.6). The immediate developmental fate of the inner cell mass is the generation of primitive endoderm and primitive ectoderm. The primitive endoderm gives rise to the visceral and parietal endoderm which constitute part of the extraembryonic tissues of the murine embryo. The primitive ectoderm, through the process of gastrulation, gives rise to the all tissues of the embryo, including germ cells, as well as contributing to extraembryonic tissues. This developmental progression is represented schematically in Figure 1.9.

The observation that X cells express pluripotential stem cell markers suggested that the differentiation event represented by the ES to X cell transition *in vitro* might reflect the embryonic ICM to primitive ectoderm transition. This was supported by several additional observations. Firstly, the expression of cytokeratin at low levels in X cells but not in ES cells was consistent with low level expression of Endo A in primitive ectoderm but not in the inner cell mass. Secondly, the distinctive cellular morphology associated with X cells, characterised by minimal cytoplasm, large nuclei and prominent nucleoli, was strikingly similar to the cellular morphology associated with primitive ectoderm cells in histological cross sections (Kaufman 1992). Thirdly, while ES cells have been reported to divide every 12-18 hours (Smith 1992), X cells were found to proliferate at a rate consistent with the cell cycle time estimated for primitive ectoderm *in vivo*, which is 6-8 hours (J. Rathjen, unpublished observations, Snow 1977). Finally, X cells possess a cellular morphology similar to the morphology exhibited by P19 EC cells, which are considered to have properties related to the cells of the primitive ectoderm (Rogers *et al.* 1991).

FIGURE 1.9 Cell lineages arising from the inner cell mass *in vivo*.

The inner cell mass gives rise to the primitive endoderm and subsequently the primitive ectoderm. The primitive endoderm forms visceral and parietal endoderm which constitute and give rise to extraembryonic tissues of the embryo. The primitive ectoderm through the process of gastrulation gives rise to extraembryonic mesoderm and the mesoderm, ectoderm, endoderm and germ cells of the embryo proper.


INNER CELL MASS
(ES CELLS)
PRIMITIVE ENDODERM
PRIMITIVE ECTODERM
GERM CELLS
ECTODERM
ENDODERM
MESODERM
EXTRA-EMBRYONIC MESODERM
VISCERAL ENDODERM
PARIETAL ENDODERM
EXTRAEMBRYONIC TISSUES
EMBRYONIC TISSUES

The ability to generate primitive ectoderm from ES cells *in vitro* would have profound importance and application to the understanding of mammalian development. The inner cell mass to primitive ectoderm transition *in vivo* is at present poorly characterised. The differentiation of ES cells into primitive ectoderm *in vitro* should allow the identification of genes and factors involved in this process. The primitive ectoderm constitutes the tissue which responds to developmental signals during gastrulation, differentiating into all the differentiated tissues constituting the murine embryo and adult. Access to pluripotential cells of the primitive ectoderm *in vitro* could therefore provide an *in vitro* assay system for the identification and functional analysis of growth factors or genes involved in gastrulation. In addition, the specific and further differentiation of primitive ectoderm *in vitro* may well lead to the generation and/or isolation of somatic stem cells which are vital for the ultimate success of gene therapy. Furthermore, the ability to generate and maintain primitive ectoderm from ES cells *in vitro* would suggest that the primitive ectoderm from the embryo itself may be isolated and maintained *in vitro*. Embryonic primitive ectoderm cells maintained *in vitro* could constitute an alternative route to generation of transgenic animals.

1.9 Aims and Approach

Appreciation of the biological significance of the ES to X cell differentiation event is largely dependent on identification of the embryonic cell equivalent of X cells. Preliminary investigations of the properties associated with X cells suggested that they represent a pluripotential cell population, possibly the *in vitro* equivalent of primitive ectoderm. The overall aim of the research described in this thesis was therefore to define the possible embryonic equivalent of X cells.

Different cell populations within the murine embryo can be defined on the basis of the genes which they express. Over recent years a number of genes with stage and tissue specific expression during early murine embryogenesis have been identified and can therefore be used for identification of cell types *in vitro* (Shen and Leder 1992, Pruitt *et al.* 1994a, 1994b, Johansson and Wiles 1995). The expression of marker genes in X cells, compared to ES cells was examined. A detailed analysis of the gene expression associated with X cells compared to ES cells would enable the biological equivalent of X

cells to be more fully defined. Identification of gene expression differences between ES and X cells would allow the nature of X cell reversion to be examined at the molecular level.

X cells express the pluripotential stem cell markers, *Oct-4*, alkaline phosphatase and SSEA-1, and spontaneously differentiate into a multitude of cell types upon withdrawal of MED2 and LIF. Thus X cells, like ES cells, appear to retain differentiation capacity. ES cells have the capacity to differentiate as embryoid bodies *in vitro* generating a variety of defined cell lineages. The ability of X cells to form and differentiate as embryoid bodies, compared to ES cells, was examined. Identification of the cell types which X cells form upon differentiation would not only be indicative of their pluripotentiality it would be suggestive of their embryonic equivalent. Knowledge of the relative differentiation potential of X cells and ES cells might be indicative of how pluripotentiality is controlled or restricted during embryogenesis.

Members of the FGF and TGFβ family have been implicated in the process of mesoderm induction during gastrulation in *Xenopus* (Sive 1993). The primitive ectoderm is the substrate for mesoderm induction during murine embryogenesis. As a pluripotent cell population, and given their proposed relationship to embryonic primitive ectoderm, the ability of X cells to differentiate in response to mesoderm inducing cytokines was examined. Differentiation of X cells with such cytokines to biologically relevant cell types *in vitro* would support a relationship between X cells and the cells of the primitive ectoderm. This would also suggest that X cells might be useful as an *in vitro* system for the identification and functional analysis of factors involved in gastrulation.

ES cells possess the capacity to reconstitute embryonic tissues upon reincorporation into murine blastocysts. X cells or reverted X cells, as pluripotential cell populations might also be capable of such contribution upon reinjection into murine blastocysts. Contribution of X cells to murine embryos would confirm their pluripotentiality. Furthermore, given their potential relationship to the cells of the primitive ectoderm, contribution of X cells to murine blastocysts might indicate that primitive ectoderm *in vivo* may have a similar potential when isolated *in vitro*. Embryonic primitive ectoderm might therefore represent an alternative target for pluripotential cell isolation *in vitro*.

CHAPTER TWO:
MATERIALS AND METHODS

CHAPTER TWO: MATERIALS AND METHODS

2.1 Abbreviations

Abbreviations are as described in "Instructions to authors" (1978) *Biochem. J.* **169**, 1-27.

In addition:

A_{260}	Absorbance at 260nm
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bisacylamide	N,N'- methylene-bisacylamide
bp	base pairs
BSA	bovine serum albumin (Fraction V)
D-MEM	Dulbecco's Modified Eagles' Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E. coli.	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
ES	embryonic stem
EtBr	ethidium bromide
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorocein isothiocyanate
Hepes	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
ITSS	Insulin-transferrin-sodium-selenite supplement
LIF	Leukaemia Inhibitory Factor
kb	kilobase pairs
MOPS	3-[N-Morpholino] propane-sulfonic acid
MQ	Milli Q

NBT	nitro blue tetrazolium chloride
NP-40	Nonidet P40
O.D.	optical density at 600nm
OSM	Oncostatin M
PBS	phosphate buffered saline
PEG	polyethylene glycol
PDGFA	Platelet Derived Growth Factor A
RA	*all trans*-Retinoic acid
rNTP	ribonucleotide phosphate
RNAase	ribonuclease
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethenediamine
tRNA	transfer RNA
Tween-20	Polyoxyethelyne-sorbitan Monolaurate
UV	ultra violet

2.2 Tissue Culture Materials

2.2.1 Chemicals

D-MEM	Gibco BRL
DMSO	Sigma
Hams F12	Gibco BRL
Heparin	Sigma
FCS	CSL
gelatin	Sigma
gentamycin	Delta West
ITSS	Boehringer Mannheim
L-glutamine	Gibco BRL
β-mercaptoethanol	Sigma

methanol	BDH Laboratories
phenylene diamine	Sigma Chemical Co.
Salmon Sperm DNA	Sigma Chemical Co.
Trypsin	Gibco BRL

2.2.2 Tissue Culture Plasticware

4 well multidishes	Nunclon
6 well trays	Corning/Falcon
24 well trays	Nunc/Corning/Falcon
96 well trays	Corning/Falcon
100mm plates	Corning/Falcon
60mm plates	Corning/Falcon
150cm^2 flasks	Corning
75cm^2 flasks	Corning
10ml plastic pipettes	Corning/Falcon
2ml plastic pipettes	Sterilin/Falcon
1700cm^2 roller bottles	Corning

2.2.3 Miscellaneous

permanox chamber slides	Nunc
glass chamber slides	Nunc
ektachrome 160T	Kodak
ektachrome 100	Kodak
freezing vials	Nunc
sterile bottle top filters	Corning
pressure filters	Sartolab
bacteriological petri dishes	Techno-plas
disposable cuvettes	Bio-rad
Optiphase 'Hisafe' 2 Scintillation fluid	LKB Scintillation Products

2.2.4 Cell Staining Kits

Alkaline Phosphatase Staining Kit Sigma Diagnostics (86-R)

2.2.5 Cytokines

Recombinant mouse LIF (ESGRO) was obtained from Amrad.

Human recombinant LIF was obtained from Amrad.

Human Oncostatin M was obtained from R&D Systems.

Human recombinant activin A was a kind gift from Dr. Ray Rogers (Flinders University, South Australia).

Human PDGFA was a kind gift from Dr. Helen Healy, C.S.I.R.O.

Purified bovine basic FGF was obtained from Sigma.

Recombinant bovine basic FGF was obtained from Boeringher Mannheim.

Human recombinant FGF-4 was a kind gift from Dr. John Heath.

Human recombinant acidic FGF was obtained from R&D Systems.

Human recombinant FGF-5 was obtained from R&D Systems.

All of the above cytokines were diluted in 0.1% BSA in PBS to specified working concentrations, aliquoted and stored at -20°C until use.

2.2.6 Antibodies

Anti-neurofilament 200 (N-4142) was obtained from Sigma Immunochemicals.

Anti-gp130 blocking antibody (RX19) was a kind gift from Dr. Tetsuya Taga (Institute for Molecular and Cellular Biology, Osaka University). The hybridoma producing this monoclonal antibody was established by Mikiyoshi Sato and purified by Atsushi Kumanogoh.

Anti-human LIF was obtained from R&D Systems.

Anti-rabbit and anti-mouse IgG-FITC conjugates were obtained from Silenus.

Anti-SSEA-1 was a kind gift from Dr. Austin Smith.

Aliquots of antibodies were stored at -20°C and a working aliquot kept at 4°C.

2.2.7 Radiochemicals

[^{3}H] Methyl Thymidine, 70Ci/mmol was obtained from ICN.

2.2.8 Solutions

Solutions were prepared using Millipore-filtered H_2O and sterilised *via* filtration through a 0.2μM filter unless otherwise specified.

Electroporation Buffer:

600μl of 18% glucose in 100ml of HeBS (Made prior to use).

Hepes buffered saline (HeBS):

20mM Hepes, 137mM NaCl, 5mM KCl, pH 7.05 filter sterilised.

L-Glutamine:

100mM L-glutamine in MQ H_2O, filter sterilised and stored as 2ml aliquots at -20°C.

ITSS:

1000x stock of 250mg of ITSS in 25ml of sterile MQ H_2O was made and stored as 200μl alliquots at -20°C.

Heparin:

Stock solution: 1μg/μl in sterile PBS, stored at 4°C.

β-mercaptoethanol/PBS:

Stock solution: 100 mM β-mercaptoethanol in sterile PBS. A fresh solution was prepared every two weeks.

Phosphate buffered saline (PBS):

136mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, pH 7.4. Sterilised by autoclaving (20 psi for 25 minutes at 140°C).

PBS/Gelatin:

0.2% (w/v) gelatin in PBS. Sterilised by autoclaving (20 psi for 25 minutes at 140°C).

phenylene diamine:

0.3g of phenylene diamine were dissolved in 50ml of HCl at room temperature. 1g of activated charcoal was added and the solution was stirred for 2hrs. The solution was filtered through Whatmann paper and the filtrate was cooled on ice. 35ml of ice-cold concentrated HCl was added to the mixture, which was then left to crystallise on ice for 6hrs. The crystals were collected in a Buchner funnel and stored at -20°C in the dark. A

1% (w/v) aqueous solution of phenylene diamine was made and the pH adjusted to 7 with NaOH. This solution was stored at -20°C in the dark.

Trypan Blue:

0.4g trypan blue, 0.81g NaCl, 0.06g KH_2PO_4 in 100ml of MQ H20, pH 7.2 filter sterilised.

Trypsin:

0.1% trypsin and 1 X Versene solution. Sterilised by filtration.

RA:

10^{-2} M RA was dissolved in DMSO and stored at in the dark at -20°C.

Versene solution (10x):

2g EDTA, 80g NaCl, 2g KCl, 2g KH_2PO_4, 11.5gNa_2HPO_4 in 1 L of MQ H_20, filter sterilised and stored at 4°C.

2.2.9 Media

Media was prepared using Millipore-filtered H_2O, and sterilised by filtration through a 0.2µM filter.

D-MEM:

1 packet (13.5g) of powdered D-MEM (high glucose, with L-glutamine, sodium pyruvate, without sodium bicarbonate), 3.7g of $NaHCO_3$, 4.8g Hepes dissolved in 1L of MQ H_2O. The media was then made to a pH of 7.5 and 1.25 ml of 40mg/ml gentamycin was added. Medium was filter sterilised.

Hams F12 medium:

1 packet (10.6g) F12 Nutrient mix (with L-glutamine, without sodium bicarbonate), 1.178g $NaHCO_3$ in 1L MQ H_2O. The medium was made to a pH of 7.5 and then 1.25 ml gentamycin (40 mg/ml) was added. Medium was filter sterilised.

Serum Free Medium:

1:1 Hams F12 medium: D-MEM, 0.1% ITSS.

ES D-MEM medium:

67.4g D-MEM (high glucose, with L-glutamine, sodium pyruvate, without sodium bicarbonate), 18.5g $NaHCO_3$ dissolved in 5 L of MQ H_2O. The medium was then made

to a pH of 7.3 and 6.25 ml (40 mg/ml) gentamicin was added. Medium was filter sterilised.

Incomplete ES cell medium:

90% ES D-MEM medium, 10% FCS, 1% glutamine, 0.1% Beta-mercaptoethanol/PBS.

Complete ES cell medium:

Incomplete ES cell medium containing 1% (w/v) COS-1 cell conditioned medium containing LIF (Refer to section 2.3.6).

X cell medium:

1:1 MED2 conditioned medium : incomplete ES cell medium.

Incomplete MED2 conditioned medium (MED2):

Medium was collected from MED2 cells cultured in incomplete ES cell medium for 5 days. Medium was filter sterilised and stored at -20°C. For MED2 preparation see 2.3.7.

All medium, unless otherwise specified, was stored at 4°C and heated to 37°C in a water bath before use.

2.2.10 Tissue Culture Cell Lines

ES Cell Lines:

ES Cell lines were from the following sources:

MBL-5 and D3 (Lindsay Williams, Ludwig Institute, Melbourne, Australia).

E14 (Anna Michelska, Murdock Institute, Melbourne, Australia).

Other Cell Lines:

Swiss albino 3T3: mouse embryonic fibroblasts obtained from ATCC (CCL92).

COS-1: monkey kidney fibroblasts obtained from ATCC (CRL1650).

MED2 cells: human hepatocarcinoma cell line (unpublished data).

2.3 Tissue Culture Methods

All centrifugation steps were carried out using a Heraeus Sepatech Megafuge 1.0, BS4402A unless otherwise specified.

2.3.1 Gelatinised Petri Dishes

All tissue culture dishes and plates used for ES and X cells were gelatinised with 0.2% (w/v) gelatin in PBS. Plates were covered with gelatin solution and left for at least 30 minutes at room temperature. The gelatin solution was removed immediately before use.

2.3.2 Determination of Cell Number

In general 100µl of cell suspension was diluted in 900µl of trypan blue. The number of cells were counted under the microscope using a haemocytometer. Trypan Blue stains dead cells blue and these were omitted from the cell count.

2.3.3 Preparation of Stem Cell Lines for Storage

Cells were harvested as described in 2.3.8 except they were resuspended in 10% DMSO, 90% FCS. In general 1 confluent plate of stem cells was resuspended in 3ml of 10%DMSO, 90% FCS and 1ml aliquoted per freezing vial. Cells were then placed in freezing racks and put at -80°C for 24 hrs, subsequently one vial was thawed to inspect the efficiency of freezing. The frozen cells were then transferred to liquid nitrogen for long term storage.

2.3.4 Thawing Frozen Stem Cell Lines

A vial of cells was removed from liquid nitrogen and placed in a 37°C water bath. As soon as the cells had thawed they were transferred to a sterile tube containing 10ml of the appropriate medium and spun at 1200 r.p.m. for 5 minutes. Cells were then resuspended in the appropriate medium and placed into two to three gelatinised 100mm dishes. Dishes were placed at 37°C, 10% CO_2 in air. The next day the medium was replaced with fresh medium.

2.3.5 Maintenance of COS-1 Cells

COS-1 cells were maintained in 150cm^2 tissue culture flasks in D-MEM containing 10% FCS at 37°C, 5% CO_2 in air. Confluent flasks were washed twice with PBS, incubated with trypsin (2ml) for 5 minutes at 37°C. Cells were detached by knocking the flask

vigorously. Approximately 8mls of medium was added and 1/10 of the volume of the resulting cell suspension was added to a fresh flask containing approximately 25mls of medium. COS-1 cells were split every 3-4 days.

2.3.6 Preparation of COS-1 Cell Conditioned Medium Containing Murine LIF

Approximately 4 sub-confluent 150cm^2 flasks of COS-1 cells were harvested by washing each flask twice with PBS and then incubated with 2mls of trypsin at 37^{o}C for 5 minutes. Approximately 5 mls of medium were added to inhibit the action of trypsin. The cells were pooled and spun at 1000 r.p.m. for 5 minutes, resuspended in 20mls of electroporation buffer and the cell number determined (2.3.2). The cell suspension was spun at 1000 r.p.m. for 5 minutes, resuspended in 10 mls of electroporation buffer and respun at 1000 r.p.m. for a further 5 minutes. While the cells were spinning 50µl of sonicated salmon sperm DNA (10mg/ml), 50µl of FCS and 10µg of DNA to be transfected (in this case murine LIF D in pXMT2) was added to sterile cuvettes. The cell pellet was then resuspended in a volume of electroporation buffer to give a cell concentration of 1x10^7 cells/ml, 500µl of cells were added to each cuvette. The cells were kept at 4^{o}C for 10 minutes and then eletroporated at 270 Volts at a capacitance of 250uD using the Bio-rad Gene Pulsar^{TM}. The cells were left at room temperature for 10 minutes before addition to 100mm petri dishes containing 10 mls of D-MEM, 10% FCS. The next day the medium was removed and 10mls of incomplete ES cell medium added. After 48hrs the medium was collected and replaced with a further 10 mls of incomplete ES cell medium. After an additional 48hrs the medium was collected and the cells discarded. The conditioned medium collected from the transfected COS-1 cells was pooled and spun at 1600 r.p.m. for 10 minutes and filter sterilised. The activity present within the medium was determined by the ability of a diluted series of the medium to maintain ES cells (2.3.13i). The medium was diluted in PBS and stored at -20^{o}C. In general a 1 in 100 dilution of this stock was able to maintain the growth of ES cells at a level comparable to 1000 U/ml of ESGRO (murine LIF obtained from Amrad). LIF preparation was generally carried out by J.Rathjen.

2.3.7 Preparation of MED2 Conditioned Medium

Confluent 150cm^2 flasks of MED2 cells were harvested by washing twice with PBS and incubating with trypsin for 5 minutes. Cells were removed from the surface of the flask by knocking the flask vigorously. Cells were transferred to a 30ml sterile plastic tube containing approximately 10mls of incomplete ES cell medium and spun at 1200 r.p.m. for 5 minutes. The cells were then resuspended and split 1:3 into 150cm^2 flasks containing 25ml of incomplete ES cell medium and incubated at 37°C, 5% CO_2 in air. After 5 days the conditioned medium was collected and the cells resplit. The conditioned medium was spun at 3000 r.p.m. for 5 minutes and then filter sterilised. For large scale preparation of MED2, 250ml of incomplete ES cell medium was placed into 1700cm^2 roller bottles. The cells harvested from two confluent flasks of MED2 cells were placed into one roller bottle. The bottles were incubated on rollers in a 37°C oven. After 5 days, the medium was collected from the bottles and 250ml of fresh incomplete ES cell medium was added. After a further 3 days the medium was collected and the cells discarded. Conditioned medium collected from the bottles was spun in 500ml centrfuge buckets at 3000 r.p.m. (Beckman J-21B centrifuge, JA-10 rotor). Conditioned medium was filter sterilised and stored at -20°C until use and kept at 4°C while in use (used for a maximum of 1 week).

2.3.8 Routine Maintenance of ES Cells

ES cells were maintained on gelatinised 100mm petri dishes in complete ES medium at 37°C, 10% CO_2 in air. A near confluent plate of ES cells was washed twice in PBS, incubated with trypsin (1 ml) at 37°C for 30 seconds and transferred into 4 ml complete ES cell medium using a pasteur pipette. The cells were spun at 1200 r.p.m. for 5 minutes, gently resuspended in 10 ml complete ES medium and re-seeded at a cell density ranging from approximately $1.5x10^3$-$3.0x10^3$ cells/cm^2 (or a dilution of 1:20-1:40). ES cells were passaged every 3-4 days. ES cells were maintained for a maximum of 34 passages before a fresh vial was thawed.

2.3.9 Routine Formation and Maintenance of X Cells

X cells were formed and maintained in gelatinised 100mm petri dishes at 37°C, 10% CO_2 in air.

i) Formation of X Cells in the Presence of LIF (Xp0+Cells)

ES cells were seeded at a cell density ranging from $2x10^3$-$6x10^3$ cells/cm^2 (1:10-1:30 dilution) in X cell medium containing 1% COS cell derived LIF (2.3.6) and incubated at 37°C, 10% CO_2 in air. X cell colonies were detected 24 hours after induction. The cells were designated Xp0+ cells where + denoted the presence of LIF and p0 equivalent to the number of passages, which was 0.

ii) Maintenance of X Cells in the Presence of LIF

X cells were maintained in X cell medium containing 1% COS cell derived LIF (2.3.6) and passaged and harvested as described for ES cells (2.3.8). X cells generally reached near confluence after 2-3 days and were reseeded at an approximate cell density ranging from $3x10^3$-$1.2x10^4$ cells/cm^2 (1:5-1:20 dilution). Cells were designated Xpx+ cells where + indicated the presence of LIF, and px indicated the number of passages.

iii) Formation of X Cells in the Absence of LIF (Xp0- Cells)

ES cells were seeded at a cell density ranging from $2x10^3$-$6x10^3$ cells/cm^2 (1:10-1:30 dilution) in X cell medium without LIF and incubated at 37°C, 10% CO_2 in air. X cell colonies were detected within 24 hours after induction. The cells were designated Xp0- cells where - denoted the absence of LIF and p0 equivalent to the number of passages, which was 0.

iv) Maintenance of X Cells in the Absence of LIF

X cells were maintained in X cell medium without LIF and passaged and harvested as described for X cells maintained in the presence of added LIF (2.3.9.ii) Cells were designated Xpx- cells where - indicated the absence of LIF, and px indicated the number of passages.

2.3.10 Routine Formation of ES Cells from X Cells (X Cell Reversion)

X cells were seeded at $2x10^3$-$6x10^3$ cells/cm^2 (1:10-1:30 dilution) in complete ES cell medium and incubated at 37°C, 10% CO_2 in air. ES cell colonies were detected after 24-48hrs. Reverted X cells were split every 3-4 days as described for ES cells (2.3.8).

2.3.11 Routine Maintenance of XB Cells

XB cells were routinely maintained in 100mm gelatinised petri dishes, in X cell medium containing 1% COS cell derived LIF (2.3.6) and incubated at 37°C, 10% CO_2 in air. XB cells were passaged and harvested as described for X cells (2.3.9). XB cells were cultured in the absence of LIF as described in 2.3.9 iv and reverted to ES cells as described in 2.3.10.

2.3.12 Harvesting Cells for RNA Preparation

Cells were washed twice with PBS and incubated with trypsin for 1 minute. Cells were transferred to 30ml tubes containing medium and spun at 1600r.p.m. for 5 minutes. The cells were then resuspended in 10ml of PBS and again spun at 1600r.p.m. for 5 minutes. The PBS was removed and the cells were stored at -80°C.

2.3.13 ES Cell Assays at Clonal Cell Density

i) Assaying for the Effect of LIF on ES cells:

0.8 ml of incomplete ES cell medium was added per well of 4 well multidishes. LIF or OSM was added at the dilutions to be tested. The dishes were kept in the incubator (37°C, 10% CO_2) while ES cells were harvested and the cell number determined (2.3.2). The cells were then resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 200 cells/cm^2. After 5 days the cells were stained for alkaline phosphatase activity (2.3.24).

ii) Assaying for X cell Formation/Maintenance

Multidishes were prepared containing incomplete ES cell medium with desired concentrations of MED2 (2.3.7) and/or LIF. COS cell derived LIF (2.3.6) was used in all experiments unless specified otherwise. The multidishes were kept in the incubator

(37°C, 10% CO_2) while ES cells were harvested and the cell number determined (2.3.2). The cells were resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 100 cells/cm^2. The medium was changed after 3 days and at day 5 the assay was stained for alkaline phosphatase activity (2.3.24).

iii) Neutralisation of OSM by Anti-gp130

0.8 ml of incomplete ES cell medium was added per well of 4 well multidishes. Anti-gp130 was added to wells at the specified concentration. ES cells were harvested and the cell number determined (2.3.2). The cells were then resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 200 cells/cm^2. The dishes were kept in the incubator (37°C, 10% CO_2) for 1.5 hours to allow antibody binding. After this time 50ng/ml OSM was added to appropriate wells and the dishes were returned to the incubator. The cells were stained for alkaline phosphatase activity (2.3.24) after 5 days.

iv) Neutralisation of Human LIF by Anti-Human LIF

0.8 ml of incomplete ES cell medium was added per well of 4 well multidishes. 1000U/ml human LIF was added to appropriate wells together with the specified concentration of anti-human LIF. The dishes were kept in the incubator (37°C, 10% CO_2) for 1.5 hours to allow antibody binding. At this time, ES cells were harvested and the cell number determined (2.3.2). The cells were resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 200 cells/cm^2. After 5 days the cells were stained for alkaline phosphatase activity (2.3.24).

In all ES cell assays, colony types were scored under magnification (10x), phase 2 using a Zeiss inverted microscope.

2.3.14 X Cell Assays at Clonal Cell Density

i) Assaying for X cell Maintenance

Multidishes were prepared containing incomplete ES cell medium with desired concentrations of MED2 and/or LIF. COS cell derived LIF (2.3.6) was used in all experiments unless specified otherwise. The multidishes were kept in the incubator (37°C, 10% CO_2) while X cells were harvested and the cell number determined. The cells were resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 100 cells/cm^2. The medium was changed after 3 days and at day 5 the assay was stained for alkaline phosphatase activity (2.3.24).

ii) Assaying for the Presence of Molecules Functionally Similar to LIF in MED2 *via* Anti-gp130

0.4 ml of incomplete ES cell medium was added per well of 4 well multidishes. Anti-gp130 (10µg/ml) was added to appropriate wells. Xp0+ cells were harvested and the cell number determined (2.3.2). The cells were resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 100 cells/cm^2. The dishes were kept in the incubator (37°C, 10% CO_2) for 1.5 hours to allow antibody binding. After this time 0.4ml of MED2, or 0.4ml of incomplete ES cell medium, human LIF (500U/ml), Amrad murine LIF(500U/ml) or OSM (25ng/ml) was added to appropriate wells and dishes were returned to the incubator. The cells were stained for alkaline phosphatase activity (2.3.24) after 4 days.

iii) Assaying for the Presence of Human LIF in MED2 *via* Anti-hLIF

0.4 ml of MED2 was added to appropriate wells of 4 well multidishes. 500U/ml human LIF or 500U/ml Amrad murine LIF and/or 10µg/ml anti-human LIF was added to appropriate wells. The dishes were kept in the incubator (37°C, 10% CO_2) for 1.5 hours to allow antibody binding. After this time, 0.4 ml or 0.8 ml of incomplete medium was added to appropriate wells. Xp0+ cells were harvested and the cell number determined (2.3.2). The cells were then resuspended in an appropriate volume of incomplete ES cell medium and seeded into prepared multidishes at a cell density of 100 cells/cm^2. At 3 days

medium was pre-incubated with anti-human LIF were appropriate and the assay medium was changed. After a total of five days the cells were stained for alkaline phosphatase activity (2.3.24).

In all X cell assays, colony types were scored under magnification (10x), phase 2 using a Zeiss inverted microscope.

2.3.15 Spontaneous Differentiation of ES Cells

ES cells were seeded at $6x10^3$-$1.2x10^4$ cells/ml (1:5-1:10 dilutions) in incomplete ES cell medium and incubated at 37°C, 10% CO_2 in air. Cells were maintained in this medium by reseeding every 2 days (where possible) at dilutions ranging from 1:2-1:5.

2.3.16 Preparation of Embryoid Bodies

i) Free Floating Embryoid Bodies

a)*Partial Trypsinisation Method*

At day 0, cells were washed twice with PBS and then incubated with trypsin (2ml) and rocked gently for 1.5 minutes. Incomplete ES cell medium was added to the plates to inhibit the action of trypsin. The cell clumps were then transferred to a sterile 30ml tube and spun at 800 r.p.m for 2 minutes. The clumps were gently resuspended in 10ml of incomplete ES cell medium, transferred to bacteriological dishes and incubated at 37°C, 10% CO_2 in air. At day 2 the aggregates were divided into 3 and placed in fresh bacteriological dishes containing fresh incomplete ES cell medium. Medium was changed every second day by carefully removing 8ml of medium and replacing it with 8ml of fresh medium.

b) *Single Cell Suspension Method*

At day 0, cells were trypsinised and resuspended at a cell density of $1x10^5$ cells/ml and 10ml placed per 100mm bacteriological petri dishes. Aggregates were cultured and maintained as described in a).

c) *Hanging Drop Method*

At day 0, cells were trypsinised and resuspended in incomplete ES cell medium at a cell density of $1.6x10^4$ cells/ml. 50µl aliquots were placed on the inside surface of tissue

culture petri dish lids. Lids were inverted over dishes containing 3ml of PBS and incubated at 37°C, 10% CO_2 in air. After 2 days lids were removed and flooded with 10ml of incomplete ES medium and transferred into bacteriological petri dishes and returned to the incubator. Medium was replaced every second day as described in a).

ii) Seeded Embryoid Bodies

At day 6 free floating embryoid bodies formed as described in a) were transferred to gelatinised tissue culture plates and allowed to attach to surfaces. Seeded embryoid bodies were incubated at 37°C, 10% CO_2 and the medium was changed every second day.

2.3.17 Scoring Beating Muscle in Embryoid Bodies

i) Free Floating Embryoid Bodies

24 well plates were coated with 1% agarose in ES D-MEM, which was allowed to set. Incomplete ES cell medium (1ml) was added to each well and the plate was equilibrated for 3 hours at 37°C, CO_2 in air. The medium was removed and 1ml of fresh incomplete ES cell medium was added to each well. Embryoid bodies were individually picked using a pasteur pipette and placed into wells (one body/well). Plates were incubated at 37°C, 10% CO_2 and the medium changed every 2 days. Each well was scored for the presence or absence of beating muscle by observation under a Nikon Diaphot Phase Contrast microscope at a magnification of 50x under Phase1.

ii) Seeded Embryoid Bodies

At Day 6 individual embryoid bodies were picked from bacteriological plates using a pasteur pipette and placed into gelatinised 96 well trays containing 200µl of incomplete ES cell medium/well. One embryoid body was placed per well. Plates were incubated at 37°C, 10%CO_2 in air and the medium was changed every second day. The presence or absence of beating muscle was assessed by inspection of each embryoid body under a Nikon Diaphot Phase Contrast microscope at a magnification of 50x under Phase1.

2.3.18 Scoring Neurons in Seeded Embryoid Bodies

At Day 6 individual embryoid bodies were picked from bacteriological plates using a pasteur pipette and placed into gelatinised 96 well trays containing 200µl of incomplete ES cell medium/well. One embryoid body was placed per well. Plates were incubated at 37°C, 10%CO_2 in air and the medium was changed every second day. The presence or absence of neurons was assessed by inspection of each embryoid body under a Nikon Diaphot Phase Contrast microscope at a magnification of 50x under Phase1.

2.3.19 Harvesting Embryoid Bodies for RNA Preparation

i) Free Floating embryoid bodies were transferred to a 30ml tube and allowed to settle. The bodies were washed with PBS and stored at -80°C.

ii) Seeded embryoid bodies were harvested *via* trypsinisation. Cells were detached from the dishes with the aid of a rubber policeman and transferred to a 30ml tube containing incomplete ES cell medium. The cells were then spun at 1600 r.p.m. for 5 minutes, washed with PBS and stored at -80°C.

2.3.20 Differentiation of Cells with Retinoic Acid

1 x 10^6 cells were placed in 100mm bacteriological petri dishes containing incomplete ES cell medium, 1.0µM RA. After 48hrs fresh RA containing medium was added to dishes and the aggregates were incubated for a further 48hrs. At this time the aggregates were transferred to 30ml sterile tubes to settle. The medium was removed and replaced with incomplete ES cell medium which did not contain RA. The aggregates were seeded into gelatinised 100mm tissue culture dishes and harvested for RNA preparation after 2 days, or individually into gelatinised 96 well trays for cell analysis after 2 days. The cell types present were assessed using a Nikon Diaphot Phase Contrast microscope at a magnification of 50x under Phase1.

Harvesting RA-treated aggregates for RNA preparation.

RA-treated aggregates were harvested by washing plates twice with PBS and adding 2ml of trypsin. Cells were detached from the dishes with the aid of a rubber policeman and

transferred to a 30ml tube containing incomplete ES cell medium. The cells were then spun at 1600 r.p.m. for 5 minutes, washed with PBS and stored at -80°C.

2.3.21 Cytokine Assays

i) Assaying for Differentiation

Assays for the affect of cytokines on ES and X cells were generally carried out in 4 well multidishes. 0.8 ml incomplete ES medium and 50% MED2:50% incomplete ES cell medium was placed per well of gelatinised multidishes for ES cells and X cells, respectively. COS cell derived LIF (2.3.6) was added at a concentration of 1%. Cytokines were added at specified concentrations and the dishes were incubated at 37°C, 10% CO_2 in air. ES, X and/or XB cells were harvested and the cell number determined (2.3.2). Cells were resuspended in an appropriate volume of medium, seeded at a cell density of 75cells/cm^2 into prepared multidishes and returned to the incubator. Medium was changed at 3 days and the assay stained for alkaline phosphatase activity (2.3.24) 2 days later.

For determination of cell number, assays were carried out as described, except scaled up 4 fold to allow sufficient cell numbers for analysis. At day 5, cells were trysinised, 1ml of medium was added and the cells spun at 1000 r.p.m. Cells were resuspended in a small volume of medium (100µl) and the number of cells for each well determined as the average of two independent cell counts using a haemocytometer. The average cell number was derived from the analysis of triplicate wells.

ii) Assaying for X Cell Reversion

Assays were carried out in 4 well multidishes. 0.8 mls of incomplete ES cell medium containing 1% COS cell derived LIF (2.3.6) was added per well. 10ng/ml of bFGF was added to appropriate wells and dishes were incubated at 37°C, 10% CO_2 in air. Xp0+ cells were harvested and resuspended in an appropriate volume of medium. Cells were seeded at a cell density of 100 cells/cm^2 in prepared multidishes and the dishes were returned to the incubator. Medium was changed at 3 days and the assay stained for alkaline phosphatase activity (2.3.24) 2 days later.

In all assays, colony types were scored under magnification (10x), phase 2 using a Ziess inverted microscope.

2.3.22 Maintenance of Swiss 3T3 Cells

Swiss 3T3 cells were maintained in 75cm^2 flasks in D-MEM, 10%FCS at 37°C, 5%CO_2. A confluent flask of cells was washed twice with PBS and incubated with 1.5ml of trypsin for 5 minutes at 37°C. Cells were detached from the flask by knocking. Approximately 8ml of medium was added to the flask and 1ml of cell suspension was added to a fresh flask containing approximately 20 ml of medium.

2.3.23 Swiss 3T3 Growth Factor Assay

A confluent flask of Swiss 3T3 cells was washed twice with PBS and incubated with trypsin (1.5ml) for 5 minutes at 37°C. Approximately 8ml of medium was added to the flask, the cell suspension transferred to a 30ml tube and the cell number determined. Cells were pelleted by centrifugation at 1200 r.p.m. for 5 minutes and resuspended in D-MEM, 10%FCS at a cell concentration of 2x10^4 cells/ml. One ml of the cell suspension was aliquoted per well of a 24 well tray and cells were incubated at 37°C, 5% CO_2. After 48hrs wells were washed twice with PBS (2ml), 1ml of serum free medium was added per well and trays were returned to the incubator. After a further 48 hours, the medium was replaced with fresh serum free medium and growth factors were added to appropriate wells at increasing concentrations (generally 0.1ng/ml to 500ng/ml). 10% FCS was used as a positive control. All variables were tested in duplicate. Six hours after growth factor addition, 10µl of [^{3}H]-methyl Thymidine diluted 1/5 in PBS was added to each well and the cells were incubated for a further 18hrs. The medium was then aspirated and each well was washed twice with 2ml of ice-cold PBS. Cell monolayers were fixed with glacial acetic acid:methanol (1:3 v/v),(1ml/well) for 10 minutes at room temperature. The fixative was aspirated and each well was washed with 2ml ice-cold 10% TCA. Two ml of 10% TCA was then added per well and the cells incubated at 4°C for 15 minutes. The 10% TCA wash and incubation was repeated, the TCA solution was aspirated and the cells washed with 2ml of H_2O. The water was aspirated and 0.2ml of 0.2MNaOH was added to each well. Plates were incubated at 37°C for 2hrs to solubilise the cell

monolayers. Solubilised cells in each well were then transferred to 3ml of scintillation fluid in scintillation vials and counted in the LKB Rackbeta scintillation counter (^3H channel).

2.3.24 Alkaline Phosphatase Staining

Staining was carried out using an alkaline phosphatase staining kit. Cells were fixed in a solution containing methanol (13ml), citrate solution (5ml) and formaldehyde (1.6ml) for 5 minutes at room temperature. A diazonium salt solution was prepared by adding 0.2ml sodium nitrate solution to 0.2ml FRV-alkaline phosphatase solution. The solution was mixed by gentle inversion and kept for 2 minutes in the dark at room temperature before addition to 9ml of deionised water. 0.2 ml of Naphthol AS-BI Alkaline solution was added to the diluted diazonium salt solution. Fixed cells were rinsed gently with deionised water and incubated with the alkaline-dye solution for 15-30 minutes in the dark at R.T. Cells were rinsed again with deionised water and placed at 37°C to dry. Counterstaining with a 1 in 10 dilution of haematoxylin stain in deionised water prior to drying was optional.

2.3.25 Detection of Neuronal Antigens by Indirect Immunofluorescence

Day 6 embryoid bodies were seeded on to gelatinised glass chamber slides in incomplete ES cell medium and incubated at 37°C, 10%CO_2. Medium was changed every 2 days. At Day 10-12 slides were fixed in methanol pre-cooled to -20°C, for 4 minutes. The slides were then immersed in acetone at -20°C for 2 minutes. Slides were air dried and then rehydrated in PBS for 15 minutes at R.T. Slides were blocked by incubation with 0.2ml 10%FCS/PBS for 30 minutes at R.T. The primary antibody, anti-neurofilament 200 (Sigma Immunochemicals N-4142) was diluted 1:100 in PBS+Tween-20 (0.05%) and 0.2ml of antibody solution was added per slide. Slides were incubated with the antibody for 45 minutes at room temperature in a humidity chamber. The slides were then washed 3 x 5 minutes in PBS+0.05%Tween-20. The secondary antibody (FITC-conjugated anti-rabbit IgG) was diluted 1/60 in PBS +0.05% Tween-20 and 0.2ml added per slide. Slides were incubated with antibody for a further 45 minutes in the dark at room temperature in a humidity chamber. Slides were washed 3 x 5 minutes in

PBS+0.05% Tween-20 and mounted in 50µl of mounting solution containing 0.9ml 80% glycerol and 0.1ml of phenylminediamine. Antibody staining was viewed under fluorescence using a Zeiss Axioplan Universal Microscope.

2.3.26 Detection of SSEA-1 by Indirect Immunofluorescence

Cells were trypsinised and resuspended in 3ml of D-MEM/10%FCS. Washes and antibody incubations were carried out in suspension using a nutator. After each treatment cells were pelleted by centrifugation at 3,000 r.p.m for 5 minutes. Cells were washed 2x5 minutes in 1:1PBS:D-MEM and resuspended in a 1:500 dilution of anti-SSEA-1 in D-MEM/10%FCS. Cells were incubated with the antibody for 45 minutes at 4°C and then washed 3x in PBS/10%FCS for 5 minutes. The cells were incubated with a 1:30 dilution of the secondary antibody (FITC-conjugated anti-rabbit IgG) diluted in D-MEM/10%FCS. The cells were washed 3x5 minutes in PBS/10%FCS and resuspended in 50µl of 1:1 D-MEM:FCS. The cells were spun onto a glass slide using a cytospin and subsequently fixed in 95:5 methanol:acteic acid at -20°C for 15 minutes. The slides were air dried and mounted in a solution containing 0.9ml 80% glycerol and 0.1ml phenylminediamine. Staining was viewed by fluorescent microscopy using the Zeiss Axioplan Universal Microscope for transmitted and incident light fluorescence. Duplicate photographs were taken of cells under fluorescent phase and normal phase at a magnification of 40x and the proportion of cells staining was determined.

2.3.27 Generation of Chimeric Mice

i) Preparation of Stem Cells for Injection into Mouse Blastocysts

Cell to be injected were washed twice with PBS, incubated with trypsin (0.5ml) and dislodged from the plate by very gentle pipetting. Trypsinisation was viewed under the microscope and 10ml of media added when a high proportion of cells existed as single cells.

ii) Blastocyst Injection and Embryo Transfer

Blastocyst injection and embryo transfer were carried out in by Anita Peura and Steven MacIlfatrick (Bresatec, Adelaide). The protocols employed were essentially as described in Hogan *et al.* 1994. Cells were injected into CBA/C57F2 blastocysts which were

transferred to pseudopregnant foster mothers. Pups were born 17-18 days later and coat colour observed.

iii) Blood GPI Analysis

Tail Blood was taken from each mouse and analysed for blood contribution by GPI analysis, as described by Bradley 1987.

2.4 Molecular Materials

2.4.1 Chemicals and Reagents

All chemicals and reagents were of analytical grade and supplied from the following manufacturers.

Acrylamide	MERCK
Agarose	Sigma Chemical Co.
Ammonium persulphate	BDH Chemicals
Ampicillin	Sigma Chemical Co.
Anti-DIG Fab antibody-AP	Boehringer Mannheim
Bacto-agar	Difco
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
BCIP	Sigma Chemical Co.
Bis-acrylamide	BIO-RAD
BSA	Sigma Chemical Co.
Cesium Chloride	Boehringer Mannheim
DTT	Diagnostic Chemicals Ltd.
Ethidium Bromide	Sigma Chemical Co.
EDTA	Sigma Chemical Co.
Formamide	BDH chemicals.
Formaldehyde	BDH chemicals
Glycogen	Boehringer Manneheim
Glutaraldehyde	Probing and Structure

Heparin	Sigma Chemical Co.
Isopropanol	Ajax
Kit Blocking reagent	Bresatec
MOPS	Sigma Chemical Co.
NBT	Boehringer Manneheim
NP-40	BDH chemicals
Phenol	BDH Chemicals
Paraformaldehyde	MERCK
PEG 6000	BDH Chemicals
RA	Sigma Chemical Co.
rNTPs	Sigma Chemical Co.
Sepharose CL-6B	Pharmacia
Salmon Sperm DNA	Sigma Chemical Co.
SDS	Sigma Chemical Co.
Sephadex G50 fine	Pharmacia
Sodium deoxycholate	Sigma Chemical Co.
TEMED	Sigma Chemical Co.
Tris base	Sigma Chemical Co.
tRNA	Sigma Chemical Co.
Tween 20	Sigma Chemical Co.

2.4.2 Radionucleotides

[α-^{32}P] dATP (3000 Ci/mmol), [α-^{35}S] dATP (1000-1500 Ci/mmol) and [α-^{32}P] UTP (3000 Ci/mmol) were supplied by Bresatec.

2.4.3 Kits

^{T7}Sequenase kit:	Pharmacia
Gigaprime kit:	Bresatec
The Geneclean II™:	Bresatec

2.4.4 Enzymes

Restriction endonucleases were supplied by Pharmacia and New England Biolabs.

Other enzymes were obtained from the following sources:

Calf Intestinal Phosphatase (CIP):	Boehringer Mannheim
DNAase 1	Bresatec
E. coli DNA polymerase I (Klenow fragment)	Bresatec
Lysozyme	Sigma
Proteinase K:	Boehringer Mannheim
Ribonuclease A (RNAase A)	Sigma.
Ribonuclease T1 (RNAase T1)	Ambion.
RNAasin	Bresatec
T3 RNA polymerase	Promega.
T4 DNA ligase	Bresatec
T7 DNA polymerase	Pharmacia
T7 RNA polymerase	Bresatec

2.4.5 Buffers

AP buffer:

100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5, 0.1% Tween 20

Denhardt's solution (50x):

1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA

FLB:

95% (w/v) deionised formamide, 0.02% bromophenol blue, 0.02% xylene cyanol

GLB(10x):

50% glycerol, 0.1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol

GTE:

50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 7.6

MOPS (10x):

23 mM MOPS pH 7.0, 50 mM NaAcetate, 10 mM EDTA

NLB(10x):

1mM EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol

RIPA:

150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40

STET:

50 mM Tris-HCl pH 8.0, 50 mM EDTA, 8% sucrose, 5% Triton X100

SSC (20x):

3M NaCl, 0.3M sodium citrate, pH 7.4

TAE (20x):

800mM Tris, 400mM NaAc, 20mM EDTA, pH 8.2

Tfb1:

30mM KAc, 100mM RbCl2, 10mM CaCl2, 50mM MnCl2, 15% glycerol. pH adjusted to 5.8 with 0.2M acetic acid.

Tfb2:

10mM MOPS, 75mM CaCl2, 10mM RbCl2, 15% glycerol. pH was adjusted to 6.5 with 1M KOH.

TBE (20x):

180 mM Tris, 180 mM boric acid, 5.0mM EDTA, pH 8.3

TBST:

136 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH 7.5, 0.1% Tween 20

TNM:

30 mM Tris-HCl pH 7.6, 150 mM NaCl, 15 mM $MgCl_2$, 0.4% NP40 (stored at -20°C)

TUNES:

10 mM Tris-HCl pH 8.0, 7 M urea, 0.35 M NaCl, 1 mM EDTA, 2% SDS

All general buffers were sterilised by autoclaving.

2.4.6 DIG Riboprobe Dot Blot Buffers

Buffer 1:

100 mM Tris-HCl pH 7.5, 150 mM NaCl

Buffer 2:

0.05g blocking reagent (Bresatec) in 10 ml Buffer 1

Buffer 3

100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$

Buffer 4:

10 mM Tris-HCl pH 8.0, 1 mM EDTA

DIG riboprobe dot blot buffers were prepared from autoclaved solutions.

2.4.7 Cloning and Expression Vectors

pBluescript KS^+ was obtained from Stratagene

LIFD in pMXT2 was obtained from Dr. Peter Rathjen.

2.4.8 Cloned DNA Sequences

AFP was kindly provided by Dr. Peter Rathjen. The plasmid contained approximately 400 bp of *AFP* cDNA in the *EcoRI* site of a plasmid of unknown origin. to overcome this problem the 400bp *EcoRI* fragment was cloned into the *EcoRI* site of pBluescript KSII+ and the orientation determined by sequencing.

Brachyury (pSK75) was kindly provided by Dr Bernard G. Herrmann. The plasmid contained a 1764bp of *Brachyury* cDNA cloned into the *EcoRI* site of pBluescript SKII (Herrmann 1991).

cNkx2.5 was a kind gift from Dr. Richard Harvey. The plasmid contained 1596 bp of the *cNkx2.5* cDNA cloned into pBluescript SK+ (Lints *et al.* 1993).

FGF-5 was kindly donated by Dr G Martin. This plasmid consisted of a fragment encoding the full length murine *FGF-5* cDNA, cloned into the in *SmaI* site of pBluescript KS+ (Hebert *et al.* 1991).

Goosecoid was kindly provided by Dr. Eddy De Robertis. This plasmid contained 909bp of *goosecoid* cDNA subcloned into the *PstI/HincII* sites of pBluescript $KSII^-$ (Blum *et al.* 1992).

H19 (LC10-8) was kindly donated by Dr Peter Rigby. This plasmid contained a fragment corresponding to 330 bp of the *H19* cDNA, cloned into pBluescript KS^+ (Poirier *et al.* 1991).

MMoxB cDNA clone 7.1 was kindly provided by Gavin Chapman. The plasmid consisted of 2.1kb fragment encoding *MMoxB* inserted into the site *EcoRI* site of pBluescript (Chapman 1994).

The *Oct-4* cDNA clone in pBluescript was a kind gift from Dr. Hans Scholer. This clone contained a 462 bp *Stu* I cDNA fragment spanning positions 491 to 953 of the *Oct-4* cDNA sequence (Scholer *et al*, 1990).

Rex-1 was kindly donated by Dr. Neil Clarke. This plasmid contained 848 bp of *Rex-1* cDNA in the *EcoR1* site of pCRTMII.

S8 was kindly provided by Dr. Frits Meijlink. The plasmid contained 576 bp of *S8* cDNA corresponding to region 51-626 of the *S8* cDNA sequence in pBluescript KS (Opstelten *et al.* 1991).

SPARC (pG43) was a kind gift from Dr. Bridgid Hogan. The plasmid contains 2036 bp of *SPARC* cDNA corresponding to regions 13-2079 *SPARC* cDNA in the *EcoRI* site of pGEM-1 (Mason *et al.* 1986).

2.4.9 Oligonucleotides

General Sequencing Primers:

T7: TAATACGACTCACTATAGGGAGA

T3: ATTAACCCTCACTAAAGGGA

2.4.10 Bacterial Strains

The *E. coli* DH5α strain (a kind gift from Dr. Helena Richardson) was used as a host for all recombinant plasmids.

DH5α: *supE*44 delta lac U169 (phi80 *lacZ*deltaM15) *hsdR*17 *recA*1 *endA*1*gyrA*96 *thi*-1*relA*1

Stock cultures of these strains (and transformed bacteria) were prepared by dilution of an overnight culture with an equal volume of 80% glycerol and storage at -80°C.

2.4.11 Bacterial Growth Media

Growth media were prepared in double distilled water and sterilised by autoclaving. Antibiotics and other labile chemicals were added after the media solution had cooled to 50°C.

FTB Broth:

Bactotryptone (12g), Bacto-yeast extract (24g), glycerol (4ml) in 900ml of MQ H_20. The medium was autoclaved and then 17ml of 1M KH_2PO_4, 72ml of K_2HPO_4 added and the medium made up to 1L.

Luria (L) broth:

1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH.

ψ broth (ψ b):

5g Bacto-yeast extract, 20g Bactotryptone, 5g $MgSO_4$, 14g Bactoagar, made up to 1L with MQ H_2O. The pH of the medium was adjusted to 7.6 with KOH.

Solid Media:

Agar plates were prepared by supplementing L Broth with 1.5% Bacto-agar.

Ampicillin (100 μg/ml) was added for growth of transformed bacteria to maintain selective pressure for recombinant plasmids.

2.4.12 DNA Markers

Eco R1 / *Hind* III lambda DNA markers were prepared by digestion of lambda DNA (New England Biolabs). Band sizes (bp): 21227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 974, 831, 564, 125.

2.4.13 Miscellaneous Materials

3MM chromatography paper: Whatman Ltd.

Nytran nylon membrane (0.45 μM): Schleicher and Schuell

X-Omat AR diagnostic film: Kodak

2.5 Molecular Methods

2.5.1 Restriction Endonuclease Digestion of DNA

Plasmid DNA was digested with 1-2 units of enzyme/μg DNA and was incubated at the appropriate temperature for 1-4 hours. Appropriate reaction buffers were determined using the NEBuffer system (New England Biolabs). Plasmid was assayed for complete digestion by agarose gel electrophoresis (2.5.2 i).

2.5.2 Gel Electrophoresis

i) Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using horizontal mini-gels. 10 ml of gel solution (1% to 2% w/v agarose in 1 x TAE, stored at 68°C) was poured onto a 7.5 cm x 5.0 cm glass microscope slide. Agarose mini-gels were submerged in 1 x TAE and samples containing 1 x GLB were electrophoresed at 80 mA. Nucleic acid was visualised by staining gels with EtBr (0.25 mg/ml in water) and exposing to medium wavelength UV light. DNA fragments to be purified were removed from preparative gels using a sterile scalpel blade.

ii) Polyacrylamide Gel Electrophoresis

6% polyacrylamide gels were prepared from a 40% acrylamide (38:2, acrylamide:bis-acrylamide), 7M urea, 0.5 x TBE stock solution. 300μl of 10% APS and 30μl of

TEMED were added to 40mls of stock gel solution prior to pouring. The gel solution was poured between clean glass plates (20 x 40 cm) separated by 0.4 mm spacers. Once the gel had set (approximately 30 minutes), the comb was removed and wells flushed with water and pre-electrophoresed for 30 minutes at 2000V/50mA. Wells were flushed with 0.5 x TBE, the samples were loaded and the gel electrophoresed at 50mA (approximately 50°C). Electrophoresis was stopped when the loading dye had reached the desired distance. The glass plates were prised apart and the gel transferred to wet 3MM Whatmann paper. The excess liquid was removed and the gel dried down at 70°C under vacuum. Radioactivity was detected by exposure to phosporimager screens at R.T. or X-ray film at -80°C.

2.5.3 Purification of Linear DNA Fragments

DNA fragments which were greater than 400bp were purified using the Geneclean II™ Kit following manufacturers instructions. Linearised vectors used for synthesising riboprobes were purified by phenol/chloroform extraction and EtOH precipitation.

2.5.4 DNA Ligation Reactions

Ligation reactions contained 25 ng purified vector, 50-100 ng DNA insert, ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and 1 unit T4 DNA ligase. Reactions were incubated at room temperature for 1-5 hours.

2.5.5 Preparation of Competent Cells

DH5α were streaked onto an LB plate and grown overnight at 37°C. 5ml of ψb medium was inoculated with a single colony of DH5α and incubated overnight at 37°C with shaking. 0.5ml of the overnight culture was subcultured into 15 ml of ψb medium and incubated at 37°C with shaking for 1.5hrs or until the culture had reached an O.D. of 0.6. 5ml of the culture was then added to 95ml of ψb medium (pre-warmed to 37°C) and incubated at 37°C for 1.5hrs (O.D. of 0.5-0.6), with shaking. The culture was chilled on ice for 5 minutes, centrifuged at 6000 r.p.m. for 5 minutes at 4°C (Beckman J-21B, JA-20 rotor) and the cells resuspended in 40 ml of Tfb1. The cells were left on ice for 5 minutes and then centrifuged again at 6000 r.p.m. for 5 minutes at 4°C. The cells were

resuspended in 4ml of Tfb2 and left on ice for 15 minutes. 100µl aliquots were placed into eppendorf tubes on ice and the cells snap frozen in dry ice/EtOH. Competent cells were stored at -80°C.

2.5.6 Transformation of Competent Cells

Approximately 10 ng ligated DNA was added to 100µl of competent cells and placed on ice for 30 minutes. The cells were heat shocked for 2 minutes at 42°C and cooled on ice for 30 minutes. 800 µl of L broth was added, and the cells were incubated at 37°C for 40 minutes. Cells were pelleted by centrifugation at 6000 r.p.m. for 2 minutes (Eppendorf centrifuge 5415C) and 800µl of L broth removed. The cells were resuspended in the remaining L Broth and spread on L plates containing ampicillin (100 µg/ml). Plates were incubated overnight at 37°C.

2.5.7 Rapid Small Scale Preparation of DNA (Mini-prep)

1.5 ml of L broth containing ampicillin 100µg/ml was inoculated with a single transformant colony and incubated overnight at 37°C with shaking. Cell cultures were transferred to eppendorf tubes and spun for 1 minute at 6000 r.p.m. The bacterial pellets were thoroughly resuspended in 200 µl STET buffer and 10 µl lysozyme (10mg/ml) was added. The bacteria were lysed by heating at 100°C for 45 seconds. Samples were then centrifuged for 15 minutes at 12,000 r.p.m. and the cell debris and chromosomal DNA removed with a sterile toothpick. Plasmid DNA was precipitated by addition of 200 µl isopropanol and incubation on ice for 5 minutes. Plasmid DNA was centrifuged for 10 minutes, washed with 70% ethanol, and resuspended in 20 µl MQ H_2O. Centrifugation steps were carried out using a Eppendorf centrifuge 5145C.

2.5.8 Double Stranded Sequencing of Plasmid DNA

i) Denaturation of Plasmid DNA

10 µl of miniprep DNA was diluted to 20 µl with MQ H_2O, 1 µl RNAase A (20mg/ml) was added and the DNA incubated at 37°C for 15 minutes. Plasmid DNA was denatured by the addition of 5 µl 1 M NaOH/1 mM EDTA and incubation at 37°C for 15 minutes.

Denatured plasmid was purified by centrifugation at 1800 r.p.m. for 3 minutes on a Sepharose CL-6B column.

ii) Sequencing of Plasmid DNA

Dideoxy sequencing reactions were carried out using the Pharmacia T7 polymerase sequencing kit according to manufacturer's instructions. Reaction products were separated on a 6% denaturing polyacrylamide gel (2.5.2 ii).

2.5.9 Large Scale Plasmid Preparation

5ml of L Broth + ampicillin (100ug/ml) was inoculated with a single bacterial colony and incubated overnight, with shaking, at 37°C. 1ml of the overnight culture was added to 500ml FTB broth + ampicillin (100 µg/ml) and the broth incubated overnight at 37°C in an orbital shaker. The cells were harvested by centrifugation at 6,000 r.p.m. for 5 minutes at 4°C (Beckman J-21B centrifuge, JA-10 rotor). The bacteria were thoroughly resuspended in 6.5 ml GTE, transferred to oakridge tube and incubated on ice for 5 minutes. 13 ml of lysis solution (0.2 M NaOH, 1% SDS) was added, the solution mixed gently and then kept on ice for 5 minutes. 6.5 ml of cold acetate solution (3 M KAc/2 M HOAc, pH 5.8) was added, the solution was mixed well and incubated on ice for a further 10 minutes. The bacterial debris was pelleted by spinning at 12,000 r.p.m. for 10 minutes at 4°C (JA-20 rotor). The supernatant was transferred to a new tube and precipitated with 15 ml isopropanol. Plasmid DNA was recovered by centrifugation (8000 r.p.m./5 minutes/4°C/JA-20) and gently resuspended in a solution containing 7 ml TE with 7g CsCl. Once the pellet was thoroughly resuspended, 700 µl EtBr (10 mg/ml) was added, and any remaining debris removed by centrifugation at 3000 r.p.m., at R.T. for 10 minutes (Jouan Centrifuge C412). The supernatant was transferred to a 10 ml Nalgene Oakridge tube and sealed with paraffin oil. Oakridge tubes were centrifuged at 45,000 r.p.m. for 24 hours (20°C/Beckman L-8-70 ultracentrifuge/ Ti50 rotor) and the super-coiled plasmid DNA was visualised under long wavelength UV light. A 1 ml syringe was used to recover the DNA and EtBr was removed by repeated extraction with NaCl/TE saturated isopropanol. The DNA solution was diluted 1:3 with MQ water, precipitated with 2 volumes of ethanol and placed at -20°C overnight. Plasmid DNA was

recovered by centrifugation (9000 r.p.m./30 minutes/4°C/JA-20 rotor) and resuspended in 400 µl MQ water. The DNA solution was transferred to an eppendorf tube, reprecipitated and resuspended in MQ water. The concentration of plasmid DNA was calculated from the A_{260} value determined by spectrophotometric analysis.

2.5.10 Isolation of Cytoplasmic RNA from Cultured Cells

Cytoplasmic RNA was isolated using the method of Edwards *et al.* (1985). Cells were harvested by trypsinisation and stored at -80°C until use. Cell pellets were thoroughly resuspended in 2 ml ice cold TNM and lysed by vigorous pipetting. After incubation on ice for 5 minutes, nuclei were pelleted by centrifugation (3000 r.p.m. for 5 minutes/Jouan C412) and the supernatant mixed thoroughly with 2 ml TUNES. This solution was extracted twice with phenol/chloroform (1:1) and the aqueous layer was transferred to a sterile corex tube. RNA was precipitated by addition of 1/10th volume of NaAc pH 5.2, 2 volumes of RNAase free ethanol and incubation at -80°C for 30 minutes (or overnight at -20°C). After spinning at 9000 r.p.m for 30 minutes at 4°C (Beckman J-21B Centrifuge/JA-20 rotor), the RNA pellet was resuspended in 450 µl of sterile water, and transferred to an eppendorf tube for re-precipitation. The RNA was centrifuged at 12,000 r.p.m. for 15 minutes in an eppendorf centrifuge, washed with 75%EtOH and dried under vacuum. The RNA was resuspended in an appropriate volume of water, and the concentration of RNA was calculated from the A_{260} determined by spectrophotometric analysis. RNA samples were stored at -20°C.

2.5.11 Isolation of RNA from Embryoid Bodies and RA-treated Aggregates

RNA from embryoid bodies and RA-treated aggregates was isolated using the acid guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Samples were thawed on ice in 1ml of solution D (4M guanidium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M β mercaptoethanol). The cell suspension was homogenised in a glass teflon homogeniser and transferred to a 10ml polypropylene tube. 0.1 ml of 2M NaAc pH 4, 1ml of phenol and 0.2ml of chloroform:isoamylalcohol was added to the homogenate (mixed with inversion after the addition of each reagent), shaken vigorously

and placed on ice for 15 minutes. The homogenate was centrifuged at 3800 r.p.m. for 5minutes (Jouan C412) and the aqueous phase was transferred to a corex tube. 1ml of isopropanol was added and the RNA precipitated by cooling at -20°C for 1 hr . RNA was pelleted by centrifugation at 9000 r.p.m. for 30 minutes (Beckman J-21B Centrifuge/JA-20 rotor) and the RNA pellet resuspended in 0.3 ml of Solution D. The RNA solution was transferred to an eppendorf tube and reprecipitated by addition of 0.3 ml of isopropanol and cooling at -20°C for 1hr. The RNA was pelleted by centrifugation at 12,000 r.p.m. for 15 minutes at 4°C in an eppendorf centrifuge, washed with 75% EtOH, vacuum dried and resuspended in MQ H_2O. The concentration of RNA isolated was calculated from the A_{260} determined by spectrophotometric analysis.

2.5.12 Northern Blot Analysis

i) Gel preparation and electrophoresis

1.2% agarose gels for northern blot analysis were prepared by dissolving 2.16g agarose in 152 ml MQ water with heat. Once the gel solution had cooled to 60°C, 18 ml 10 X MOPS and 10 ml 20% formaldehyde (freshly prepared by dissolving 4 g paraformaldehyde in 20 ml MQ H_2O) were added and then the gel was poured. RNA samples and a control RNA sample (approximately 30µg) were prepared for electrophoresis. Each sample contained 11.25 µl RNA /MQ H_2O, 5µl 10 X MOPS, 8.75 µl formaldehyde (37%, pH 4.5) and 25 µl deionised formamide. RNA samples were denatured by heating at 65°C for 15 minutes and snap cooled on ice. 5µl of NLB was added to each sample and the samples loaded onto the gel. 5 µg *Eco* RI/*Hind* III lambda DNA markers were also loaded. Northern gels were run at 150V in 1 X MOPS until the bromophenol blue dye had reached 4/5 to the bottom of the gel.

ii) Transfer of Northern

The lanes containing the lambda DNA markers and control RNA sample were removed from the gel, stained in ethidium bromide for 45 minutes and destained in water overnight. The gel fragment was then photographed under medium wavelength UV light. The remainder of the gel was blotted onto Nytran nylon membrane using capillary transfer. Two pieces of Whatmann 3MM paper were pre-wetted in 20 X SSC and placed over a platform so that the edges of the paper were submerged in 20 X SSC. The gel was

placed wells facing down on the damp Whatmann paper (avoiding bubbles) and Parafilm placed around the edge of the gel to avoid short circuiting. The nylon membrane was pre-wetted in 20 X SSC and carefully placed over the gel ensuring no air bubbles were trapped between the filter and the gel. Two pieces of Whatmann paper, pre-wetted in 20 X SSC, were then placed on top of the membrane, followed by a 2 cm stack of dry Whatmann paper. A 5 cm stack of paper towel was then added and a glass plate placed on top of the paper towel. A 0.8 kg weight was placed on the glass plate and the RNA transferred for at least 24 hours. Following transfer, the RNA was covalently cross-linked to the filter by exposure to 240 mJ of UV radiation in a Stratagene UV Stratalinker^{TM} before pre-hybridisation.

2.5.13 Ribonuclease Protection

RNAase protection analyses were essentially performed using the method of Krieg and Melton (1987). Transcription reactions contained 240 µCi α-^{32}PdUTP (2.5.15). 150,000 counts/minute of single stranded *MMoxB* riboprobe and 3700counts/minute of *mGAP* riboprobe were added to each sample. After hybridisation, samples were digested with RNAase A and RNAase T1 for 30 minutes at 37^{o}C. Samples were phenol/chloroform extracted, ethanol precipitated and resuspended in FLB. RNAase digestion products were separated on a sequencing gel (2.5.2) and visualised using phosphorimager analysis (2.5.20).

MMoxB specific probe was generated by T3 polymerase transcription of clone 7.1 linearised with *Bgl*II.

The murine gluteraldehyde phosphate dehydrogenase (*mGAP*) loading control probe was generated by SP6 RNA polymerase transcription of a 300 bp cDNA clone linearised with *Bam* H1 (Rathjen *et al.*, 1990).

2.5.14 Synthesis of Radioactive DNA Probes

Single stranded DNA probes were prepared using a Gigaprime labelling kit with 10-60 µCi α–^{32}PdATP. Unincorporated label was removed from probe reactions by diluting the reactions with 80 µl of MQ H_2O, loading onto a Sephadex G-50 column and

centrifuging at 2800 r.p.m. for 4 minutes (Jouan C412). Probes were prepared with the following DNA fragments.

FGF-5: 800bp fragment containing the *FGF-5* cDNA isolated by digestion of the plasmid with *EcoRI/BamHI.*

H19: 778bp fragment containing *H19* cDNA sequence isolated by digestion of the plasmid with *PvuII.*

Oct-4 : 462 bp fragment containing Oct-*4* cDNA sequence isolated by digestion of the plasmid with *XhoI/HindIII.*

Rex-1: 848 bp fragment containing *Rex-1* cDNA isolated by digestion of the plasmid with EcoRI.

SPARC: 570bp fragment containing *SPARC* cDNA sequence isolated by digestion of the plasmid with *EcoRI.*

S8 : 580bp fragment containing *S8* cDNA sequence isolated by digestion of plasmid with *BamHI/HindIII.*

mGAP: whole plasmid containing 300bp of *mGAP* cDNA sequence.

2.5.15 Synthesis of Radioactive RNA Probes

Riboprobes were synthesised as described by Krieg and Melton (1987). RNAase protection probes contained 240 μCi α–^{32}PUTP and 0.15 mM UTP. Northern probes contained 60 μCi α–^{32}PUTP and 12 μM UTP. Unincorporated label was removed using a Sephadex G-50 column as for DNA probes (2.5.14).

Antisense *AFP* riboprobes were prepared by T3 RNA polymerase transcription of *Hind* III linearised template.

Antisense *Brachyury* riboprobes were prepared by T7 RNA polymerase transcription of *BamHI* linearised template.

Antisense *cNkx2.5* riboprobes were prepared by T3 RNA polymerase transcription of *Hind* III linearised template.

Antisense *goosecoid* riboprobes were prepared by T3 RNA polymerase transcription of *BamHI* linearised template.

2.5.16 Hybridization of Radioactive Probes to Nylon Filters

Hybridisation reactions were carried out in a Hybaid Hybridisation Oven.

i) DNA probes

The prehybridisation solution contained 1 M NaCl, 40% deionised formamide, 1% SDS, 10 % PEG, 50 mM Tris-HCl pH 7.4 and 5 X Denhardt's. Filters were prehyridised for a minimum of 4 hours at 42°C in 10 ml prehybridisation solution/filter. DNA probes were boiled for 2 minutes with 3 mg sonicated salmon sperm DNA, snap-cooled on ice and then added to Hybaid cylinders. Filters were hybridised overnight at 42°C and washed in 2x SSC/ 0.1% SDS at 42°C, 0.2x SSC/0.1% SDS at 42°C and 0.2x SSC/0.1% SDS at 65°C. All washes were for 1/2 hr. Depending on the level of radioactivity present on the filter an additional wash at 65°C with 0.1xSSC/0.1% SDS was included.

ii) RNA probes

Northern filters were prehybridised for a minimum of 4 hours at 65°C in 10 ml of hybridisation solution containing 5 X SSC, 60% formamide, 5 X Denhardt's, 20 mM sodium phosphate pH 6.8, 1% SDS, 100 µg/ml sonicated salmon sperm DNA and 100 µg/ml denatured tRNA. RNA probes were denatured by heating at 85°C for 2 minutes, snap-cooled on ice and then added to Hybaid cylinders. Filters were hybridised overnight at 65°C and washed in 2 X SSC / 1% SDS at 50°C for 3 X 15 minutes and then in 0.2 X SSC/1% SDS at 75°C for 1 hour or as specified.

Before reprobing, filters were stripped in a solution of 10mM Tris, 7.5, 0.1% SDS for 15 minutes at 100°C.

2.5.17 Synthesis of Digoxygenin (DIG) Labelled RNA Probes

Reactions contained 0.5 µg linearised plasmid, 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM DTT, 1 unit RNAasin, 1 X DIG labelling mix and 20 units RNA polymerase in a total volume of 20 µl. Transcription reactions were incubated at 37°C for 2 hours. Riboprobes were precipitated by addition of 80 µl MQ H_20, 20 µl 100 mM EDTA, 10 µl 3 M Sodium Acetate pH 5.2, 250 µl 100% ethanol and 20 µg glycogen and incubation overnight at -20°C. After centrifugation at 12,000 r.p.m for 15 minutes in an Eppendorf centrifuge, the probe was resuspended in 100 µl of MQ H_2O with 0.5 units

RNAasin and stored at -20°C. Antisense DIG ribprobes were obtained from the following templates by transcription with the described enzymes.

AFP : *HindIII* linearised template, transcription with T3 polymerase

Brachyury : *BamHI* linearised template, transcription with T7 polymerase

FGF-5 : *BamHI* linearised template, transcription with T3 polymerase

goosecoid : *BamHI* linearised template, transcription with T3 polymerase

H19 (LC10-8): *BamHI* linearised template, transcription with T3 polymerase

Oct-4: *HindIII* linearised template, transcription with T7 polymerase

Rex-1 : *XbaI* linearised template, transcription with SP6 polymerase

S8 : *HindIII* linearised template, transcription with T7 polymerase

SPARC (pG43) : *BamHI* linearised template, transcription with SP6 polymerase

2.5.18 Dot Blot Assay for DIG Probe Reactivity

Nitrocellulose membrane was soaked in 20 X SSC and air-dried. A 2 µl sample of DIG labelled probe was serially diluted 10 fold to 1/10000, heated at 100°C for 5 min and snap-cooled on ice. 1 µl of each dilution was spotted onto the filter and UV cross-linked by exposure to 120 mJ of UV radiation in a Stratagene UV Stratalinker™. The filter was washed for 1 minute in buffer 1, 30 minutes in buffer 2 (blocking reagent), and 1 minute in buffer 1. Anti-DIG Fab antibody-alkaline phosphatase conjugate was diluted to 1:5000 in buffer 1 and incubated with the filter for 30 minutes. Unbound antibody was removed by washing 2 x 15 minutes in buffer 1 and the membrane equilibrated in buffer 3 for 2 minutes. Alkaline phosphatase reaction solution was prepared by mixing 4.5 µl NBT and 3.5µl BCIP with 1 ml buffer 3. This solution was added to the filter and developed for 30 minutes in the dark. The reaction was terminated by washing the membrane in buffer 4 for 5 minutes. Probes used for *in situ* hybridisation were reactive at a 1/1000 dilution.

2.5.19 *In Situ* Hybridisation of Cell Monolayers

Cell monolayers were rinsed twice with PBS and fixed overnight in 4% paraformaldehyde (PFA)/PBS solution. The following day, the cells were rinsed twice on ice with PBT (0.1% Tween 20/PBS solution) and dehydrated on ice by the addition of 25% methanol/PBS for 5 minutes, 50% methanol/PBS for 5 minutes, 75% methanol/PBS

for 5 minutes and stored in 100% methanol for up to 3 months. Cells were rehydrated to PBS, on ice, using the methanol series and rinsed three times at room temperature with PBT. Cells were then washed for 3 x 20 minutes with RIPA buffer and fixed in 4% PFA/0.2% gluteraldehyde in PBT for 20 minutes. Plates were rinsed 3 x 5 minutes in PBT and washed for approximately 10 minutes in 1:1 hybridisation buffer:PBT (hybridisation buffer, 50% deionised formamide, 5 X SSC, 0.1% Tween-20, 50 µg/ml heparin). Cells were washed for a further 10 minutes in hybridisation buffer, and prehybridised at 68°C in a sealed container containing 50% formamide in hybridisation buffer containing 10 µg/ml denatured salmon sperm DNA and 10 µg/ml denatured yeast tRNA for 1-6 hours. DIG-labelled riboprobes were denatured at 80°C for 10 minutes, added to fresh hybridisation buffer containing denatured salmon sperm DNA and yeast tRNA, and allowed to hybridise overnight at 68°C. Probes were diluted 1:100-1:200 for the hybridisation step. The following day, cell monolayers were washed in 50% formamide/2 X SSC/0.1% Tween 20 for 5 minutes and 3 x 30 minutes at 68°C. The cells were then washed three times at room temperature in TBST (136 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH 7.5, 0.1% Tween 20) followed by incubation in 10% FCS/TBST for one hour at room temperature. Anti-digoxygenin Fab fragments-AP conjugate (Boehringer Mannheim) was diluted 1:2000 in 1% FCS/TBST and incubated with cells overnight at 4°C. The next day, cells were washed for 3 x 5 minutes and 3 x 45 minutes in TBST and 3 x 10 minutes in AP buffer. The cells were developed in AP buffer containing 0.45 mg/ml NBT and 0.18 mg/ml BCIP in the dark until purple staining appeared (3-24 hours). The staining reaction was terminated by rinsing several times with PBT/1 mM EDTA.

2.5.20 Phosphorimager Analysis and Photographic Scanning

Gels and filters were exposed to Storage Phosphor Screens (Molecular Dynamics) and processed using a Molecular Dynamics PhosphorImager running the ImageQuant software package, expression levels were determined using this package. Photographs were scanned using a Hewlett Packard *ScanJet IIcx* scanner running the DeskScan II 2.0 software package. PhosphorImager and DeskScan files were manipulated using the

AdobePhotoshop™ and Powerpoint programs, and printed using a Hewlett Packard LaserJet4 printer.

2.5.21 Containment Facilities

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

CHAPTER THREE:
DEFINITION OF CULTURE CONDITIONS FOR X CELL FORMATION AND MAINTENANCE

CHAPTER THREE: DEFINITION OF CULTURE CONDITIONS FOR X CELL FORMATION AND MAINTENANCE

3.1 Introduction

Factors within MED2 induce the specific and uniform differentiation of ES cells into X cells in the presence or absence of exogenous LIF (J. Rathjen, unpublished observations). X cells grow as monolayer colonies which are phenotypically different from the compact, dome-shaped colony morphology characteristic of ES cells (Figure 1.8). Maintenance of X cells requires the continual presence of MED2. In the absence of MED2, in the presence of LIF, X cells phenotypically revert to ES cells. In the absence of MED2 and LIF X cells differentiate spontaneously. This observation, coupled with the expression of pluripotential cell specific markers (1.8.2), suggests that X cells retain differentiation capability.

A range of growth conditions affect the stability of ES cells in culture, including the concentration of exogenous LIF present, the cell density and serum batch (Smith 1991). X cells, like ES cells, retain differentiation capability, therefore X cell stability is also likely to be dependent on culture conditions employed for their formation and maintenance. Analysis of X cell gene expression and differentiation potential requires the uniform conversion of X cells from ES cells, and maximal stability of X cells in culture. Background differentiation and incomplete conversion will hamper the interpretation of such analyses.

X cells are routinely formed in sufficient quantities for gene expression analysis by culture of ES cells in the presence of MED2 at high cell density ($2x10^3$-$6x10^3$cells/cm^2). X cell differentiation in response to cytokines is examined at clonal cell density (50-200cells/cm^2) where cell interactions are minimised. Hence definition of the optimal conditions for the formation and maintenance of X cells at both high and low cell density was required.

This chapter details the differentiation of ES cells into X cells at both clonal cell density and at high cell density. The optimal concentration of MED2 for X cell formation and maintenance, and the requirement for LIF in such processes is described.

3.2 MED2 Induces the Differentiation of ES Cells into X Cells in the Presence of LIF at Clonal Cell Density

D3 ES cells were seeded at clonal cell density (100 cells/cm^2) into 50% MED2/50% incomplete ES medium containing LIF, and compared with ES cells seeded into incomplete ES medium containing LIF alone (2.3.13 ii). The concentration of LIF in all experiments, unless otherwise indicated, was 1000U/ml. Four duplicate wells were examined for each variable. X cells were evident in MED2 supplemented conditions as early as 48 hours. The assay was continued for 5 days, at this time small dome-shaped colonies characteristic of ES cells were only observed in wells containing LIF alone (Figure 3.1A). Wells which contained MED2 in addition to LIF did not contain any colonies with an ES cell morphology but contained larger monolayer colonies characteristic of X cells (Figure 3.1B). Cells within X cell colonies, in contrast to those within ES cell colonies, could clearly be discerned and were characterised by relatively large nuclei containing prominent darkly staining nucleoli. X cells were similar in morphology to P19 EC cells (1.8, Rudnicki and McBurney 1987).

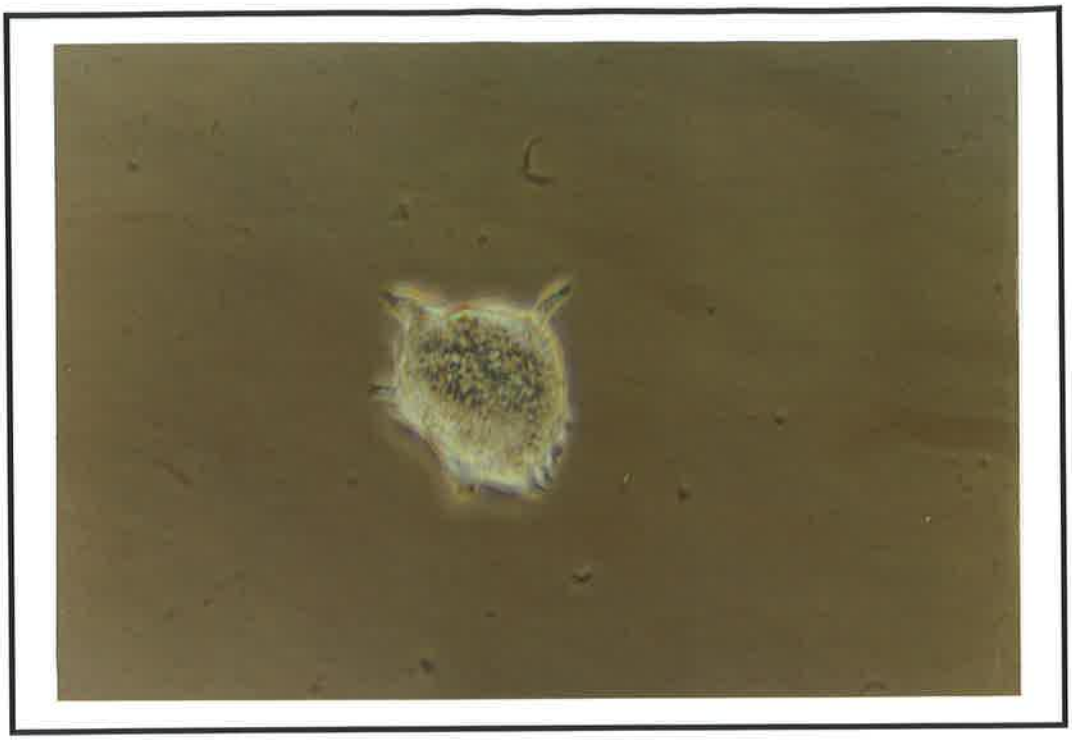
The assays were stained for alkaline phosphatase which stains ES and X cells but does not stain their differentiated derivatives (J. Rathjen, unpublished observations, Adamson 1988, Pease *et al.* 1990). ES cell colonies were readily distinguished from X cell colonies based on colony morphology and intensity of alkaline phosphatase staining. ES cell colonies were observed as small, compact, dome-shaped colonies which stained deep-red due to alkaline phosphatase activity, while X cell colonies were larger monolayer colonies which stained less intensely for alkaline phosphatase. Both ES and X cell colonies could be distinguished from differentiated colonies which did not stain for alkaline phosphatase.

The percentage of ES, X and differentiated (D) colonies present in wells containing LIF alone and LIF + MED2 were determined. The results of this analysis are presented in Figure 3.2. The majority of colonies present after 5 days when ES cells were seeded into LIF alone had an ES cell colony morphology. When ES cells were cultured in the presence of MED2 and LIF, colonies with an ES cell morphology were not detected. Approximately 94% of the colonies stained for alkaline phosphatase and had a colony morphology characteristic of X cell colonies. The plating efficiencies of ES cells under

FIGURE 3.1 ES cells converted to X cells when cultured in MED2 in the presence of LIF.

At 5 days ES cells cultured in the presence of 50% MED2 and LIF had converted to X cells, a representative colony is pictured in B. This contrasts with the morphology of ES cells cultured in the presence of LIF, a representative colony is pictured in A. Photographs were taken at a magnification of 50x using phase contrast optics.

A



B

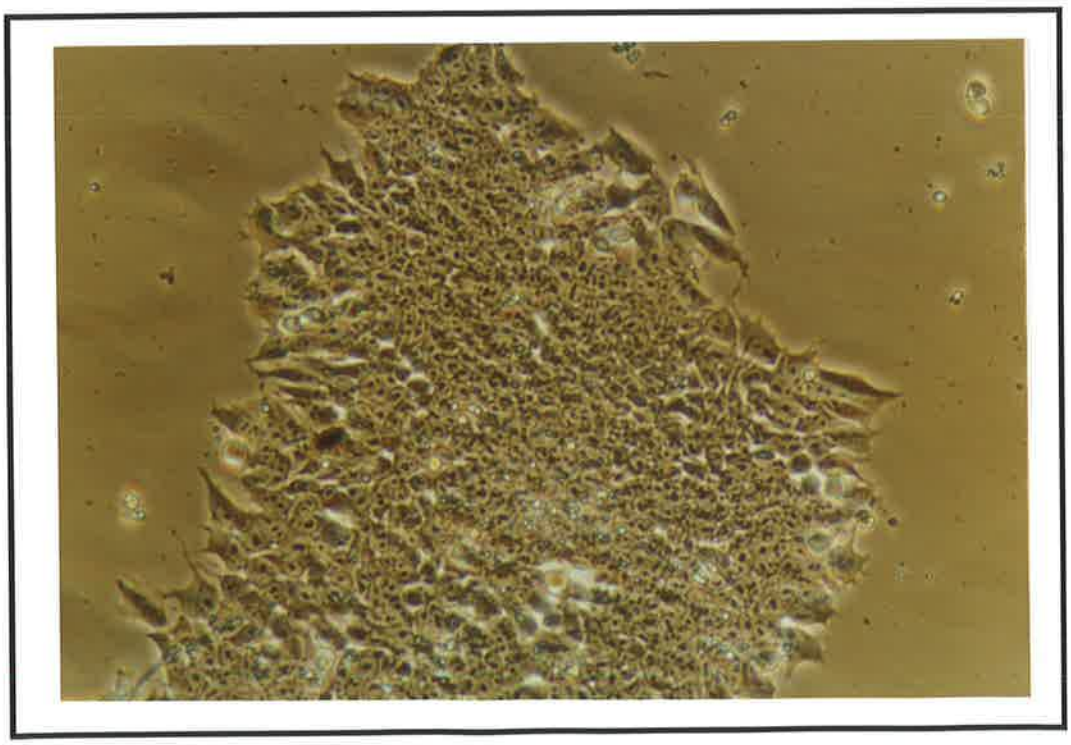


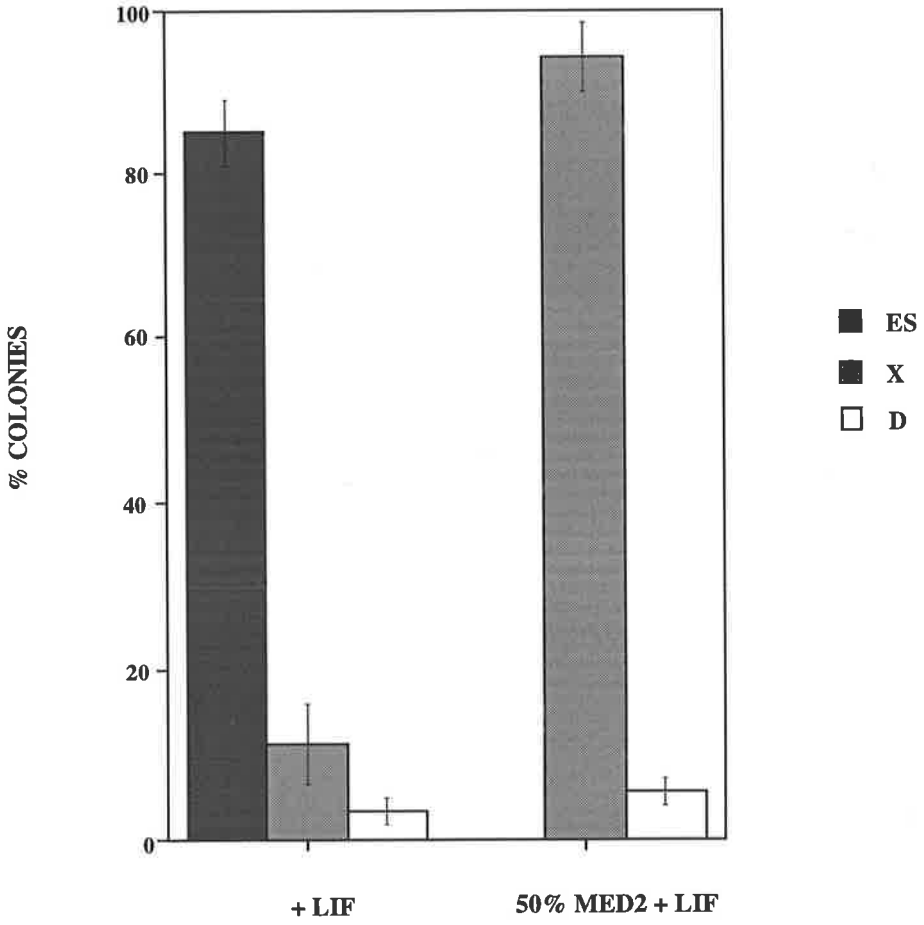
FIGURE 3.2 Colony types present when ES cells are cultured in the presence of LIF with and without MED2.

ES cells were cultured in the presence of LIF with and without MED2 (2.3.13 ii). At day 5 cells were stained for the presence of alkaline phosphatase (2.3.24). Colony types were scored as ES cell colonies (ES), X cell colonies (X) and differentiated colonies (D) based on morphology and alkaline phosphatase staining. The total number of colonies scored in each class were expressed as a percentage of the total number of colonies present in each well. The mean and standard deviation of the percentages of ES, X and D in 50% MED2 + LIF and LIF alone were derived from colony analysis of four wells. The results are tabulated in A) and represented graphically in B). The plating efficiency of ES cells under each condition was expressed as a percentage, determined by dividing the total number of colonies present at day 5, in each well, with the number of cells originally seeded into each well. The mean plating efficiency and standard deviation were derived from analysis of 4 wells and is tabulated in C).

A

CONDITIONS	% COLONIES		
	ES	X	D
LIF	85.2+/-4.1	11.5+/-4.9	3.3+/-1.6
50% MED2 + LIF	0	94.3+/-4.5	5.7+/-1.7

B



C

PLATING EFFICIENCY (%)	
LIF	50% MED2 + LIF
42.8+/-8.7	47.0 +/- 2.6

the two conditions were similar, approximating 45% (Figure 3.2, Table C). Therefore the differentiation observed when ES cells were cultured in the presence of MED2 was not due to an alteration in cell viability. Thus culture of ES cells in the presence of MED2 and LIF induced their uniform conversion to X cells at clonal cell density.

Interestingly a low proportion (11%) of the colonies observed in ES cell cultures had a colony morphology and alkaline phosphatase staining more reminiscent of an X cell colony, however these colonies were not as large as those observed in X cell cultures. During routine culture of ES cells, cells with the morphology of X cells have been observed at low frequencies (data not shown, J. Rathjen, personal communication). These observations, coupled with the transient appearance of X cells when ES cells were spontaneously differentiated by culture in the absence of LIF (4.3.1), suggest that X cells represent a natural pluripotential cell intermediate between ES cells and their differentiated derivatives.

3.3 The Stability of X Cells Cultured in 50% MED2 and LIF

A relatively low proportion of X cell colonies observed in 50% MED2 and LIF at Day 5 (3.2) were surrounded by overtly differentiated cells. Differentiated cells within these colonies did not stain for alkaline phosphatase activity. Thus X cells exhibit a degree of instability when cultured in 50% MED2 in the presence of LIF.

In further experiments, aimed at defining the optimal conditions for X cell formation and maintenance, colonies were partitioned into four categories based on colony morphology and alkaline phosphatase staining; ES cell colonies (ES), X cell colonies (X), semi-differentiated X cell colonies (SD), and differentiated colonies (D). A representative colony of each category is depicted in Figure 3.3.

To define X cell formation and stability more precisely, ES cells were seeded at clonal cell density into duplicate wells containing 50% MED2 and LIF and cultured for periods of three days and five days. At these times cultures were stained for alkaline phosphatase activity and the percentage of ES, X, SD and D within duplicate wells were determined. Results of this analysis are presented in Figure 3.4.

ES cell colonies could not be detected under these conditions at either day 3 or day 5. At three days while 89% of colonies were X cell colonies, 74% were classified as

FIGURE 3.3 Categories of colony types based on alkaline phosphatase staining and colony morphology.

Colonies types were divided into categories based on the following criteria and are pictured in:-

A) ES cell colonies (ES): colonies which have dome-shaped morphology and stain deep red for alkaline phosphatase.

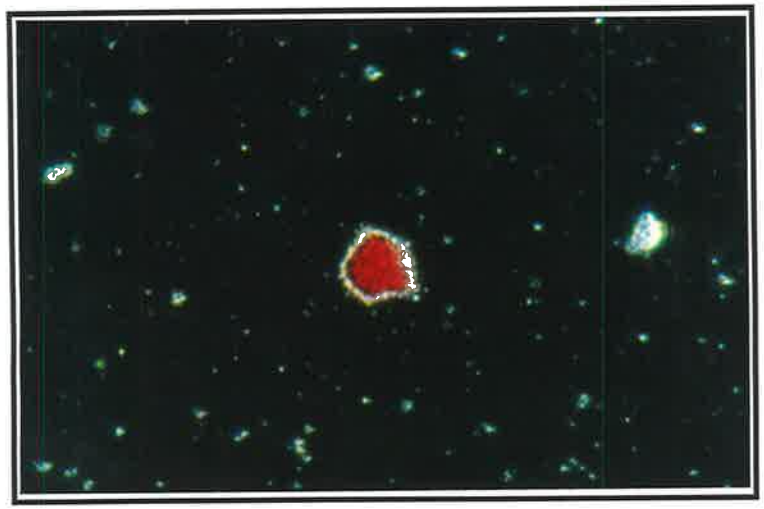
B) X cell colonies (X): colonies which have a flatter monolayer morphology and stain red-pink for alkaline phosphatase.

C) Semi-differentiated colonies (SD): colonies which consisted of X cells staining for alkaline phosphatase and non-staining differentiated cells.

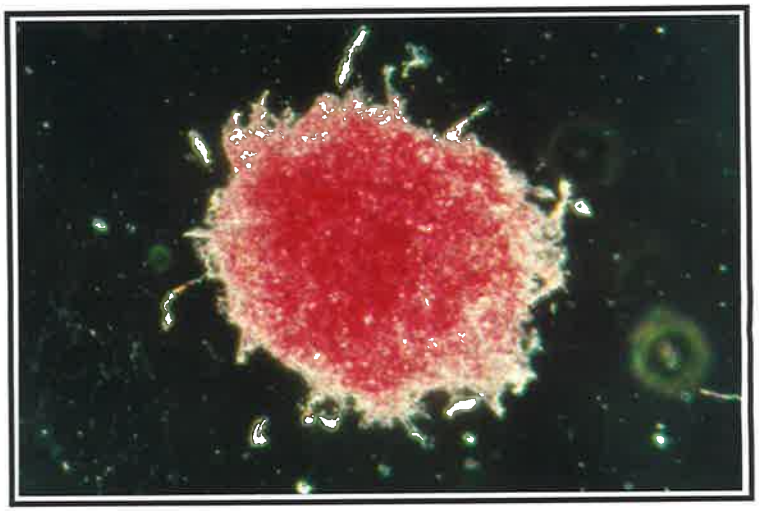
D) Fully differentiated colonies (D): colonies which did not stain for alkaline phosphatase.

Photographs were taken at a magnification of 25x using phase contrast microscopy

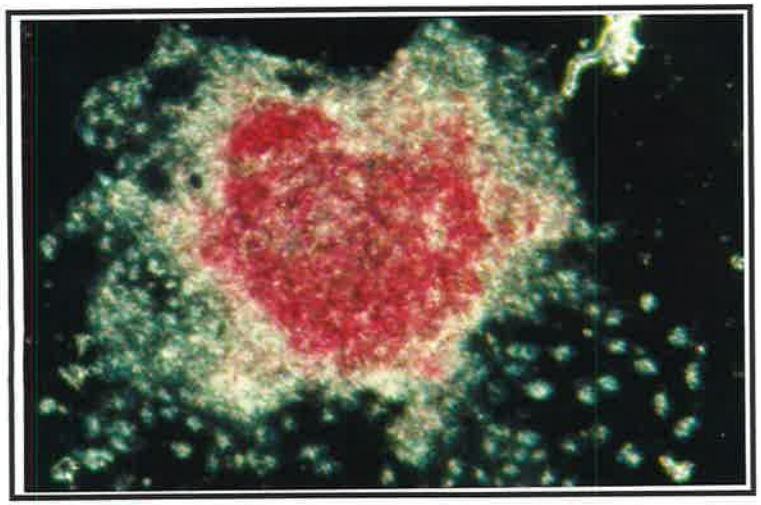
A



B



C



D

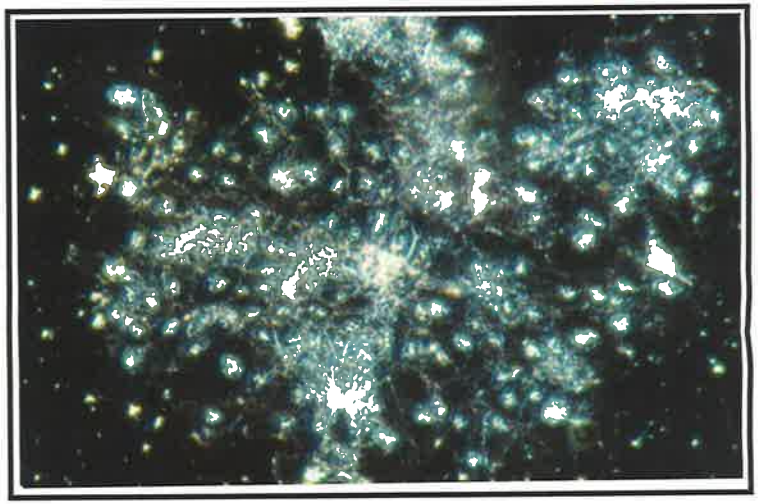


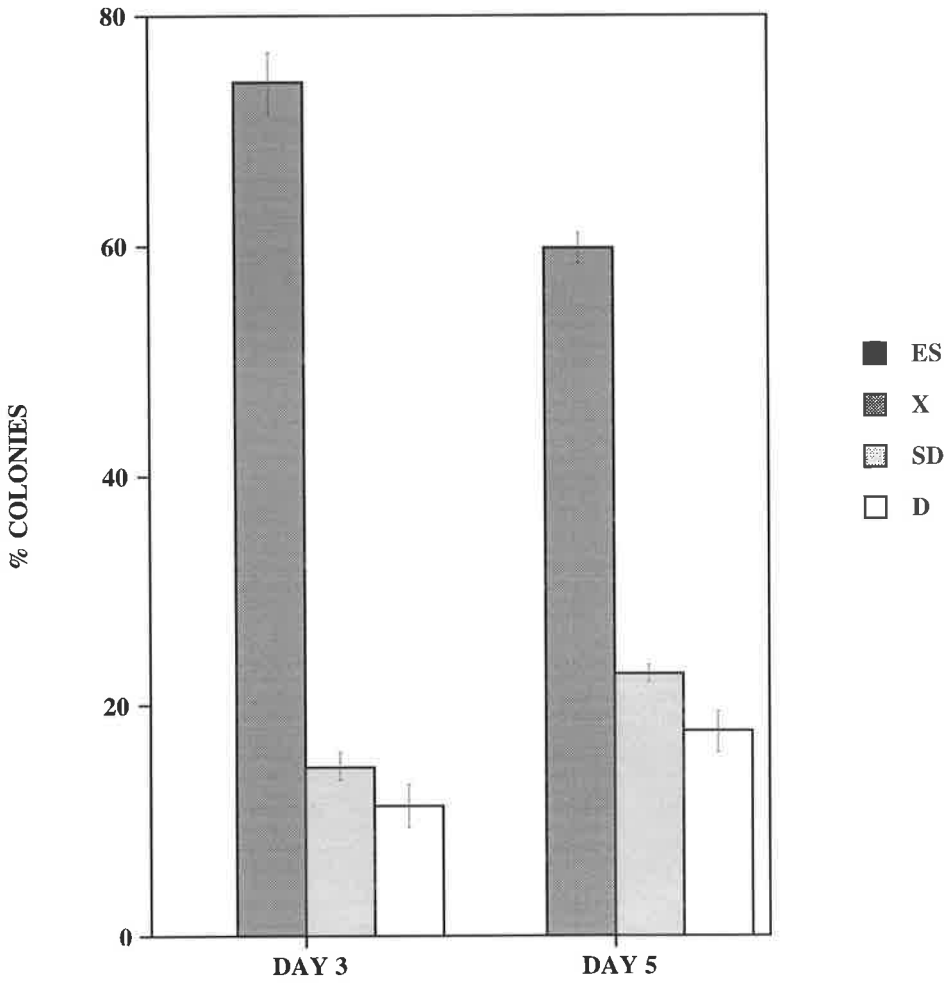
FIGURE 3.4 Colony types present at day 3 and day 5 when ES cells are cultured in the presence of MED2 and LIF.

ES cells were cultured in the presence of 50% MED2 + LIF (2.3.13 ii). Assays were stained for the presence of alkaline phosphatase at day 3 and day 5 (2.3.24). Colony types were scored as ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) based on criteria outlined in Figure 3.3. The total number of colonies scored in each class were expressed as a percentage of the total number of colonies present in each well. The mean and standard deviation of the percentages of ES, X, SD and D at day 3 and day 5 were derived from colony analysis of four wells. The results are tabulated in A) and represented graphically in B).

A

% COLONIES				
	ES	*X*	*SD*	*D*
DAY 3	*0*	*74.2+/-2.6*	*14.6+/-1.2*	*11.2+/-1.8*
DAY 5	*0*	*59.7+/-1.3*	*22.7+/-0.7*	*17.6+/-1.8*

B



undifferentiated X cell colonies and 15% were classified as semi-differentiated colonies. Fully differentiated colonies constituted 11% of the population. At five days the level of undifferentiated X cell colonies (X) had decreased to 60%, while the level of semi-differentiated and fully differentiated colonies had increased to 23% and 17%, respectively. Therefore while MED2 induced relatively uniform and immediate differentiation of ES cells into X cells at clonal cell density, X cells exhibited a degree of instability when cultured under these conditions.

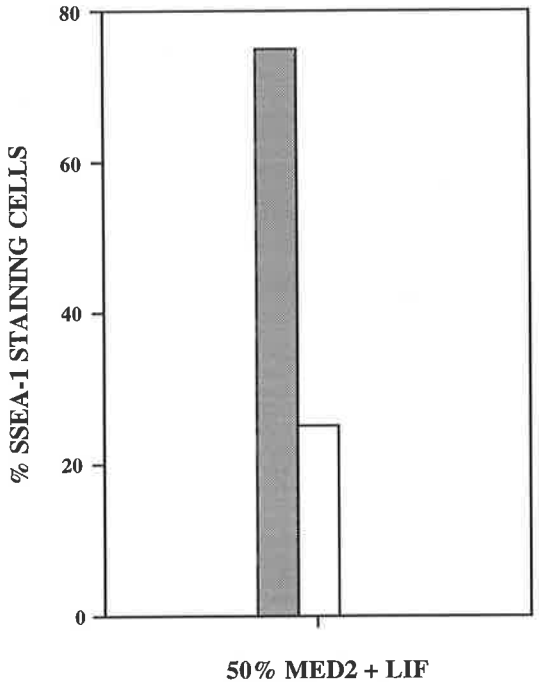
A separate experiment was carried out with the aim of defining the proportion of X cells present compared to differentiated cells. D3 ES cells were seeded at clonal cell density (100 cells/cm^2) into 10cm^2 duplicate wells containing 50% MED2 and LIF. After 5 days the cells in one duplicate well were trypsinised to single cells and stained for the presence of the stem cell specific surface marker SSEA-1 using immunofluorescence antibody staining. SSEA-1 is expressed on the surface of ES and X cells but is not present on the surface of differentiated cells (J. Rathjen, unpublished observations, Richa and Solter 1988, Smith *et al.* 1988). The proportion of cells staining with anti-SSEA-1 was determined as the number of fluorescent cells compared to the total number of cells viewed under normal phase. Colonies in the remaining well were stained for alkaline phosphatase activity as previously described. Results of this experiment are presented in Figure 3.5.

The proportion of pluripotent cells as assessed by SSEA-1 present within MED2 supplemented conditions after 5 days was approximately 75%. ES cell colonies were not observed within MED2 supplemented conditions (Fig. 3.5B) and suggests that if ES cells were present they could only be present at low levels. Therefore while the presence of SSEA-1 does not discriminate between ES cells and X cells, it can be concluded that the SSEA-1 staining observed in this experiment was largely representative of X cells. Notwithstanding X cell instability, a high proportion of the cells present after 5 days in the presence of MED2 and LIF constituted undifferentiated X cells.

FIGURE 3.5 The proportion of X cells at day 5.

ES cells were seeded into duplicate wells containing 50% MED2 with LIF (1000U/ml) (2.3.13.ii). After 5 days the cells in one well were harvested and stained for the presence of SSEA-1 (2.3.26). The proportion of X cells present in the well was determined by counting the number of cells staining with anti-SSEA-1 under fluorescence and dividing this by the total number of cells viewed under normal phase. The proportion of X cells present compared to differentiated cells is tabulated and represented graphically in A). The remaining well was stained for alkaline phosphatase (2.3.24) and the colony types present determined as previously described (Fig. 3.4). Results are tabulated and represented graphically in B).

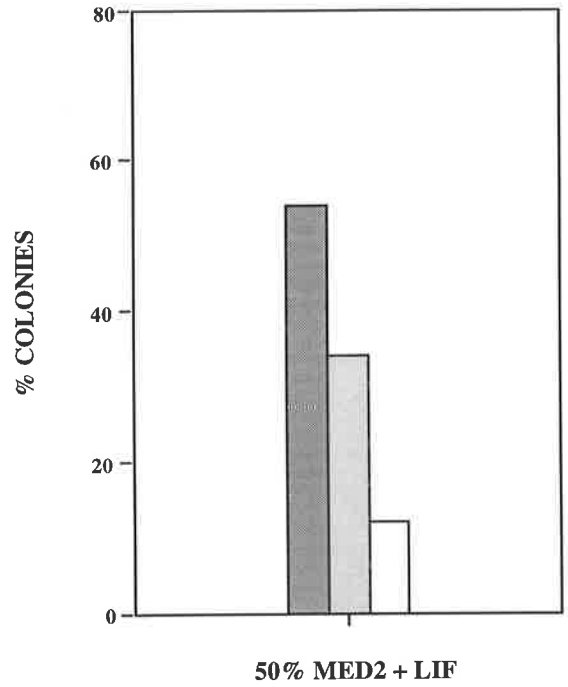
A



X
D

SSEA-1 STAINING		
TOTAL	STAINING	%
536	404	75

B



X
SD
D

% X	%SD	%D
54	34	12

3.4 50% MED2 is the Optimal Condition for X Cell Maintenance at Clonal Cell Density

X cell formation and maintenance is dependent on the presence of the conditioned medium MED2. Conditioned medium is likely to contain additional factors not involved in X cell formation and maintenance that reduce the stability of X cells in culture. The level of differentiation seen in X cells at clonal cell density may be improved by alteration of the level of MED2 present.

Although X cells were formed when ES cells were cultured in higher percentages of MED2, colonies were small, unhealthy and decreased plating efficiencies were evident (data not shown). Conditioned medium is inherently depleted of essential nutrients required for cell survival in culture and high percentages of conditioned medium will therefore inhibit cell growth. To determine whether lower concentrations of MED2 might improve X cell stability D3 ES cells were seeded at clonal density into duplicate wells containing increasing percentages of MED2 in incomplete ES cell medium containing LIF. After 5 days assays were stained for alkaline phosphatase activity and the percentages of ES, X, semi-differentiated and fully differentiated colonies were determined. Results from a representative assay are presented in Figure 3.6.

When ES cells were cultured in the presence 10-30% MED2 in the presence of 1000U/ml LIF, conversion to X cells was incomplete and ES cell colonies were present. The percentage of ES cell colonies decreased as the percentage of MED2 increased approaching 0% at 40% MED2. In two other assays (data not shown) the presence of ES cell colonies approached 0% at 30% MED2. Therefore, 50% MED2 as a compromise between conversion, X cell stability and cell health was standardised as the optimum percentage of conditioned medium for X cell formation and maintenance throughout this work.

3.5 LIF is Required for the Maintenance of X Cells at Clonal Cell Density

Further definition of the optimal conditions for X cell formation/maintenance required the investigation of the role of LIF in this process. D3 ES cells were seeded at clonal density into 25% MED2 and 50% MED2 in the presence and absence of 1000U/ml

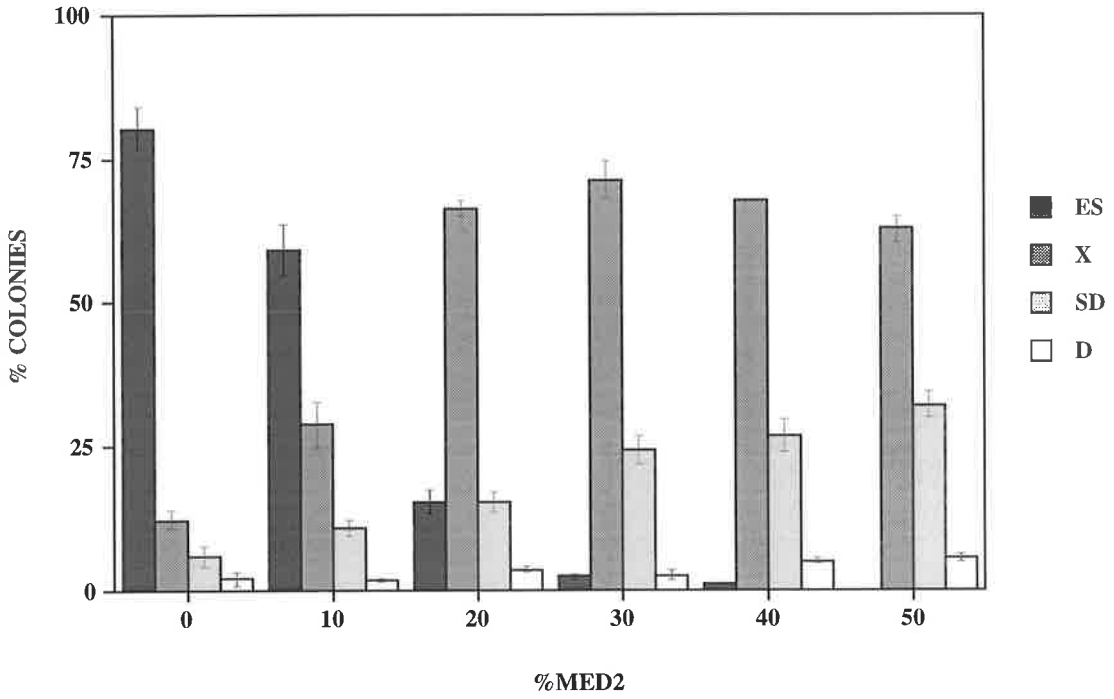
FIGURE 3.6 The optimal percentage of MED2 for X cell formation and maintenance at clonal cell density.

ES cells were cultured in duplicate wells containing increasing percentages of MED2, in the presence of LIF (2.3.13 ii). The assay was stained for alkaline phosphatase activity at five days (2.3.24) and the colony types present were determined as previously described (Fig. 3.4). The mean and standard deviation of the percentage of ES, X, SD and D was derived from the analysis of four wells, and is tabulated in A and represented graphically in B. The mean plating efficiency for cells cultured under the described conditions is tabulated in A.

A

% MED2	% COLONIES				PLATING EFFICIENCY (%)
	ES	X	SD	D	
0	80.2 +/- 3.6	12.2 +/- 1.7	5.8 +/- 1.6	1.8 +/- 1.2	42.4+/-3.3
10	58.9 +/- 4.4	28.6 +/- 3.9	10.8 +/- 1.4	1.7 +/- 0.4	46.9+/-3.2
20	15.1 +/- 2.0	66.3 +/- 1.4	15.2 +/- 1.7	3.4 +/- 0.5	52.7+/-3.2
30	2.2 +/- 0.5	71.1 +/- 3.3	24.2 +/- 2.5	2.4 +/- 0.8	54.5+/-2.9
40	0.8 +/- 0.3	67.7 +/- 0.3	26.6 +/- 2.7	4.9 +/- 0.5	48.9+/-5.1
50	0	62.6 +/- 2.2	32.0 +/- 2.2	5.4 +/- 0.6	51.7+/-2.2

B



LIF and cultured for 5 days before being stained for alkaline phosphatase activity. The results of this experiment are shown in Figure 3.7.

When ES cells were cultured in the presence of 25% MED2 + LIF, 61% of the colonies were classified as undifferentiated X cell colonies and 2% as ES cell colonies. At 25% MED2 in the absence of LIF ES cell colonies were not detected, but undifferentiated X cell colonies were only maintained at a level of 6% and the majority of colonies were semi-differentiated. The level of undifferentiated X cell colonies increased to 21% when ES cells were cultured in 50% MED2 in the absence of LIF. However, a much higher level of undifferentiated X cell colonies was observed in the presence of MED2 and LIF (64%). It therefore appears that addition of LIF is required for effective maintenance of X cells at clonal cell densities.

It has been reported that very small concentrations (1U/ml) of LIF can affect ES cell maintenance (Johansson and Wiles 1995). Therefore the concentration of LIF required for X cell maintenance was investigated. D3 ES cells were seeded at clonal density and cultured in 50% MED2 in the presence of increasing concentrations of recombinant murine LIF (Amrad). Each variable was tested in duplicate and the assay was stained for alkaline phosphatase activity after 5 days. The percentage of X cell colonies observed at each concentration of LIF was determined and is depicted in Figure 3.8. When ES cells were seeded into 50% MED2 in the absence of LIF only 21% of the resulting colonies contained undifferentiated X cells and the majority of colonies were semi-differentiated. The percentage of undifferentiated X cell colonies increased steadily as the concentration of LIF increased, nearing maximal levels at 100U/ml. This contrasts with the level of LIF (500U/ml) which is required for substantial ES cell maintenance (Refer to Figure 3.14). The lower level of LIF required for X cell maintenance suggests either that X cells require less LIF than ES cells or that MED2 may contain LIF.

3.6 Addition of Murine LIF is not Required for X Cell Formation at High Cell Density but Improves X Cell Maintenance

X cells are routinely formed for analysis by culturing ES cells at high cell densities ($2x10^3$-$6x10^3$ cells/cm^2) in the presence of MED2 and LIF. X cells can subsequently be maintained at high cell density by passage in the presence of MED2 and LIF (J. Rathjen,

FIGURE 3.7 The effect of LIF on X cell formation and maintenance.

ES cells were cultured in duplicate wells containing 25% and 50% MED2 with and without LIF (2.3.13 ii). At day 5 the assay was stained for the presence of alkaline phosphatase (2.3.24). The colony types present were determined as previously described (Fig. 3.4). The mean and standard deviation of the percentage of ES, X, SD, and D is tabulated in A and represented graphically in B. The mean plating efficiency for cells under each condition is tabulated in A.

A

	% COLONIES				PLATING EFFICIENCY (%)
	ES	*X*	*SD*	*D*	
25% MED2	*0*	*5.9+/-2.0*	*69.6+/-3.0*	*24.5+/-3.0*	*38.0+/-2.5*
25% MED2 + LIF	*1.9+/-0.7*	*60.6+/-8.2*	*27.0+/-5.9*	*10.5+/-4.3*	*42.8+/-3.8*
50% MED2	*0*	*21.3+/-0*	*55.6+/-3.1*	*23.1+/-3.1*	*40+/-0*
50% MED2 + LIF	*0*	*64.3+/-0.2*	*24.1+/-1.9*	*11.6+/-1.8*	*45.5+/-0.5*

B

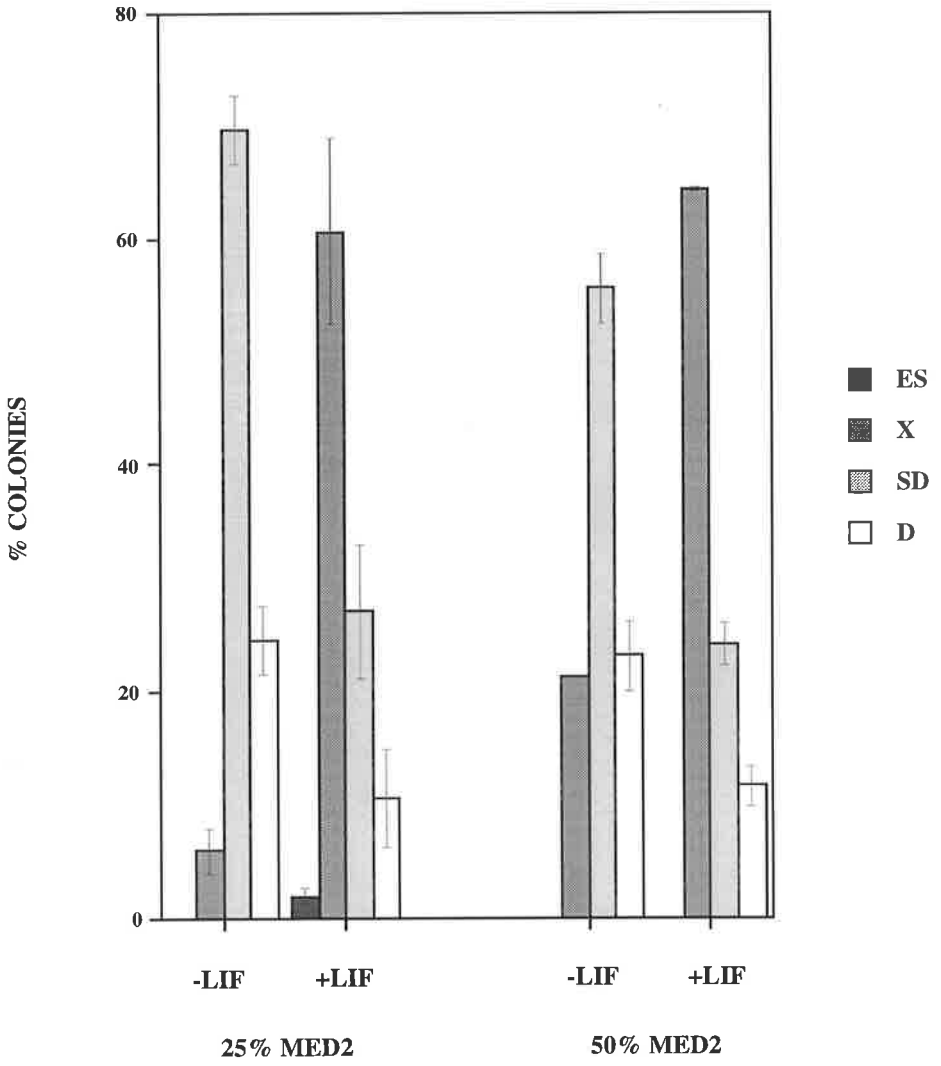


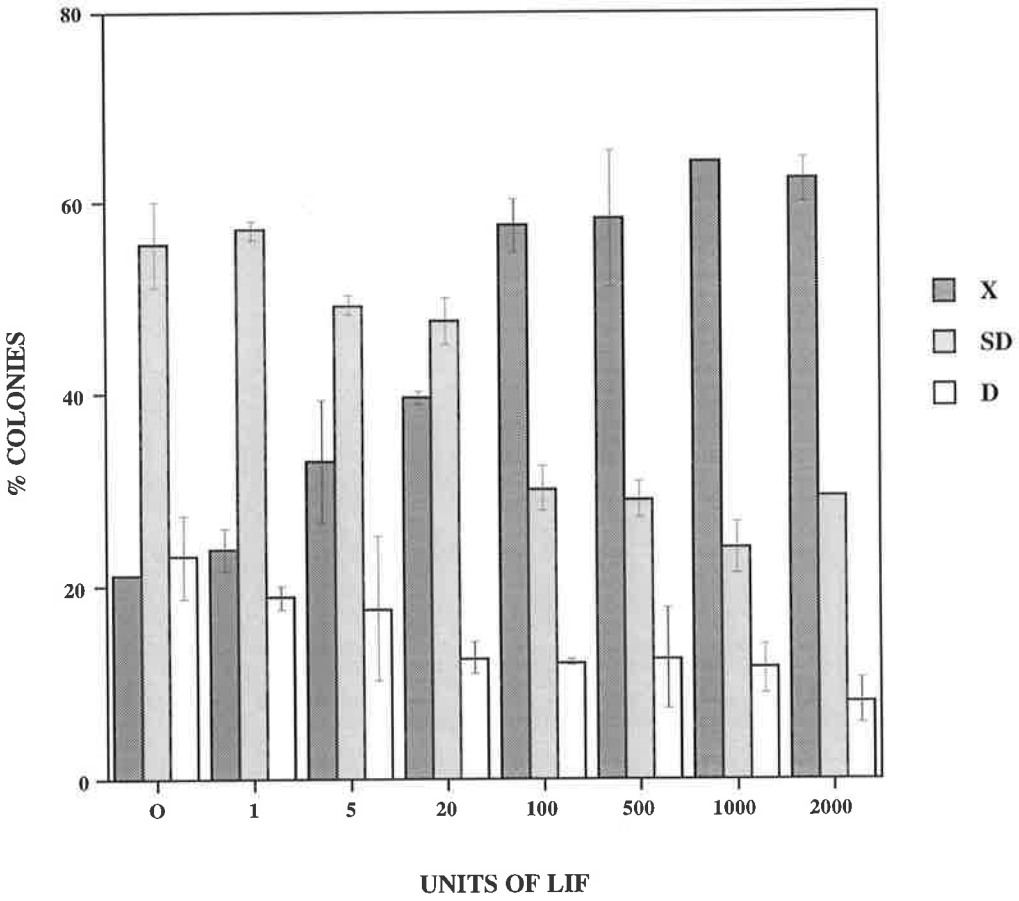
FIGURE 3.8 The concentration of LIF required for X cell maintenance.

ES cells were cultured in duplicate wells, with increasing concentrations of LIF in the presence of MED2 (2.3.13 ii). The colony types present at day 5 were determined as described previously (Fig. 3.4). ES cell colonies were not seen, the mean percentage and standard deviation of X, SD and D is tabulated in A and represented graphically in B. The plating efficiency is described in A.

A

LIF (U/ml)	% COLONIES			PLATING EFFICIENCY (%)
	X	SD	D	
0	21.3+/-0	55.6+/-3.1	23.1+/-3.1	40.0+/-0
1	24+/-1.6	57.1+/-0.8	18.9+/-0.8	42.5+/-4.5
5	32.9+/-4.6	49.3+/-0.7	17.7+/-5.2	42.5+/-5.5
20	49.7+/-0.5	47.7+/-1.7	12.6+/-1.2	41.5+/-2.0
100	57.6+/-1.9	30.2+/-1.6	12.2+/-0.3	43.0+/-1.0
500	58.3+/-5.0	29.1+/-1.3	12.6+/-3.7	45.5+/-0.5
1000	64.3+/-0.2	24.1+/-1.9	11.6+/-1.8	45.5+/-0.5
2000	62.5+/-1.6	29.4+/-0	8.1+/-1.6	46.0+/-0

B



unpublished observations). X cells formed and maintained under these conditions are relatively homogeneous, ES cells are not observed and differentiation levels are low. This contrasts with the stability of X cells at clonal cell density under these conditions. ES cells are also less stable at low or clonal cell densities than at high cell densities (Smith 1992).

To determine the effect of LIF on X cells formed and passaged at high cell densities, ES cells were seeded at $6x10^3$cells/cm^2 in 50% MED2 with and without LIF. X cells formed at high cell density from ES cells cultured in MED2 in the presence of LIF were termed Xp0+ cells, where 'p0' indicates the number of passages and '+' indicates the presence of LIF. X cells formed by culture of ES cells at high cell density in 50% MED2 in the absence of LIF were therefore termed Xp0-. Conversion of ES cells to X cells was apparent after 24 hours and X cells were confluent after 48 hours. Conversion of ES cells to X cells in the presence and absence of LIF was highly uniform, ES cell colonies could not be detected and the level of fully differentiated cells was extremely low. No observable difference could be seen in the level of differentiated cells between Xp0+ cells and Xp0- cells when viewed under magnification (Figure 3.9).

Oct-4 is expressed by ES and X cells but is not expressed in differentiated cells (Scholer 1991, Thomas 1994). This finding was confirmed by carrying out *in situ* hybridisation (2.5.19), utilising an antisense *Oct-4* DIG labelled riboprobe on ES cells, Xp0+ cells and passaged X cells which contained differentiated cells. The results are pictured in Figure 3.10. *Oct-4* expression was detected in ES and X cells but was not detected in differentiated cells. Differences in the levels of differentiation in Xp0+ and Xp0- cells could therefore be defined by quantitation of *Oct-4* expression within the culture. Expression of the murine glyceraldehyde phosphate dehydrogenase (*mGAP*) gene serves as a loading control in such experiments (Rathjen *et al.* 1990, Conover *et al.* 1993).

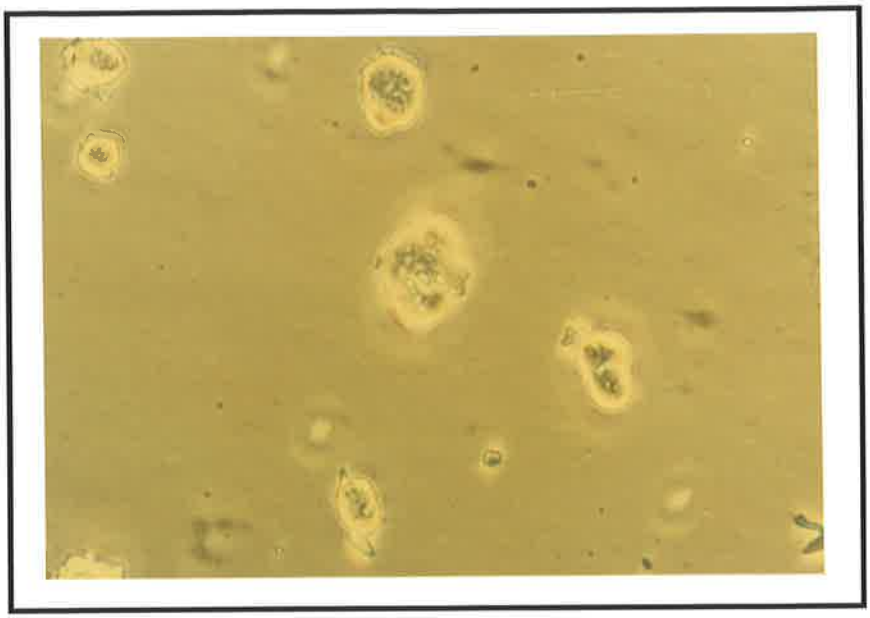
RNA from ES, Xp0+ and Xp0- cells was analysed for *Oct-4* expression by Northern blot analysis and the level of *Oct-4* expression was normalised against *mGAP* expression (Figure 3.11). The levels of *Oct-4* expression in Xp0+ and Xp0- cells were comparable, therefore the levels of differentiation in X cells cultured at high cell density with and without LIF were similar.

FIGURE 3.9 ES cells convert to X cells when cultured in MED2 in the presence and absence of LIF at high cell density.

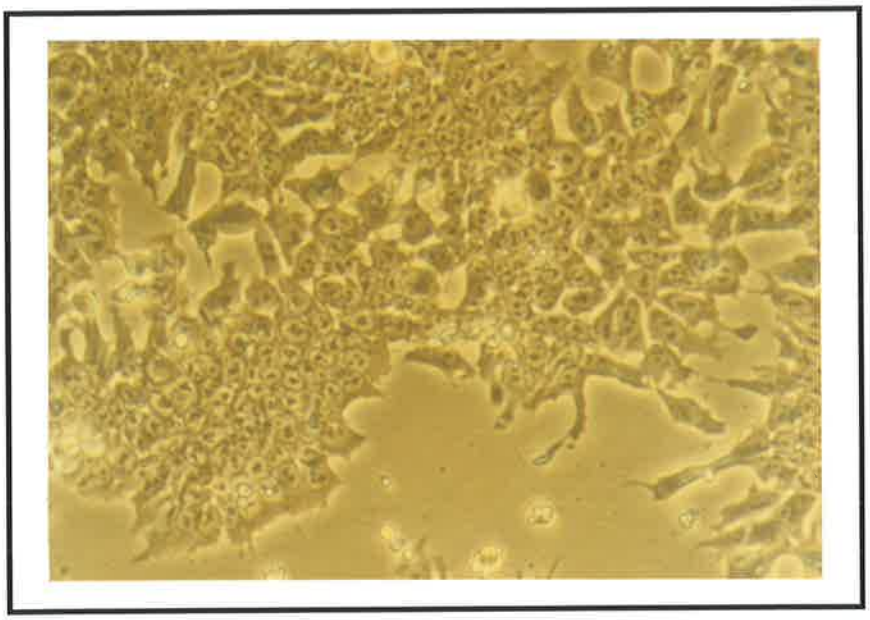
ES cells were cultured at high cell density in 50% MED2 in the presence and absence of LIF (2.3.9). X cells formed at high cell density in the presence of 50% MED2 and LIF (Xp0+) after 2 days are pictured in B. X cells formed from ES cells at high density in MED2 in the absence of LIF (Xp0-) after 2 days are pictured in C. ES cells cultured at high cell density (2.3.8) in LIF alone after 2 days are shown in A.

Photographs were taken at a magnification of 50x using phase contrast microscopy.

A



B



C

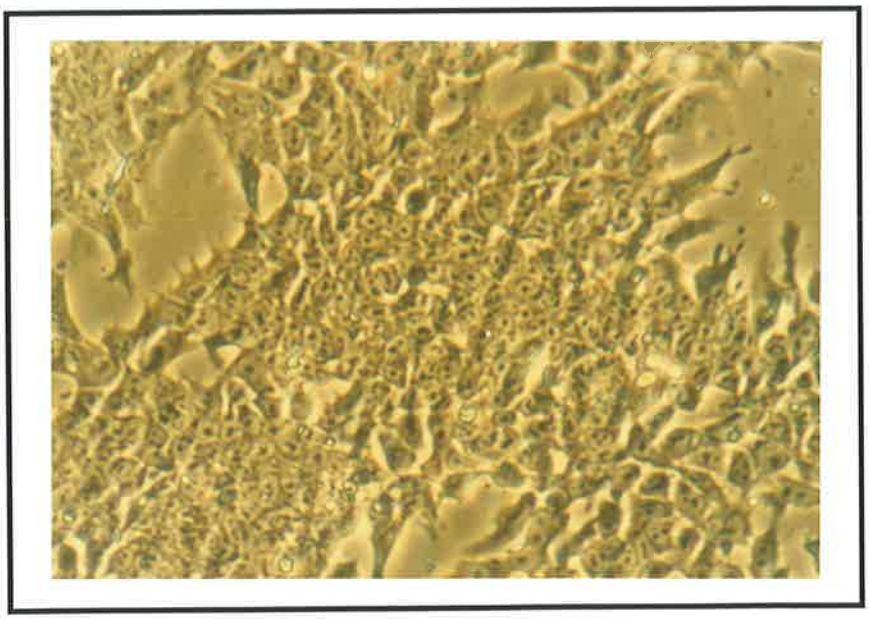
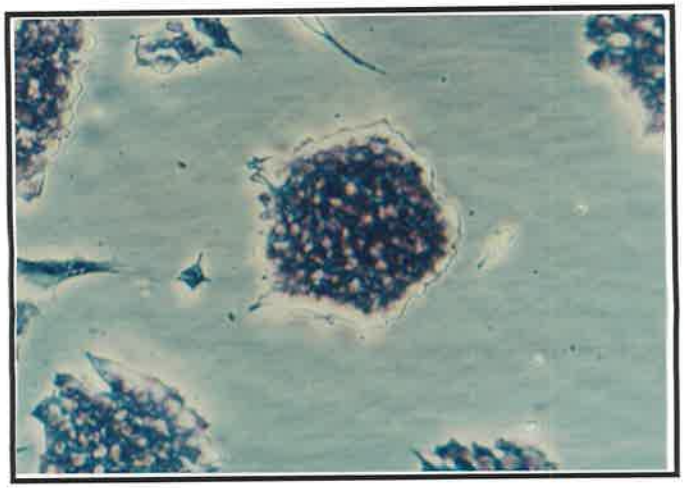


FIGURE 3.10 *In situ* analysis of *Oct-4* expression in ES cells, X cells and differentiated cells.

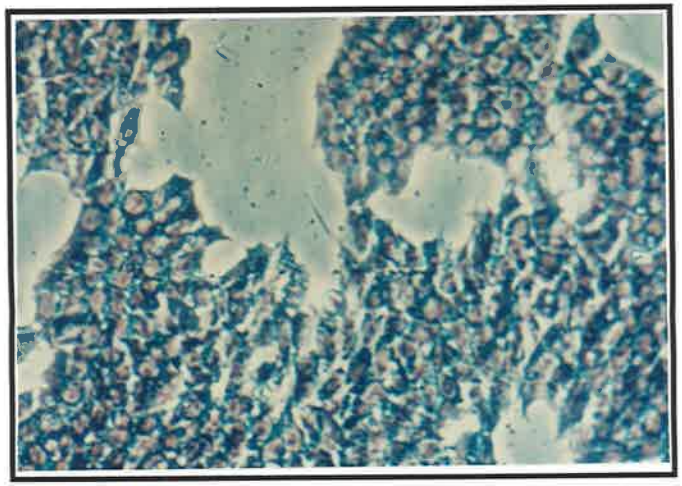
ES cells were cultured in the presence of LIF at high cell density for three days (2.3.8) (A, B). X cells were formed by culturing ES cells in the presence of MED2 and LIF for two days (2.3.9i) (C, D). X cells which have been passaged in the presence of MED2 and LIF contain a low level of differentiated cells (Bettess 1993) and were obtained by passaging X cells at high density in MED2 and LIF for six days (2.3.9 iii) (E, F). *Oct-4* expression was detected using a 394bp DIG labelled antisense riboprobe (2.5.17). Plates were developed for 8 hours before the colour reaction was stopped (2.5.19). Cells which express *Oct-4* are detected by purple staining. Note that differentiated cells (d) did not stain for *Oct-4*.

Photographs were taken under phase contrast (A, C, E) and bright field optics (B, D, F) at a magnification of 50x.

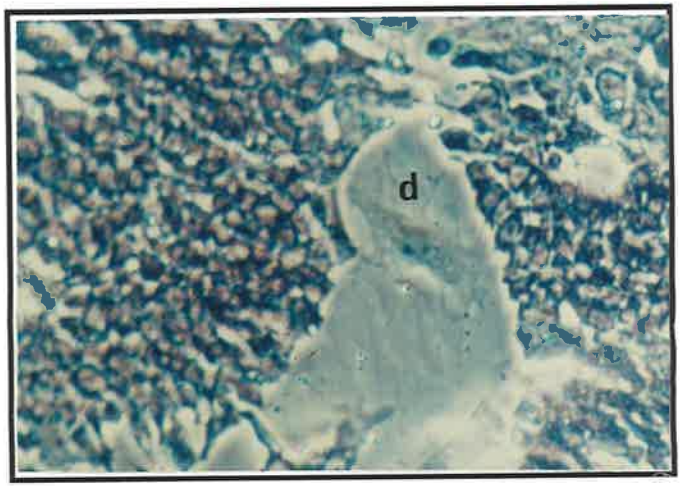
A



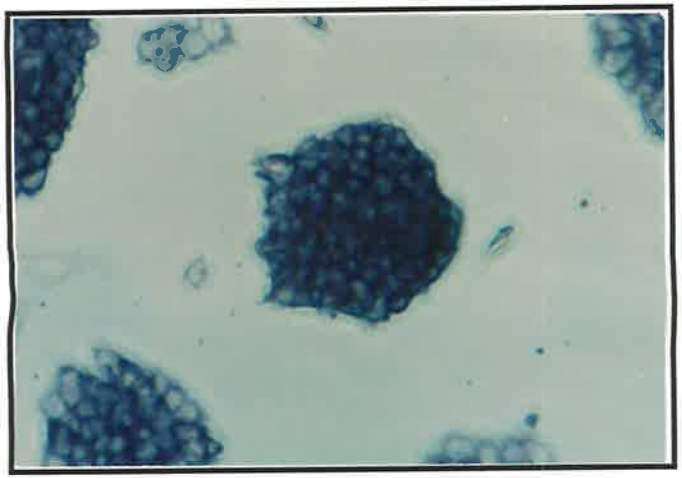
C



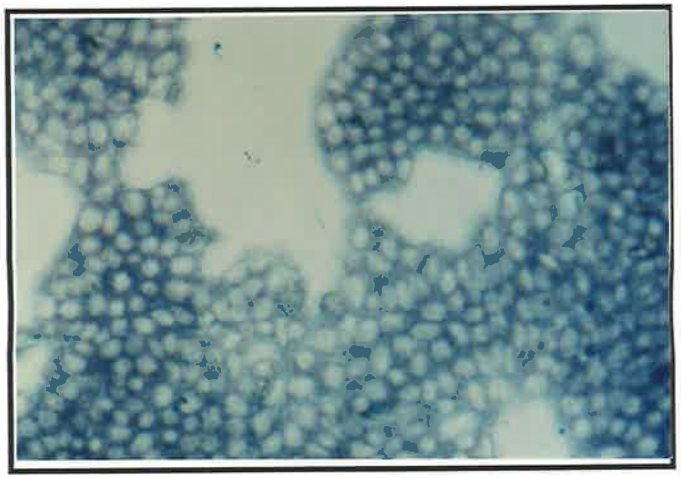
E



B



D



F

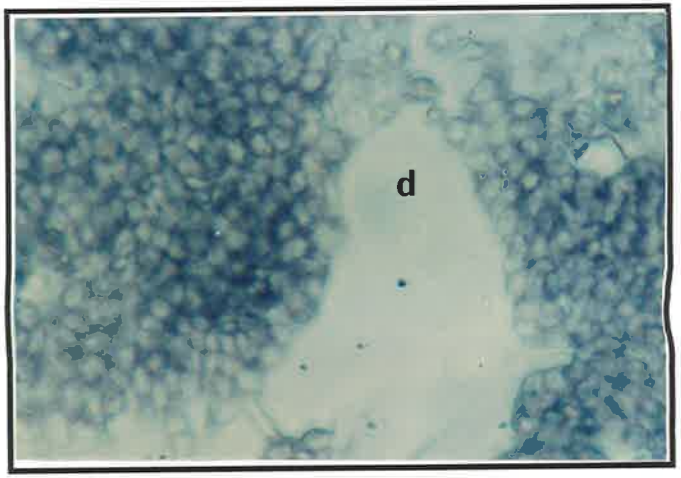
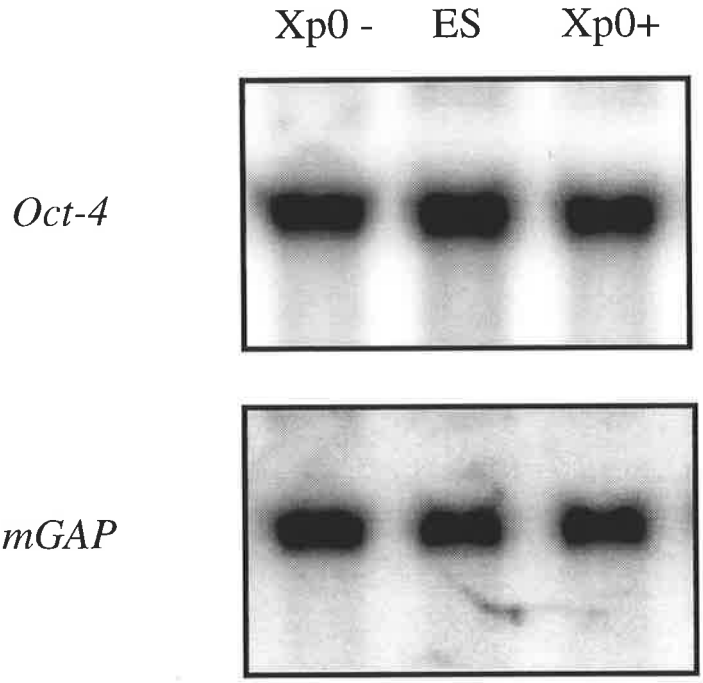


FIGURE 3.11 Northern blot analysis of *Oct-4* expression in ES, Xp0+ and Xp0- cells.

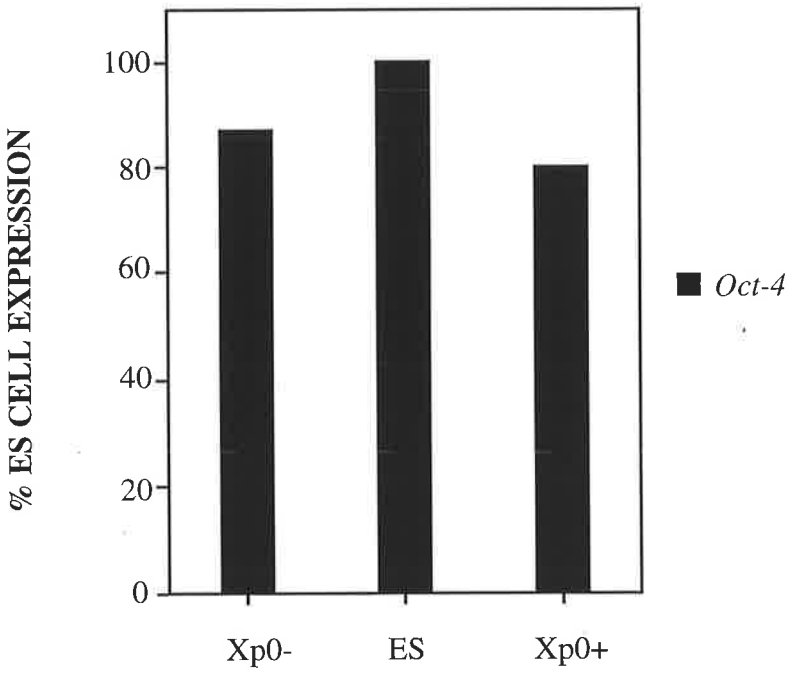
RNA was isolated from ES, Xp0+ and Xp0- cells (2.5.10). Northern analysis of *Oct-4* expression was carried out using an *Oct-4* DNA probe (2.5.12, 2.5.14). The filter was washed in 2xSSC/0.1% SDS at 42°C, then 0.2xSSC/0.1%SDS at 42°C, followed by a final wash in 0.2x SSC/0.1% SDS at 65°C. All washes were for a duration of 30 minutes. The filter was exposed to a phosphorimager screen for 15 hours (2.5.20). The filter was subsequently stripped and reprobed with a *mGAP* loading control (2.5.14, 2.5.16), exposure time 22 hours. Bands corresponding to the 1.6kb *Oct-4* transcript and 1.5kb *mGAP* transcript were detected. The expression of *Oct-4* and *mGAP* in ES, Xp0+ and Xp0- cells is shown in A.

The expression of *Oct-4* compared to *mGAP* was quantitated by volume integration (2.5.20). Expression of *Oct-4* in Xp0+ and Xp0- cells is expressed as a percentage of ES cell expression and represented graphically in B.

A


Xp0 -
ES
Xp0+
Oct-4
mGAP

B


% ES CELL EXPRESSION
100
80
60
40
20
0
Xp0-
ES
Xp0+
Oct-4

Xp0+ and Xp0- cells have been cultured in MED2 supplemented conditions for two days. When ES cells are seeded at low or clonal cell density the level of X cell differentiation increases from three days to five days (3.3). This has also been established for ES cells seeded at clonal cell density in 50% MED2 in the absence of LIF (data not shown). Thus the possibility arises that although the presence of LIF may be unimportant for X cells which have been at high density in MED2 for two days, it may be important for the maintenance of X cells during passaging. X cells were passaged every two days in the presence and absence of LIF to passage 8 (p8) which was equivalent to 18 days in culture. RNA was isolated from ES cells, Xp0+ cells through to Xp8+ cells and Xp0- cells through to Xp8- cells and analysed *via* Northern blot analysis for *Oct-4* expression.

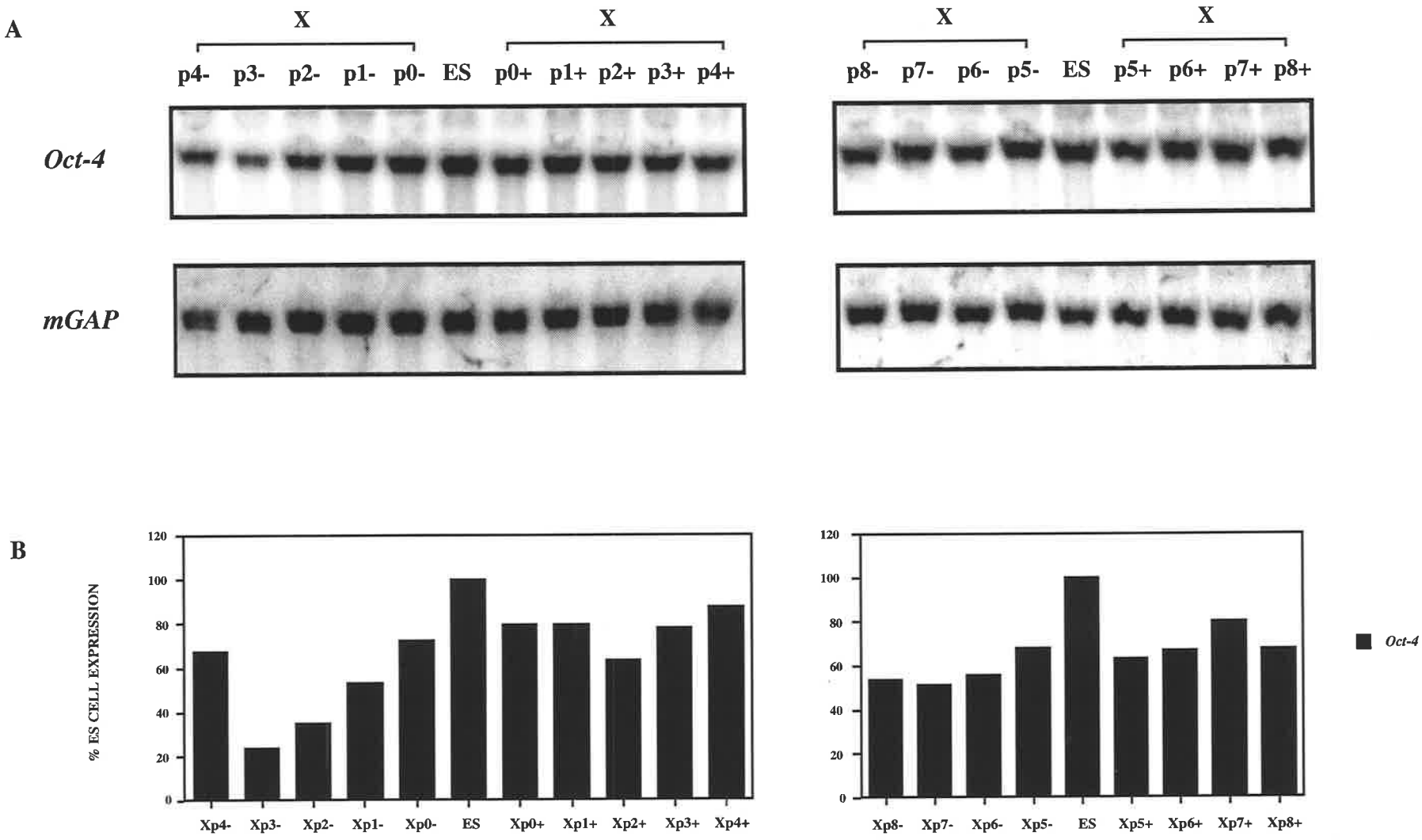
The levels of differentiation observed in X cells maintained in MED2 in the presence of LIF were low and relatively consistent throughout passage. In contrast the level of differentiation observed in X cells maintained in MED2 in the absence of LIF increased significantly from passage 1 to 3. After this period, the level of differentiation appeared to decrease and remained at a consistent level through the remainder of passage, similar to the level observed in X cell cultures maintained in the presence of LIF.

These observations were reflected in the levels of *Oct-4* expression (Figure 3.12). Consistent with previous observations Xp0+ and Xp0- cultures exhibited similar levels of *Oct-4* expression. In X cells passaged in MED2 in the absence of LIF a significant decrease in *Oct-4* expression occurred from Xp1- through to Xp3-. The level of *Oct-4* expression increased at Xp4- and remained at a consistent level for the remainder of passaging. In contrast, although slight fluctuations were observed, *Oct-4* expression levels in X cells passaged in MED2 in the presence of LIF were higher and at more consistent levels throughout passaging. The broad implications of this finding are discussed in Chapter 5 which contains a detailed analysis of passaged X cell gene expression. The correlation between the level of differentiated cells within cultures and *Oct-4* expression suggests that the level of *Oct-4* expression reflects the proportion of X cells compared to differentiated cells within the population and does not result from differential expression in X cells themselves. This is particularly important for the analysis of gene expression described in subsequent chapters.

FIGURE 3.12 Northern blot analysis of *Oct-4* expression in ES cells and X cells passaged in MED2 in the presence and absence of LIF.

RNA was isolated from the following (2.5.10):- ES cells, X cells passaged in the presence of MED2 and LIF (Xp0+ through to Xp8+) and X cells passaged in the presence of MED2 in the absence of LIF (Xp0- through to Xp8-). Northern analysis of *Oct-4* expression was carried out as previously described (Fig. 3.11). The filters were exposed to phosphorimager screens for 15 hours (2.5.20) and subsequently stripped and reprobed with a *mGAP* loading control (2.5.14, 2.5.16), exposure time 21 hours. The expression of *Oct-4* and *mGAP* in these cells is shown in A.

Expression of *Oct-4* compared to *mGAP* was quantitated by volume integration (2.5.20). The expression of *Oct-4* in X cells passaged in the presence of MED2 and LIF and X cells passaged in MED2 in the absence of LIF is expressed as a percentage of ES cell expression and represented graphically in B.


A
X
X
X
X
p4- p3- p2- p1- p0- ES p0+ p1+ p2+ p3+ p4+
p8- p7- p6- p5- ES p5+ p6+ p7+ p8+
Oct-4
mGAP
B
% ES CELL EXPRESSION
120
100
80
60
40
20
0
Xp4- Xp3- Xp2- Xp1- Xp0- ES Xp0+ Xp1+ Xp2+ Xp3+ Xp4+
Xp8- Xp7- Xp6- Xp5- ES Xp5+ Xp6+ Xp7+ Xp8+
Oct-4

Xp0+ and Xp0- cultures constitute homogeneous X cell cultures, ES cell colonies are not observed and the levels of differentiated cells are low. The expression of *Oct-4* at a similar level in Xp0+ and Xp0- cultures suggests that LIF is not required for X cell formation. The presence of exogenous LIF improves X cell maintenance during initial passage (p1-p3). However, the morphology and *Oct-4* expression levels associated with X cells after passage three demonstrates that exogenous LIF is not absolutely required for continued maintenance of X cells in MED2 during passaging at high cell density.

3.7 X Cells Revert to ES Cells when Cultured in the Presence of LIF and the Absence of MED2

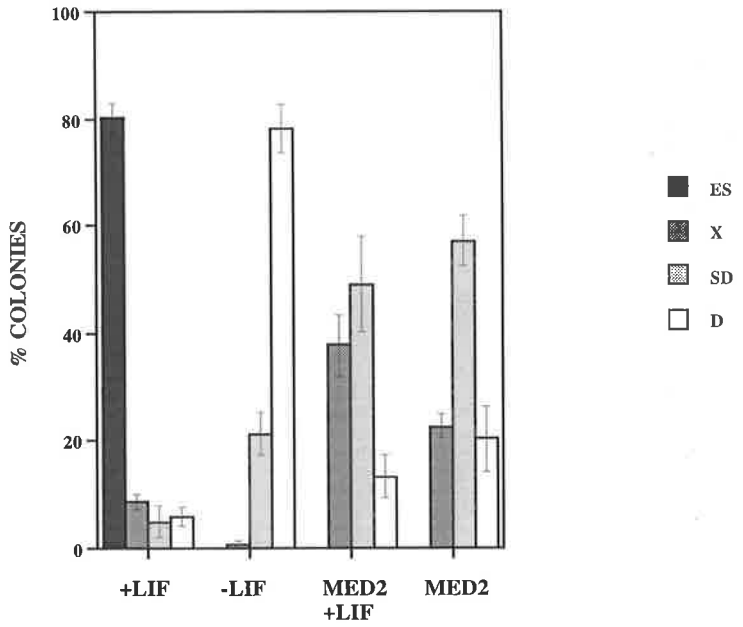
Previous observations had suggested that X cells seeded at high density revert to an ES cell morphology when cultured in the absence of MED2 and the presence of LIF (J. Rathjen, unpublished observations). To analyse this observation more closely Xp0+ were seeded at clonal density into wells containing incomplete medium alone and incomplete medium supplemented with 1) 50% MED2, 2) 50% MED2 + LIF and 3) LIF alone. These were compared to ES cells cultured under the same conditions. The assay was stained at 5 days for alkaline phosphatase activity and the colony types were determined. The results of the experiment are shown in Figure 3.13. When Xp0+ cells were seeded into LIF alone, 80% of the colonies at day 5 had an ES cell morphology. This was similar to the level of ES cell colonies detected when ES cells were cultured in the presence of LIF and indicates that X cells had reverted to ES cells at a high frequency. ES and Xp0+ cells cultured in the absence of MED2 and LIF underwent spontaneous differentiation. This was reflected in a large decrease in pluripotent cells and a large increase in the percentages of semi-differentiated and fully differentiated colonies. X cell colonies were maintained in all MED2 supplemented conditions. The mean plating efficiencies of ES and Xp0+ cells under the various conditions were not statistically different.

Clonal X cell lines possess the ability to revert to ES cells when cultured in the presence of LIF, in the absence of MED2 (J. Rathjen, unpublished observations). This observation, coupled with the high reversion frequency of X cells in this experiment, indicates that reversion is a genuine property of X cells, and is not due to the presence of

FIGURE 3.13 The behaviour of Xp0+ cells at clonal cell density in MED2 and LIF supplemented conditions.

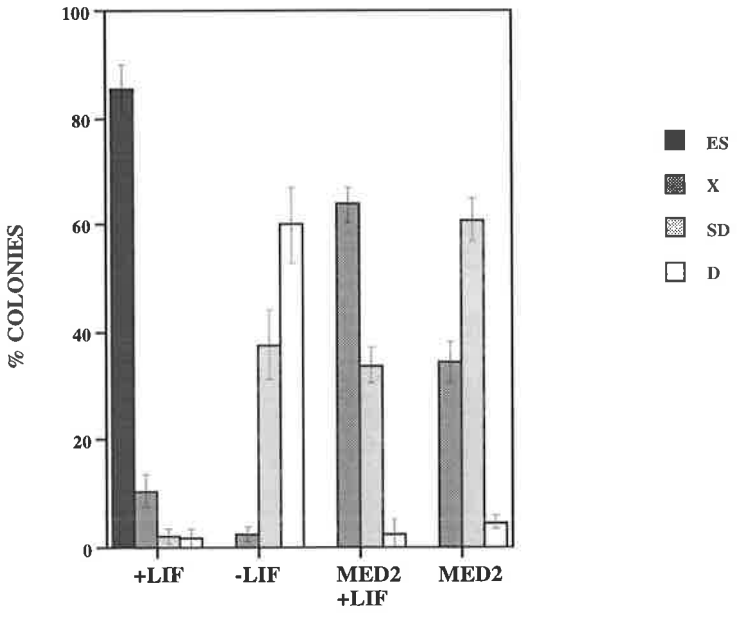
ES cells and Xp0+ cells were cultured in the presence and absence of MED2 and/or LIF (2.3.13ii, 2.3.14i). The assay was stained for alkaline phosphatase activity on day five (2.3.24) and the percentage of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD), and differentiated colonies (D) present in each well was determined as previously described (Fig. 3.4). The mean percentage and standard deviation for each variable was derived from colony analysis of four duplicate wells. Results obtained for Xp0+ cells under the outlined conditions are tabulated and represented graphically in A. Results obtained with ES cells under identical conditions are tabulated and represented graphically in B. Note: the mean plating efficiency for Xp0+ and ES cells in the described conditions is shown in tables A and B respectively.

A **Xp0+**



Xp0+ CELLS	*% COLONIES*				*PLATING EFFICIENCY (%)*
	ES	*X*	*SD*	*D*	
+ LIF	*80.4+/-2.8*	*8.8+/-1.4*	*5.0+/-2.9*	*5.8+/-4.4*	*32.4+/-7.4*
- LIF	*0*	*0.7+/-0.7*	*21.3+/-4.1*	*78.0+/-4.4*	*41.4+/-6.6*
+ MED2 + LIF	*0*	*37.7+/-5.7*	*49.0+/-8.9*	*13.3+/-4.0*	*41.9+/-5.0*
+ MED2	*0*	*22.7+/-2.3*	*57.0+/-4.7*	*20.3+/-6.2*	*40.5+/-5.9*

B **ES**



ES CELLS	*% COLONIES*				*PLATING EFFICIENCY (%)*
	ES	*X*	*SD*	*D*	
+ LIF	*85.6+/-4.6*	*10.6+/-3.0*	*2.1+/-1.3*	*1.7+/-1.7*	*42.6+/-6.7*
- LIF	*0*	*2.4+/-1.5*	*37.7+/-6.4*	*59.9+/-7.1*	*43.3+/-7.5*
+ MED2 + LIF	*0*	*63.8+/-3.4*	*33.8+/-3.2*	*2.4+/-2.7*	*47.6+/-0.4*
+ MED2	*0*	*34.4+/-3.7*	*60.9+/-4.1*	*4.7+/-1.1*	*45.9+/-1.7*

residual ES cells. The very low level of background differentiation observed in X cells cultured in LIF alone suggests that reversion to ES cells occurred relatively early during culture.

3.8 MED2 Contains Biologically Active Human LIF

The observations that LIF is required for optimal X cell maintenance, and that X cells required less LIF than ES cells, suggest that MED2 may contain LIF activity. RNAase protection of RNA derived from the human cell line from which MED2 is produced shows that LIF transcripts are detectable (B. Haines, unpublished observations). In addition, MED2 may contain factors such as OSM, CNTF, Interleukin (IL)-6, Interleukin (IL)-11 and CT-1, which like LIF signal *via* the gp130 signalling receptor and therefore could function like LIF (Gearing *et al.* 1992, Gearing and Bruce 1992, Ip *et al.* 1992, Conover *et al.* 1993, Yoshida *et al.* 1994, Pennica *et al.* 1995).

A neutralising anti-murine gp130 antibody was obtained from Tetsuya Taga (Institute for Molecular and Cellular Biology, Osaka University). This antibody was able to neutralise the activity of human OSM, IL-6, IL-11 and murine LIF on myeloid leukemic M1 cells. However it was unable to inhibit the biological function of human LIF on mouse cells (T. Taga, personal communication). A neutralising anti-human LIF antibody was obtained from R&D Systems.

Biological activities of anti-gp130 and anti-human LIF antibodies were determined by establishing the level required to inhibit the action of OSM and human LIF respectively on ES cells. Human LIF, murine LIF and human OSM are able to promote ES cell growth and renewal (Williams *et al.* 1988, Gearing and Bruce 1992, Piquet-Pellorce *et al.* 1994, Yoshida *et al.* 1994). The ability of recombinant human LIF (Amrad) and recombinant human OSM (R&D Systems) to maintain ES cells was compared to recombinant murine LIF (Amrad) and COS cell derived murine LIF (2.3.6, Smith 1991). D3 ES cells were seeded at clonal cell density into incomplete medium containing increasing levels of human OSM, human LIF, murine LIF and COS cell derived murine LIF. The assays were stained for alkaline phosphatase activity after 5 days and growth curves for ES cells cultured in increasing concentrations of each factor are depicted in

Figure 3.14. Both OSM and human LIF were able to maintain ES cells in culture at recommended concentrations.

D3 ES cells were seeded into 1000U/ml human LIF with increasing concentrations of neutralising anti-human LIF antibody. In a separate experiment D3 ES cells were cultured in 50ng/ml OSM, which had activity equivalent to 1000U/ml of LIF (refer to Figure 3.14), with increasing concentrations of anti-gp130. After 5 days the assays were stained for alkaline phosphatase activity and the proportion of ES cell colonies determined. Results are presented in Figure 3.15. A concentration of 10μg/ml of anti-human LIF completely blocked the action of 1000U/ml human LIF on ES cells. Although 10μg/ml anti-gp130 did not completely block the maintenance of ES cells by OSM, it resulted in a significant decrease in the percentage of undifferentiated ES cell colonies (76% to 22%). Due to the limited quantity of gp130 antibody available, the effects of higher antibody concentrations were not investigated.

Xp0+ cells were seeded at clonal cell density into duplicate wells containing incomplete medium with the following additions A) MED2 + murine LIF (500U/ml), B) MED2 + human LIF (500U/ml), C) MED2 + OSM (25ng/ml), D) MED2 + OSM (25ng/ml) + anti-gp130 (10μg/ml), E) MED2 + anti gp130 (10μg/ml), F) MED2 + human LIF (500U/ml) + anti-gp130 (10μg/ml), G) MED2 alone, H) human LIF (500U/ml) and I) no addition. The assay was stained at four days and colony types were assessed (Figure 3.16). OSM and human LIF had the same effect as murine LIF on X cell maintenance (compare B and C with A and G). Addition of anti-gp130 abolished the increase in X cell stability seen with OSM addition (compare C with D and G). However, it did not inhibit the stabilising effect of human LIF on X cell maintenance (compare D and F). Addition of anti-gp130 to MED2 alone had no effect on the presence or maintenance of X cells (E). Therefore, it could be concluded that functionally active human OSM was not present in MED2. The anti-gp130 antibody is also able to block signalling by human IL-6 and IL-11 (T. Taga, personal communication) therefore it is unlikely that these molecules were present in MED2.

The presence of human LIF in MED2 was investigated in a second experiment. Xp0+ cells were seeded at clonal density into duplicate wells containing incomplete medium supplemented with A) MED2 + murine LIF (500U/ml), B) MED2 + human LIF

FIGURE 3.14 The effect of human LIF, human OSM and murine LIF on ES cell maintenance.

ES cells were seeded into duplicate wells containing incomplete ES cell medium containing increasing concentrations of COS cell derived murine LIF, Amrad murine LIF, human LIF or human OSM (2.3.13 i). The assay was stained for alkaline phosphatase activity at day 5 (2.3.24) and the number of ES cell colonies were counted and expressed as a percentage of the total number of colonies present in each well. The mean percentage and standard deviation of ES cell colonies in the described conditions is tabulated in A and represented graphically in B.

Note: 1000U/ml is equivalent to 50ng/ml of OSM.

murine LIF (C) = COS cell derived LIF (2.3.6).

murine LIF (A) = recombinant murine LIF obtained from Amrad.

A

UNITS/ML	% ES CELL COLONIES			
	murine LIF(C)	murine LIF (A)	human LIF	OSM
0	0	0	0	0
20	1.3+/-0.1	8.3+/-4.1	27.8+/-6.4	5.7+/-0.1
100	15.0+/-2.12	54.0+/-9.8	59.7+/-1.5	36.3+/-1.0
500	60.5+/-5.2	71.2+/-2.6	63.9+/-0.9	55.1+/-0.1
1000	71.3+/-4.2	81.6+/-0.4	74.4+/-0.1	73.0+/-1.3
2000	82.5+/-2.9	85.9+/-3.8	83.6+/-1.9	82.8+/-1.5

B

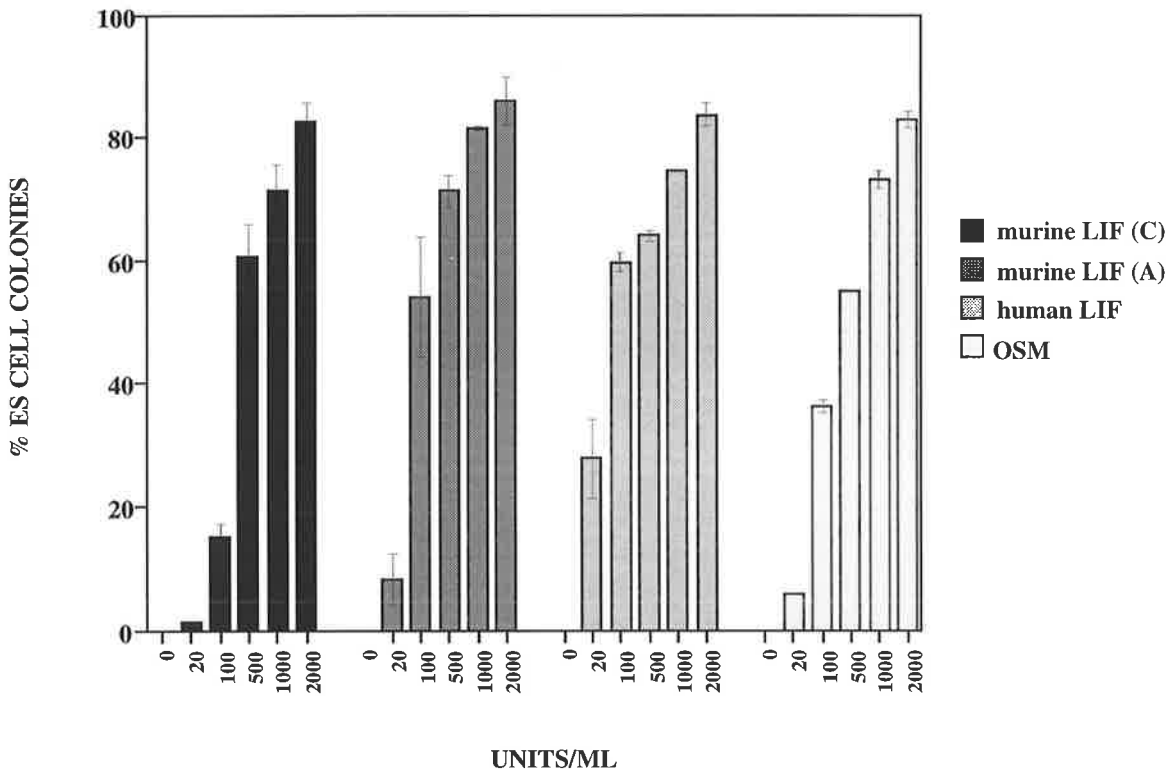


FIGURE 3.15

A) The concentration of anti-human LIF required to inhibit the action of human LIF on ES cells.

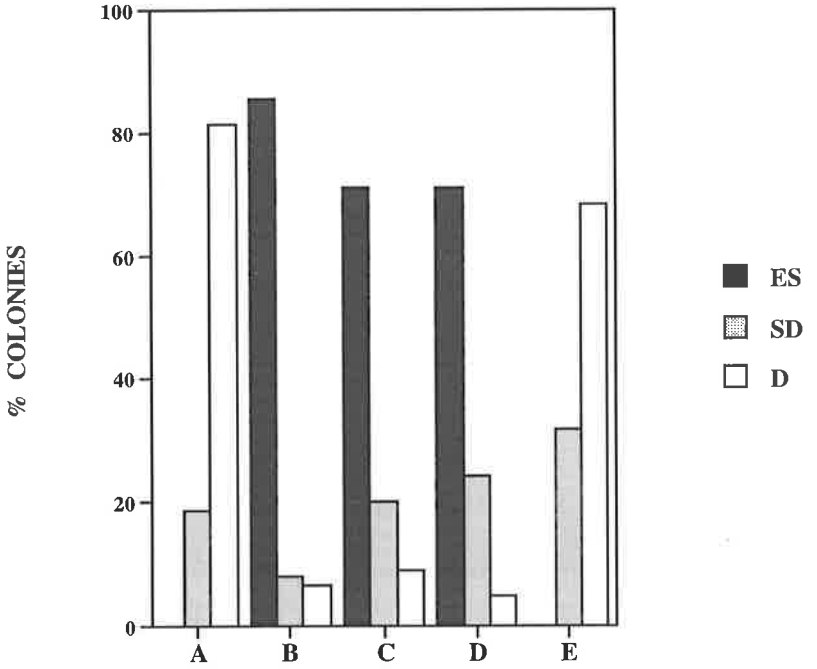
ES cells were cultured in incomplete ES cell medium containing A) no addition, B) human LIF (1000U/ml), C) human LIF (1000U/ml) + 1μg/ml anti-human LIF, D) human LIF (1000U/ml) + 5μg/ml anti-human LIF and E) human LIF + 10μg/ml anti-human LIF (2.3.13 iv). The assay was stained for alkaline phosphatase activity at 5 days (2.3.24) and the percentage of ES cell colonies (ES), semi-differentiated colonies (SD) and differentiated colonies (D) were determined as previously described (Fig. 3.4). The percentages of colonies observed in the described conditions are represented graphically and tabulated in A.

B) The concentration of anti-gp130 required to neutralise the effect of human OSM on ES cells.

ES cells were cultured in incomplete ES cell medium containing A) no addition, B) OSM (50 ng/ml), C) OSM (50 ng/ml) + 1μg/ml anti-gp130, D) OSM (50 ng/ml) + 5μg/ml anti-gp130 and E) OSM (50 ng/ml) + 10μg/ml anti-gp130 (2.3.13 iii). The assay was stained for alkaline phosphatase activity at 5 days (2.3.24) and the percentage of ES cell colonies (ES), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig 3.4). The percentages of colonies observed in the described conditions are represented graphically and tabulated in B.

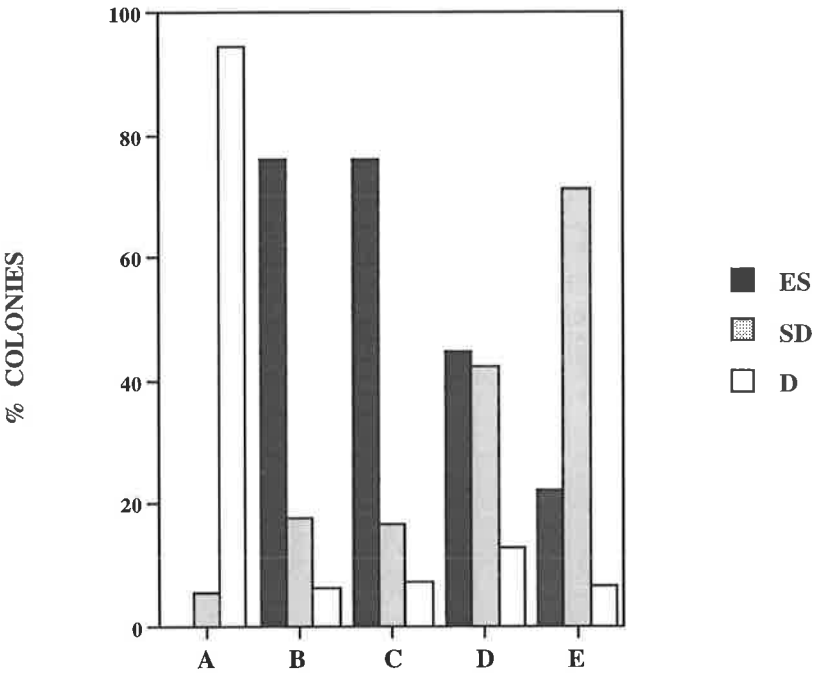
X cell-like colonies were omitted from the analysis, hLIF = human LIF, ahLIF = anti-human LIF, agp130 = anti-gp130.

A



	CONDITIONS	% COLONIES			PLATING EFFICIENCY(%)
		ES	SD	D	
A	No addition	0	18	82	32.5
B	+hLIF	86	8	7	38
C	+hLIF +1ug/ml ahLIF	71	20	9	45
D	+hLIF +5ug/ml ahLIF	71	24	5	31
E	+hLIF +10ug/ml ahLIF	0	32	68	22

B



	CONDITIONS	% COLONIES			PLATING EFFICIENCY(%)
		ES	SD	D	
A	No addition	0	5	95	18.5
B	+OSM	76	18	6	39.5
C	+OSM +1ug/ml agp130	76	17	7	42
D	+OSM +5ug/ml agp130	45	42	13	39
E	+OSM +10ug/ml agp130	22	71	7	22.5

FIGURE 3.16 The effect of anti-gp130 on X cell maintenance.

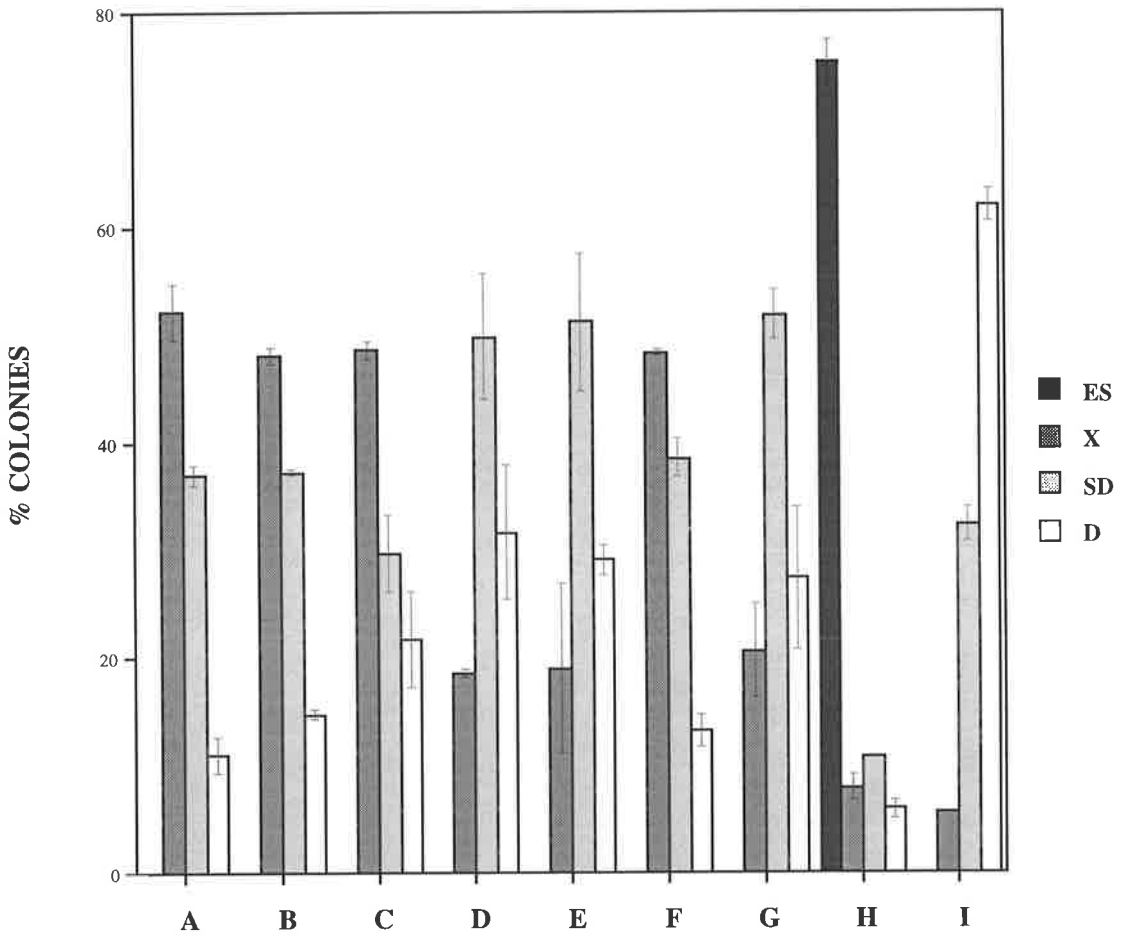
Xp0+ cells were cultured in duplicate wells containing incomplete ES cell medium with A) MED2 + murine LIF (500U/ml), B) MED2 + human LIF (500U/ml), C) MED2 + OSM (25ng/ml), D) MED2 + OSM (25ng/ml) + anti-gp130 (10µg/ml), E) MED2 + anti-gp130 (10µg/ml), F) MED2 + human LIF (500U/ml) + anti-gp130 (10µg/ml), G) MED2 alone, H) human LIF alone and I) no addition (2.3.14 ii). The assay was stained for alkaline phosphatase activity at four days (2.3.24) and the percentage of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was assessed as previously described (Fig. 3.4). The mean percentage and standard deviation of colonies present under the outlined conditions is tabulated in A and represented graphically in B.

hLIF = human LIF, mLIF = murine LIF (Amrad), agp130 = anti-gp130.

A

	CONDITIONS	% COLONIES				PLATING EFFICIENCY(%)
		ES	X	SD	D	
A	MED2+mLIF	0	52.1+/-2.6	37.0+/-1.0	11.0+/-1.7	42.5+/-5.0
B	MED2+hLIF	0	48.0+/-0.8	37.2+/-0.3	14.8+/-0.5	39.0+/-3.0
C	MED2+OSM	0	48.5+/-0.9	29.7+/-3.6	21.7+/-4.4	36.5+/-4.0
D	MED2 + OSM +agp130	0	18.6+/-0.4	49.8+/-5.8	31.6+/-6.2	32.2+/-0.8
E	MED2+agp130	0	19.1+/-7.8	51.1+/-6.4	29.1+/-1.5	36.8+/-3.3
F	MED2+hLIF+agp130	0	48.3+/-0.2	38.5+/-1.8	13.2+/-1.5	36.3+/-2.3
G	MED2	0	20.7+/-1.2	51.8+/-2.4	27.4+/-6.6	40.8+/-4.8
H	hLIF	75.2+/-2.0	7.9+/-1.2	10.9+/-0.1	6.0+/-0.9	25.2+/-0.3
I	No addition	0	5.7+/-0.1	32.4+/-1.6	61.9+/-1.5	26.25+/-0.3

B



(500U/ml), C) MED2 + human LIF (500U/ml) + anti-human LIF antibody (10μg/ml), D) MED2 + murine LIF (500U/ml) + anti-human LIF antibody (10μg/ml), E) MED2 + anti-human LIF (10μg/ml), F) MED2 in the absence of LIF and G) no addition. The assay was stained at 5 days for alkaline phosphatase activity and the colony types determined. The results of this experiment are shown in Figure 3.17. Addition of murine LIF (A) or human LIF (B) to MED2 resulted in increased stability of X cells (F). Addition of anti-human LIF to MED2 alone (E) or MED2 which contained added human LIF (C) abolished undifferentiated X cell colonies. The same effect of anti-human LIF on X cell maintenance was seen if the assay was analysed at three days (data not shown). Therefore human LIF is present in MED2 and is required for the maintenance of X cells in MED2 alone. The level of human LIF present in MED2 is likely to be at relatively low levels since addition of LIF at 20U/ml had a significant effect on the maintenance of X cells (Figure 3.8).

The results of this experiment show that human LIF is present in MED2 and suggest that it acts to maintain X cells in the undifferentiated state. The presence of human LIF in MED2 also explains the reduced requirement of X cells for LIF when compared to ES cells (3.5). Partial purification of the active components of MED2 indicates that X cell formation requires the presence of two factors which are distinct from human LIF (M. Bettess, J. Washington, unpublished observations). Therefore while the presence of human LIF in MED2 is required for X cell maintenance it is not intrinsically involved in X cell formation.

3.9 Discussion

The conditioned medium MED2 induces uniform differentiation of ES cells into a morphologically distinct, intermediate, pluripotent cell population termed X cells. MED2 induces the differentiation of ES cells to X cells both at clonal cell density and at high cell density. Levels of MED2 lower than 50% resulted in incomplete conversion while levels higher than 50% resulted in reduced cell viability. Therefore a level of 50% MED2 as a compromise between conversion, stability and viability was found to be optimal for the formation and maintenance of X cells. The addition of LIF was required for optimal X cell maintenance at both clonal cell density and high cell density, however its addition was

FIGURE 3.17 The effect of anti-human LIF on X cell maintenance.

Xp0+ cells were cultured in duplicate wells containing incomplete ES cell medium with A) MED2 + murine LIF (500U/ml), B) MED2 + human LIF (500U/ml), C) MED2 + human LIF (500U/ml) + anti-human LIF (10μg/ml), D) MED2 + murine LIF (500U/ml) + anti-human LIF (10μg/ml), E) MED2 + anti-human LIF (10μg/ml), F) MED2 alone and G) no addition (2.3.14 iii). The assay was stained for alkaline phosphatase activity at five days (2.3.24) and the percentage of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was assessed as previously described (Fig. 3.4). The mean percentage and standard deviation of colonies present under the outlined conditions is tabulated in A and represented graphically in B.

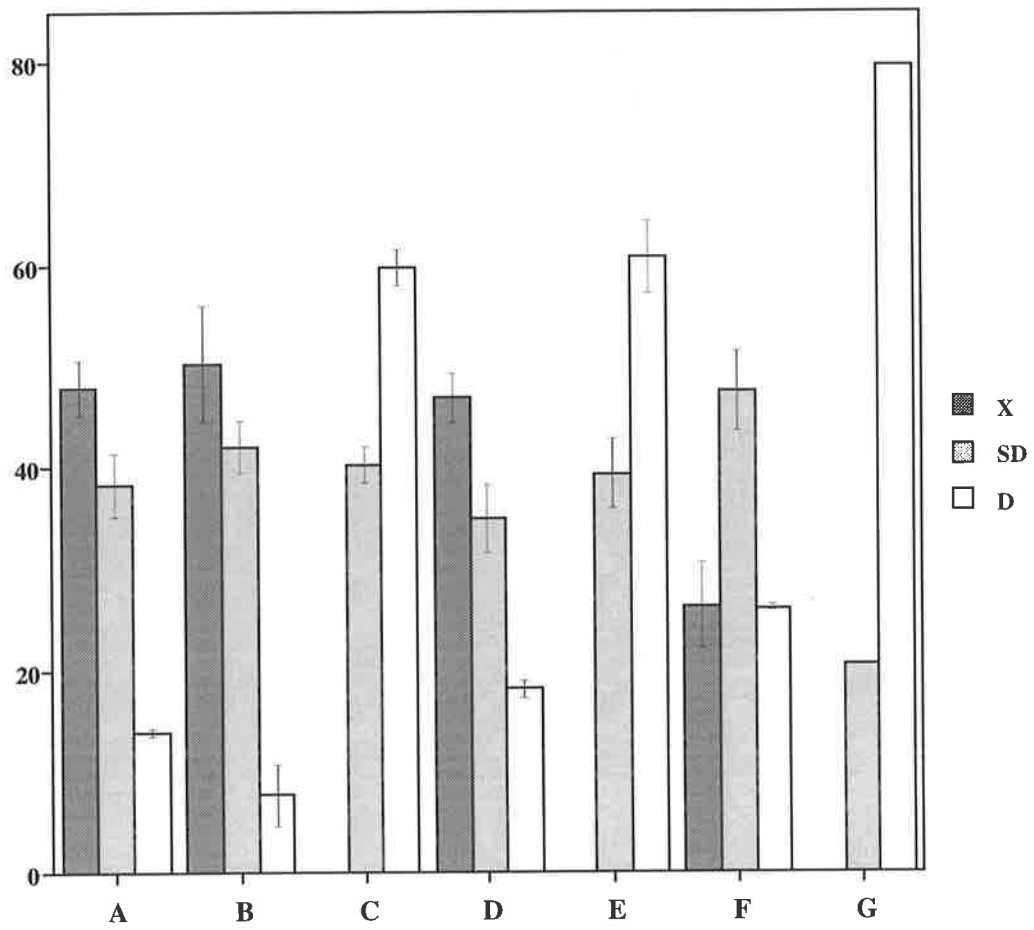
Note ES cell colonies were not detected.

hLIF = human LIF, mLIF = murine LIF (Amrad), ahLIF = anti-human LIF.

A

	CONDITIONS	% COLONIES			PLATING EFFICIENCY(%)
		X	SD	D	
A	MED2+mLIF	47.9+/-2.8	38.3+/-3.2	13.9+/-0.4	39.8+/-1.3
B	MED2+hLIF	50.3+/-5.7	42.0+/-2.6	7.7+/-3.1	35.0+/-2.0
C	MED2+hLIF +ahLIF	0	40.2+/-1.8	59.8+/-1.8	28.8+/-3.8
D	MED2 + mLIF +ahLIF	46.9+/-2.5	35.0+/-3.3	18.1+/-0.9	40.0+/-0.5
E	MED2+ahLIF	0	39.4+/-3.5	60.7+/-3.5	25.5+/-1.0
F	MED2	26.4+/-4.3	47.5+/-4.0	4.29+/-0.3	32.5+/-1.5
G	No addition	0	20.5+/-0.1	79.5+/-0.1	20.8+/-3.8

B



not an absolute requirement for X cell maintenance. Maintenance of X cells in the absence of added LIF appeared to be dependent on the presence of low levels of biologically active human LIF within MED2. Thus while LIF is not intrinsically involved in X cell formation (M. Bettess, J. Washington, unpublished results) its presence appears to be absolutely required for X cell maintenance. LIF and OSM exert their effect *via* gp130 receptor signalling. OSM has a similar effect on X cell maintenance as LIF, therefore X cell maintenance appears to be dependent on gp130 signalling. In the presence of LIF in the absence of MED2 X cells reverted at high frequency to ES cells. The significance of X cell reversion will be discussed in Chapter 5.

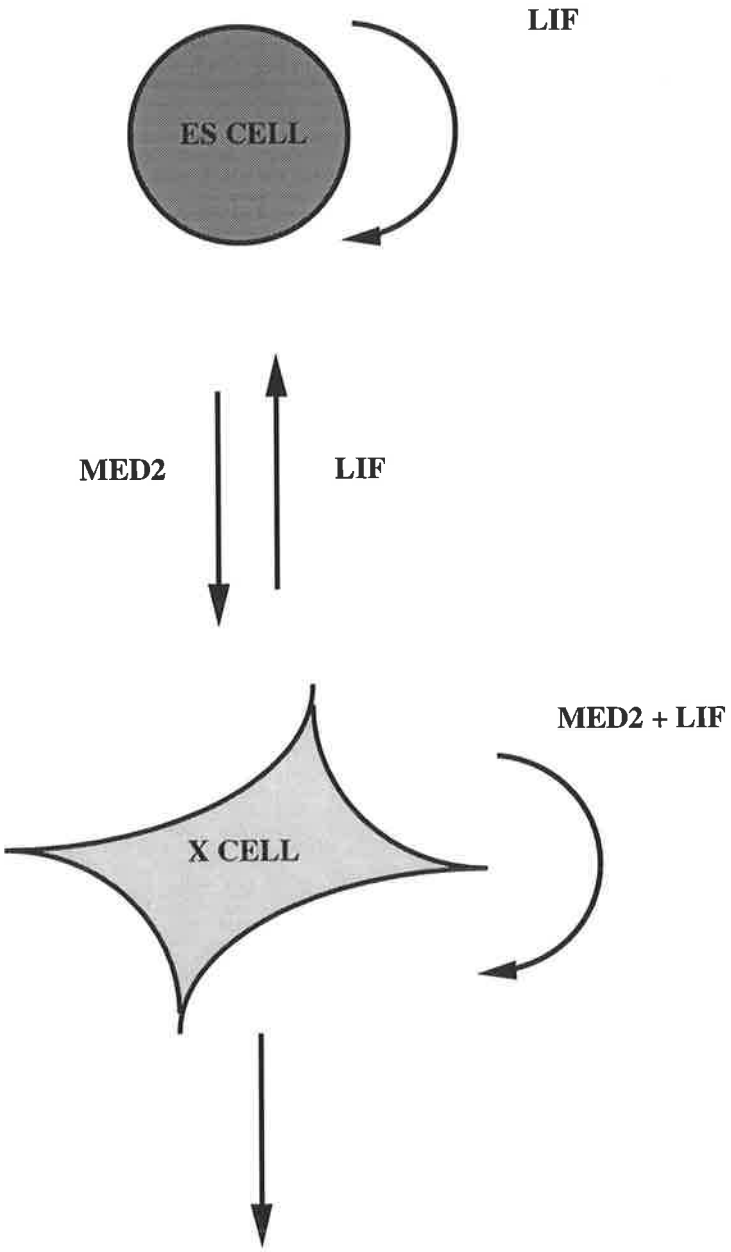
The results presented in this chapter can be summarised in the model presented in Figure 3.18. X cells represent an intermediate pluripotent cell population which exists between ES cells and differentiated derivatives. Factors within MED2 are responsible for the formation of X cells. LIF is required for ES cell maintenance and both MED2 and LIF are required for X cell maintenance. If MED2 is removed and LIF is present, X cells will revert to an ES cell phenotype. If both MED2 and LIF are removed ES and X cells differentiate spontaneously.

While the role of LIF in maintenance of pluripotential cell populations during murine embryogenesis remains unclear (1.5.3), LIF is required for the maintenance of both ES cells and primordial germ cells *in vitro* (Matsui *et al.* 1991, Pesce *et al.* 1993 Cheng *et al.* 1994, Smith *et al.* 1988). In addition, LIF has been shown to inhibit mesodermal differentiation during gastrulation (Conquet *et al.* 1992) and during the differentiation of P19 EC cells *in vitro* (Pruitt and Natoli 1992, Vidricare *et al.* 1994). The requirement of LIF for X cell maintenance is consistent with these observations.

Alternatively, the requirement for LIF may be due to the current growth conditions. *In vitro* growth conditions require the presence of serum which contains ill-defined factors which may affect X cell stability. In addition, the factors within MED2 responsible for the differentiation of ES to X cells have not been fully purified, hence current growth conditions rely on the addition of a conditioned medium which could also contain factors that may reduce the stability of X cells. Complete analysis of the role that LIF plays in X cell maintenance *in vitro* will ultimately require purification of the active constituents in MED2. Recently, greater X cell stability has been found when X cells are

FIGURE 3.18 A model for X cell formation, maintenance and differentiation.

The presence of LIF is required for ES cell maintenance. ES cells are converted to X cells when cultured in the presence of MED2. The presence of MED2 and LIF is required for X cell maintenance. If MED2 is removed and LIF is present X cells will revert to ES cells. If MED2 and LIF are removed ES cells and X cells will spontaneously differentiate.


LIF
ES CELL
MED2
LIF
MED2 + LIF
X CELL
DIFFERENTIATED CELL

grown on laminin, fibronectin and collagen IV substrates (J. Rathjen, personal communication), which are present in the murine embryo *in vivo* (Adamson and Ayers 1979, Wartiovaara *et al.* 1979, Leivo *et al.* 1980). The requirement of LIF for X cell maintenance under these conditions has not been investigated.

CHAPTER FOUR:
GENE EXPRESSION IN X CELLS

CHAPTER FOUR: GENE EXPRESSION IN X CELLS

4.1 Introduction

X cells represent a novel cell population derived from ES cells. ES cells are pluripotential and have the capacity to give rise to all tissues which constitute the developing embryo (Bradley *et al.* 1984, Robertson 1987). Therefore X cells may represent the *in vitro* equivalent of a cell population within the murine embryo (Figure 1.9). During embryogenesis different cell lineages can be defined by the unique repertoire of genes that they express. A variety of genes expressed at specific stages in specific tissues during murine embryogenesis have been identified. These genes can be used as markers to assess the presence of particular cell types *in vitro*. One way to determine the possible biological equivalent of X cells is to investigate the expression of marker genes.

Oct-4 expression is restricted to the pluripotential cell populations within the murine embryo including the cells constituting the inner cell mass, primitive ectoderm and the primordial germ cells (Rosner *et al.* 1990, Scholer *et al.* 1990a, 1990b, Yeom *et al.* 1991). At the outset of this research, preliminary evidence suggested that X cells expressed *Oct-4* (Thomas 1994). This observation was confirmed in Section 3.6. These observations, coupled with the expression of alkaline phosphatase and SSEA-1(1.8.2, 3.3), suggested that X cells may represent a pluripotential cell population within the murine embryo.

In further work in collaboration with M. Bettess (1993), X cell gene expression was analysed using a variety of marker genes. X cells were found to express *uvomorulin*. *Uvomorulin* is expressed in all pluripotential cell populations within the murine embryo with the exception of the primordial germ cells (Damjanov *et al.* 1986, Burdsal *et al.* 1993). Since X cells expressed *uvomorulin* they could not be considered equivalent to primordial germ cells. X cells did not express genes that are expressed in the primitive streak of the embryo such as *Brachyury, goosecoid* and *evx-1* (Herrmann. 1991, Blum *et al.* 1992, Bastian and Gruss 1990). Nor did they express *H19*, *SPARC* and *Alpha fetoprotein* (*AFP*) which are expressed by extraembryonic tissues in the early murine embryo (Poirier *et al.* 1991, Holland *et al.* 1987, Dziadek and Adamson, 1978,

Dziadek and Andrews 1983). These observations restricted the likely biological equivalent of X cells to either the inner cell mass (ICM), primitive ectoderm or an unrecognised pluripotential cell population.

Investigation of differential gene expression during the blastocyst and egg cylinder stages of early murine embryogenesis has been hampered, not only by the *in vivo* context in which the embryo develops, but also by its extremely small size. Consequently alterations of gene expression in the pluripotential cells as the ICM forms the primitive ectoderm prior to day 6.0 are not well characterised. Despite these limitations the expression of a limited number of genes during these stages has been defined and can be used to differentiate between the ICM and primitive ectoderm in early murine embryogenesis.

The importance of fibroblast growth factors and their receptors in early developmental processes such as mesoderm induction has been demonstrated in *Xenopus* (reviewed in Sive 1993). The role that these factors play in murine embryogenesis is less clear. FGF-5 is a member of the fibroblast growth factor (FGF) family. The expression of *FGF-5* during early development is confined to the primitive ectoderm prior to and during gastrulation, from day 5.25 to day 7.75. *FGF-5* expression could not be detected in the ICM of preimplantation embryos at day 3.5 (Haub and Goldfarb, 1991). In this report early expression analyses were only carried out on embryos at 3.5 and 5.25 days of development. Therefore the exact developmental stage at which *FGF-5* expression begins has not been ascertained.

Rex-1 is a zinc finger protein which is highly expressed in the ICM of the pre-implantation murine blastocyst. *Rex-1* expression decreases as the ICM forms the primitive ectoderm and is not detected in the primitive ectoderm at day 6.0 of development (Rogers *et al.* 1991). Consistent with the relationship between ES cells and the ICM, ES cells express *Rex-1*. *Rex-1* expression is down regulated when ES cells are spontaneously differentiated in the absence of LIF (Hosler *et al.* 1989, Rogers *et al.* 1991).

The homeobox gene *Gbx-2* (*MMoxB*) was initially identified in PCR screens of ES cell cDNA (Thomas 1994) and the telecephalon of the 13.5 day mouse embryo (Murtha *et al.* 1991). Further characterisation of *Gbx-2* expression revealed that it was expressed in

ES cells and down regulated as ES cells spontaneously differentiated in the absence of LIF (Chapman 1994, Thomas 1994). *Gbx-2* expression is not detected in the primitive ectoderm of the murine embryo at day 6.0 (J. Rathjen, unpublished results, Chapman 1994). Preliminary evidence suggests that *Gbx-2* is expressed in the ICM of preimplantation murine blastocyst at day 4.0 (G. Chapman, unpublished observations). The exact developmental stage at which *Gbx-2* expression is down regulated as the ICM forms the primitive ectoderm has not been determined.

This chapter describes the gene expression of X cells with respect to the aforementioned pluripotential cell markers.

4.2 Expression of Marker Genes that Discriminate Between the ICM and Primitive Ectoderm in X Cells Passaged with and without Added LIF

Results in Chapter three indicated that X cells cultured at high cell density in the presence of MED2 and LIF (1000U/ml) were more stable than X cells cultured in MED2 in the absence of LIF (1000U/ml) (3.6). The expression of the pluripotential cell marker genes, *FGF-5*, *Rex-1*, *Gbx-2* and *Oct-4*, were examined in X cells cultured and passaged at high cell density in MED2, with and without LIF supplementation.

ES cells were seeded at high cell density ($6x10^3$ cells/cm^2) into MED2 with and without LIF (1000U/ml). After two days uniform conversion of ES cells into X cells had occurred. X cell cultures at this stage were termed Xp0+ and Xp0- (3.6). Xp0+ cell cultures were continually passaged in MED2 with added LIF and Xp0- cells were continually passaged in MED2 without added LIF. Cultures were reseeded at high cell density every two days (2.3.9). Investigation in this instance was confined to passages up to and including passage two.

4.2.1 Northern Blot and RNAase Protection Analysis of Pluripotential Cell Marker Gene Expression

RNA was harvested from ES cells, X cells passaged in the presence of MED2 and added LIF (Xp0+ to Xp2+) and X cells passaged in the presence of MED2 in the absence of added LIF (Xp0- to Xp2-). The expression of *FGF-5*, *Rex-1* and *Oct-4* relative to a *mGAP* loading control in ES and X cells was determined by Northern blot analysis of

total RNA. The results of this analysis are presented in Figure 4.1. The levels of *FGF-5*, *Rex-1* and *Oct-4* relative to a *mGAP* loading control were quantitated by volume integration and expressed as a percentage of ES cell expression (Figure 4.2). *Oct-4* is expressed in ES and X cells but is not expressed in differentiated cells. Therefore *Oct-4* expression can be considered representative of the level of X cells compared with differentiated cells within the population (3.6). An estimate of the proportion of pluripotential stem cells within the population allows alterations in gene expression levels to be interpreted more clearly.

Expression of *FGF-5* was not detected at appreciable levels in Xp0+ and Xp1+ but was detected at increased levels in Xp2+. The expression of *Rex-1* decreased steadily relative to *Oct-4* expression as X cells were passaged in MED2 and LIF (Xp0+ to Xp2+). This indicated that the decrease in *Rex-1* expression reflected a decrease of *Rex-1* expression in X cells themselves and not simply a decrease in the level of X cells within the population.

X cells formed in MED2 in the absence of added LIF (Xp0-) had an increased level of *FGF-5* expression when compared to ES cells and Xp0+ cells. The level of *FGF-5* expression increased dramatically as X cells were passaged in MED2 without added LIF, with the highest expression level observed at Xp2-. The levels of *Rex-1* expression decreased steadily relative to *Oct-4* expression from ES to Xp2-. Decreases in *Rex-1* expression were greater in X cells cultured in MED2 in the absence of LIF than in X cells cultured in MED2 in the presence of LIF. The level of *Oct-4* expression was at a significantly decreased level in Xp2- this reflected the higher proportion of differentiated cells within this population (3.6).

Consistent alterations in gene expression were observed in similar experiments described in Sections 5.2 and 5.3, however in these and other experiments Xp0+ and Xp1+ cells clearly exhibited increases in *FGF-5* expression when compared to ES cells. Thus while expression levels varied between experiments, alterations in gene expression were similar.

Northern analysis of total RNA was not sensitive enough for determining *Gbx-2* expression levels, therefore RNAase protection was carried out on RNA derived from ES cells, Xp0+ to Xp2+ cells, and Xp0- to Xp2- cells. The results are presented in Figure

FIGURE 4.1 Northern blot analysis of *FGF-5*, *Rex-1* and *Oct-4* expression in X cells passaged in the presence and absence of added LIF.

X cells were passaged at high cell density in the presence of MED2 with and without added LIF (2.3.9). RNA was isolated from ES cells, Xp0+/-, Xp1+/- and Xp2+/- (2.5.10) and 30μg of total RNA was analysed by Northern blot (2.5.12). The filter was probed sequentially with *FGF-5*, *Rex-1*, *Oct-4* and finally with a *mGAP* loading control. DNA probes were used in this analysis (2.5.14) and the filter was stripped after each hybridisation (2.5.16). Washes were in 2xSSC/0.1%SDS at 42°C, 0.2xSSC/0.1% SDS at 42°C, with a final wash in 0.2xSSC/0.1%SDS at 65°C. All washes were for a minimum duration of 30 minutes. The expression of each gene was determined by phosphorimager analysis (2.5.20). The exposure time for *FGF-5* was 22 hours, *Rex-1* 5 hours, *Oct-4* 2.5 hours and *mGAP* 16 hours. *FGF-5* transcripts were of 2.7 and 1.8kb, *Rex-1* 1.9kb, *Oct-4* 1.6kb and *mGAP* 1.5kb.

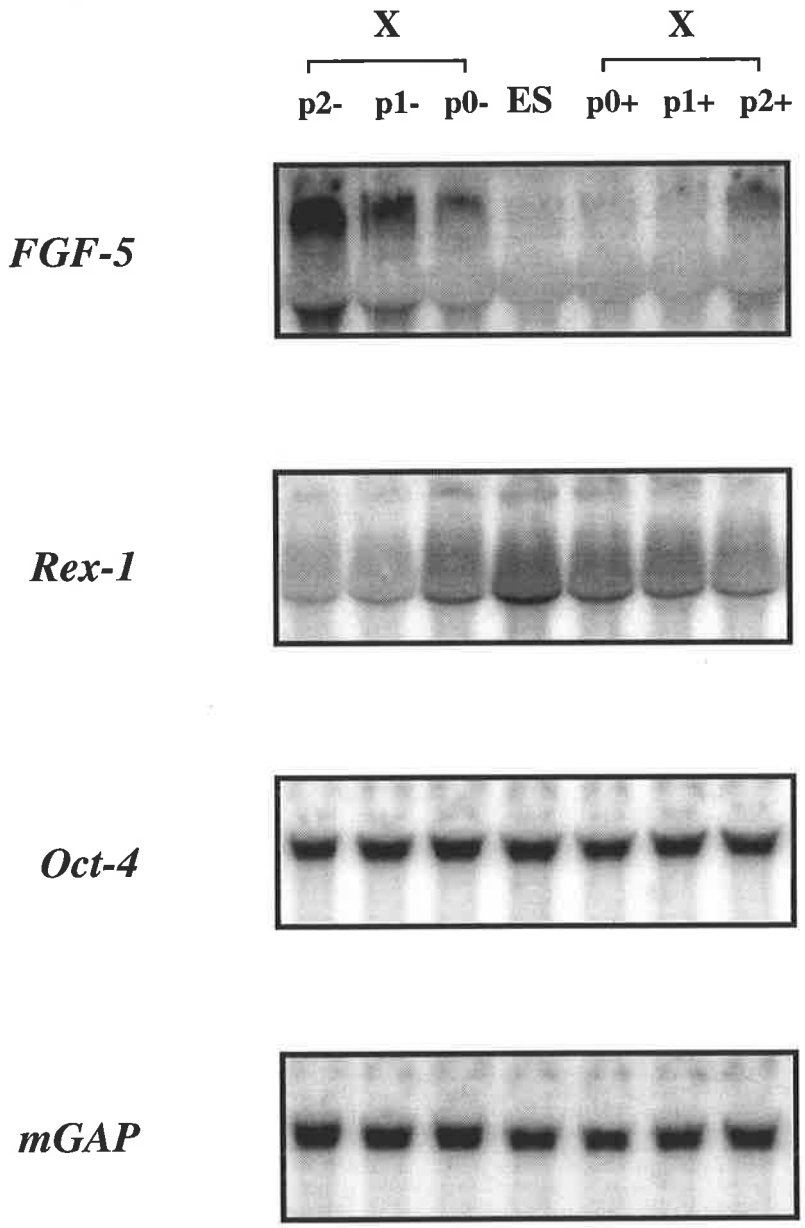
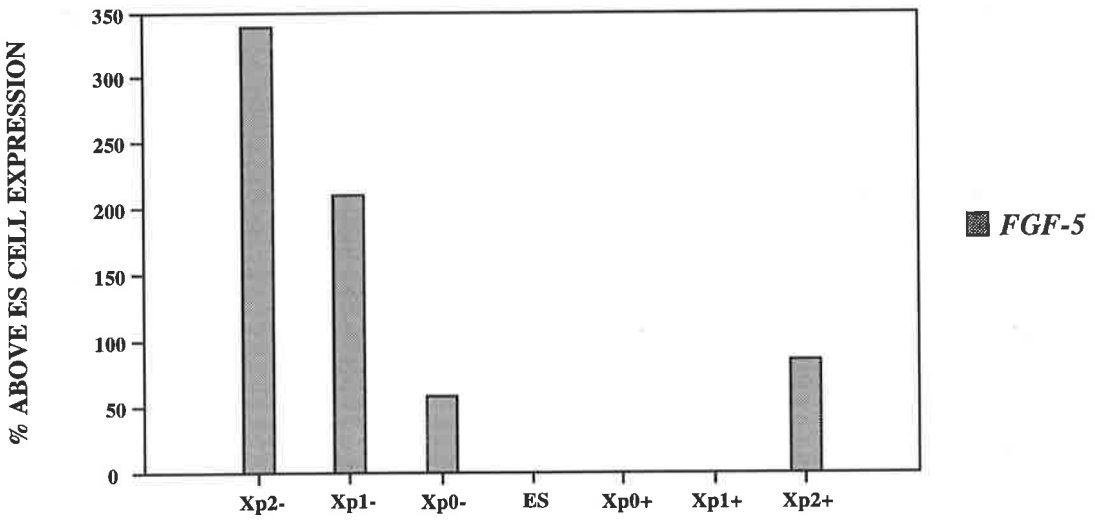
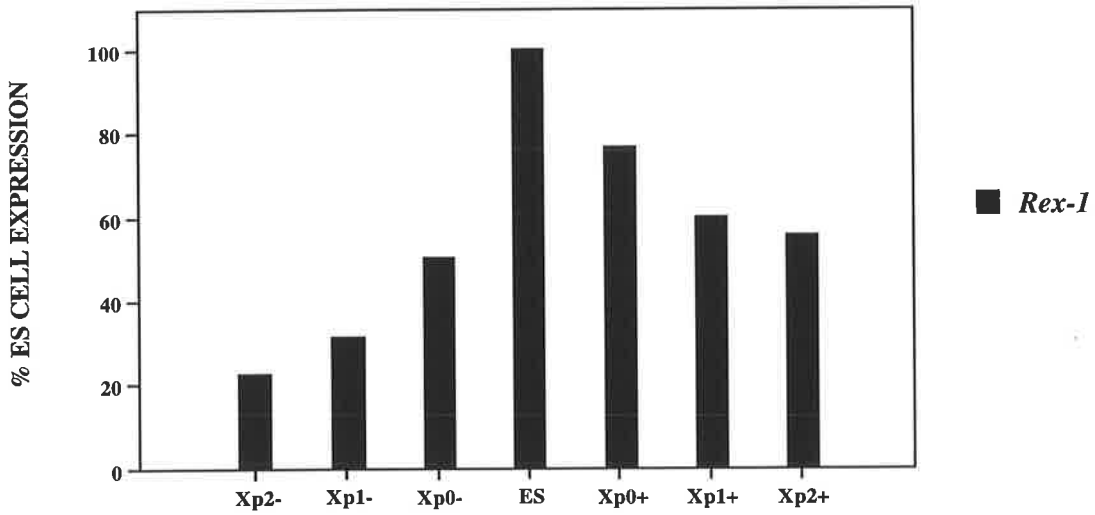
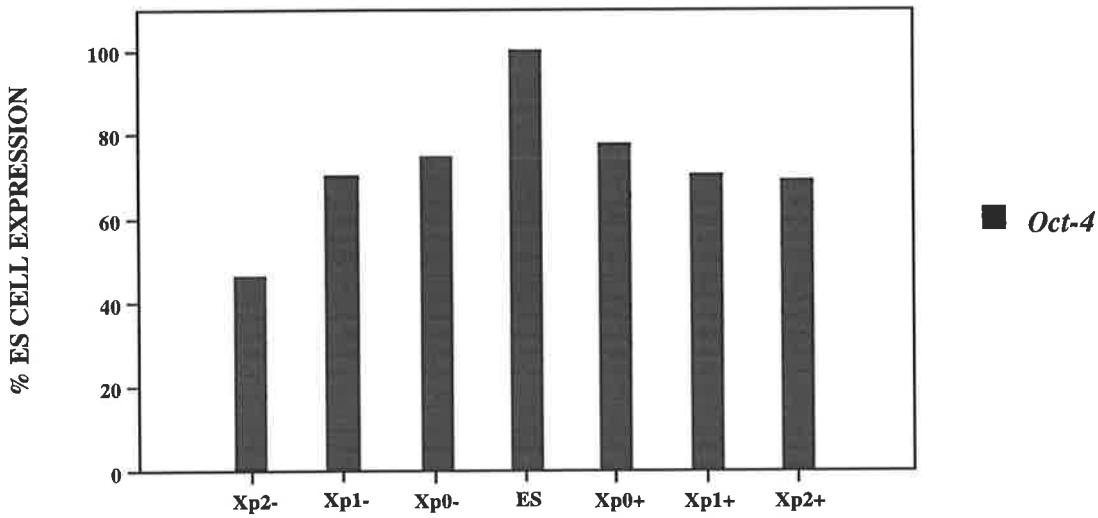

X
X
p2- p1- p0- ES p0+ p1+ p2+
FGF-5
Rex-1
Oct-4
mGAP

FIGURE 4.2 Comparison of *FGF-5*, *Rex-1* and *Oct-4* expression in X cells passaged in MED2 with and without added LIF.

The level of *FGF-5*, *Rex-1* and *Oct-4* compared to *mGAP* in (Fig. 4.1) was quantitated by volume integration (2.5.20). *FGF-5* expression in ES cells was assigned a value of 0% and the level of *FGF-5* expression in each X cell culture is shown as a percentage above ES cell expression. *Rex-1* and *Oct-4* expression in ES cells were assigned values of 100%. The level of *Rex-1* and *Oct-4* in each X cell culture is represented as a percentage of ES cell expression.


% ABOVE ES CELL EXPRESSION
350
300
250
200
150
100
50
0
Xp2-
Xp1-
Xp0-
ES
Xp0+
Xp1+
Xp2+
FGF-5


% ES CELL EXPRESSION
100
80
60
40
20
0
Xp2-
Xp1-
Xp0-
ES
Xp0+
Xp1+
Xp2+
Rex-1


% ES CELL EXPRESSION
100
80
60
40
20
0
Xp2-
Xp1-
Xp0-
ES
Xp0+
Xp1+
Xp2+
Oct-4

4.3A. *Gbx-2* expression was quantified compared to a *mGAP* loading control by volume integration and was expressed as a percentage of ES cell expression. This is represented graphically in Figure 4.3B.

Overall, *Gbx-2* expression decreased as X cells were passaged in MED2. Expression of *Gbx-2* was down regulated earlier in X cells passaged in the absence of added LIF than it was in X cells passaged in the presence of added LIF. *Gbx-2* expression increased transiently in Xp0+ and then subsequently decreased from Xp1+ to Xp2+. Similar observations have been made in previous experiments where *Gbx-2* expression has been analysed in X cells cultured in MED2 and added LIF (Chapman 1994). The possibility exists that *Gbx-2* expression in the embryo may reach maximal levels after the inner cell mass stage and then subsequently decrease during primitive ectoderm formation. Evidence for this will require a more detailed analysis of *Gbx-2* expression *in vivo*.

X cells expressed *FGF-5*. *FGF-5* expression is detected in the primitive ectoderm from day 5.25. Both *Rex-1* and *Gbx-2* are expressed in the inner cell mass at day 4.0 but are not expressed in late primitive ectoderm at day 6.0. These observations suggest that X cells are most likely to represent primitive ectoderm prior to *Rex-1 and Gbx-2* down regulation at day 6.0.

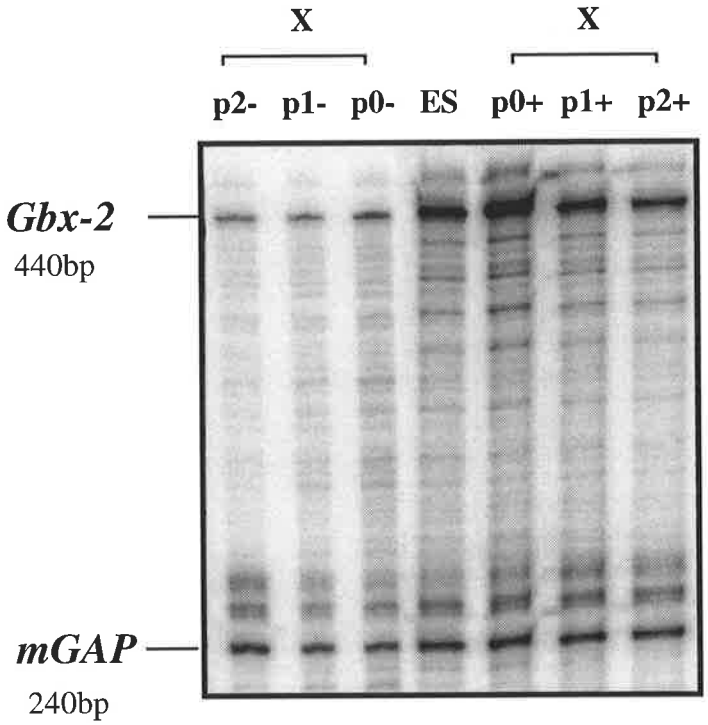
The gene expression changes observed as X cells were cultured in MED2 represents the transition of cells from a gene expression profile characterised by low *FGF-5*, high *Rex-1* and high *Gbx-2*, to one which is characterised by higher *FGF-5*, lower *Rex-1* and lower *Gbx-2*. The inner cell mass expresses *Rex-1* and *Gbx-2* and expression of these genes is down regulated as the primitive ectoderm is formed (Rogers *et al.* 1991, G. Chapman, unpublished observations, Chapman 1994). *FGF-5* is expressed in the primitive ectoderm at approximately day 5.25 but not in the ICM (Haub and Goldfarb 1991). The gene expression profiles as ES cells formed X cells and X cells were passaged in MED2 were therefore consistent with the predicted gene expression profile of the inner cell mass as it forms the primitive ectoderm *in vivo*. The addition of LIF to X cell culture conditions slowed this transition. In the absence of LIF, transition to cells with higher *FGF-5*, lower *Rex-1* and lower *Gbx-2* expression occurred faster and to a greater extent than observed in the presence of added LIF. An inhibitory effect of

FIGURE 4.3 RNAase protection analysis of *Gbx-2* expression in ES cells and X cells passaged in MED2 with and without added LIF.

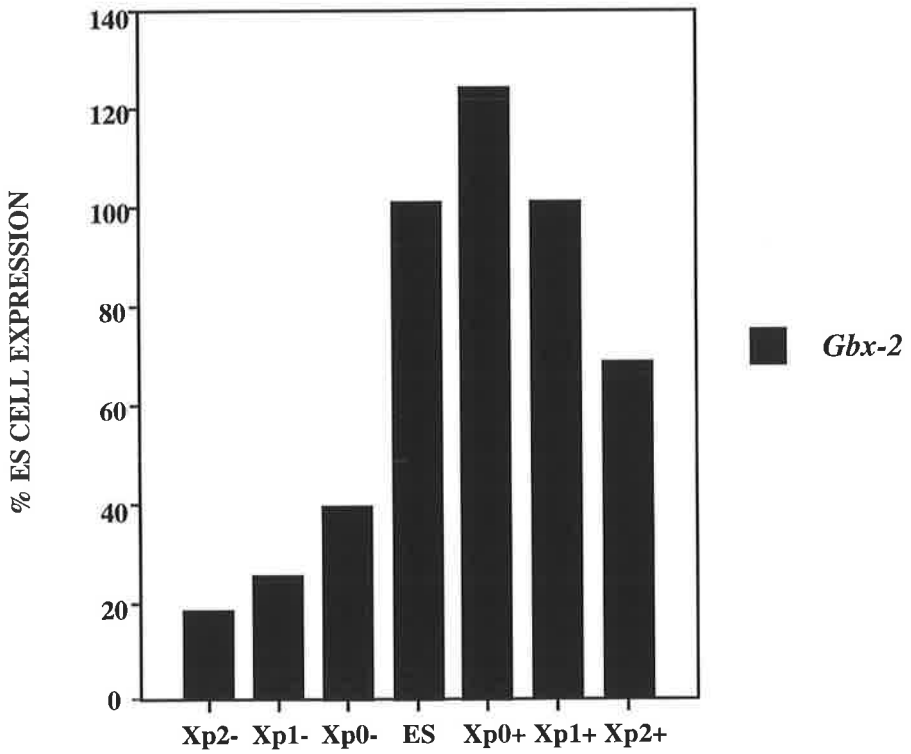
RNAase protection analysis was carried out on 20µg of RNA, using a *Gbx-2* antisense riboprobe and a *mGAP* loading control (2.5.13). RNAase protection products were separated on a 6% polyacrylamide gel (2.5.2) and exposed to a phosphorimager screen for 12 hours (2.5.20). *Gbx-2* expression as determined by RNAase protection is shown in A.

The level of *Gbx-2* compared to *mGAP* was quantitated by volume integration (2.5.20) The level of expression for each culture is shown as a percentage of ES cell expression. *Gbx-2* expression in ES cells was assigned a value of 100%. Results of this analysis are represented graphically in B.

A


X
X
p2- p1- p0- ES p0+ p1+ p2+
Gbx-2
440bp
mGAP
240bp

B


% ES CELL EXPRESSION
140
120
100
80
60
40
20
0
Xp2- Xp1- Xp0- ES Xp0+ Xp1+ Xp2+
Gbx-2

LIF on primitive ectoderm formation *in vitro* has also been demonstrated by others (Shen and Leder 1992).

4.2.2 *In Situ* Hybridisation Analysis of *FGF-5*, *Rex-1* and *Gbx-2* Expression

Xp0+ and Xp0- cultures contain low levels of differentiated cells. While X cells can be maintained by passage in the presence of MED2 in the absence of added LIF (3.6), the level of differentiated cells is higher and is particularly evident in Xp2- cultures. Interpretation of gene expression in 4.2.1 relies on the assumption that it was the X cells within the population that were expressing *FGF-5*, *Rex-1* and *Gbx-2* and not the differentiated cells. The cellular expression of *FGF-5*, *Rex-1* and *Gbx-2* was visualised by *in situ* hybridisation to X cell monolayers.

Xp0- and Xp2- cultures were analysed for *FGF-5* expression using DIG labelled antisense riboprobes. Results of these analyses are presented in Figure 4.4. *In situ* hybridisation indicated that *FGF-5* was expressed by X cells and not by their differentiated derivatives. This is particularly important as it shows that the large increase in *FGF-5* expression observed in Xp2- cultures can be attributed to the X cells within the population rather than the differentiated cells.

ES and Xp0- cultures were analysed for the expression of *Rex-1* and *Gbx-2* using antisense DIG labelled riboprobes. The results of this analysis for *Rex-1* and *Gbx-2* are presented in Figure 4.5 and Figure 4.6 respectively. *Rex-1* and *Gbx-2* expression was also detected in X cells but was not detected in differentiated cells.

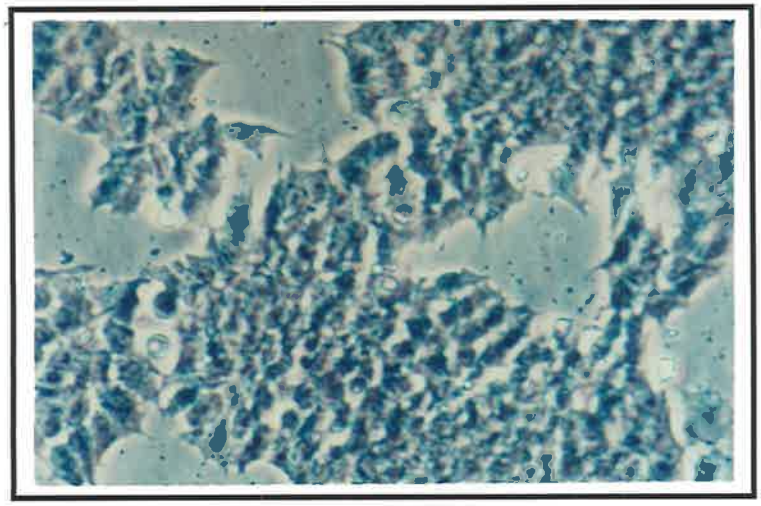
While *Gbx-2* expression appeared relatively uniform within ES cell populations, it appeared to be differentially expressed between X cells within X cell populations. X cells expressing higher levels of *Gbx-2* were estimated to constitute approximately 20% of the population. In contrast *Rex-1* and *FGF-5* expression appeared more uniform within X cell populations. However, the level of staining and therefore the expression of *FGF-5* and *Rex-1* did appear higher in certain cells and may reflect small variations in expression levels between cells. The implications of differential expression of *FGF-5*, *Rex-1* and *Gbx-2* within X cell populations will be discussed in Chapter 5.

FIGURE 4.4 *In situ* analysis of *FGF-5* expression in X cells passage in MED2 without LIF.

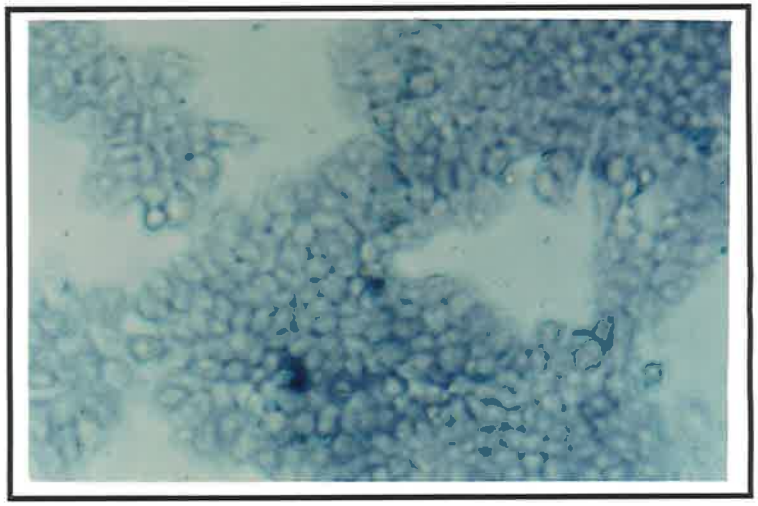
In situ analysis of *FGF-5* expression in Xp0- (A, B) and Xp2-(C, D) cultures (2.5.19) *FGF-5* expression was detected using a 800bp DIG labelled antisense riboprobe (2.5.17). Plates were developed for 24 hours before the colour reaction was stopped. Cells expressing *FGF-5* were detected by purple staining. Staining could not be detected in differentiated cells (d).

Photographs were taken under phase contrast (A, C) and bright field optics (B, D) at a magnification of 50x.

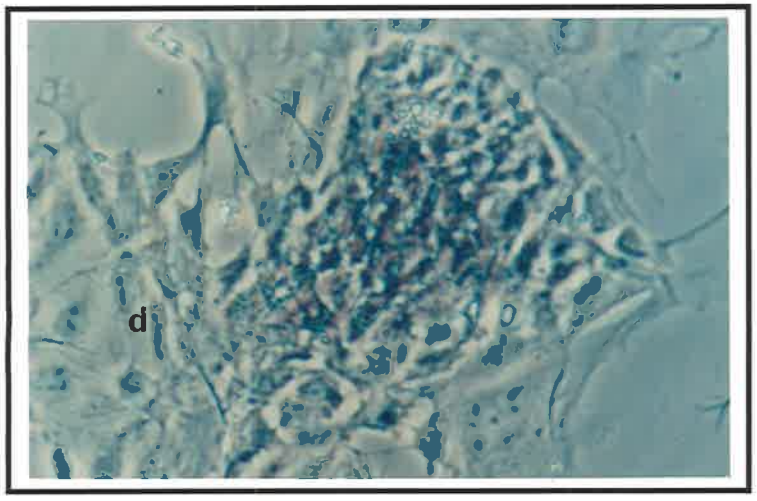
A



B



C



D

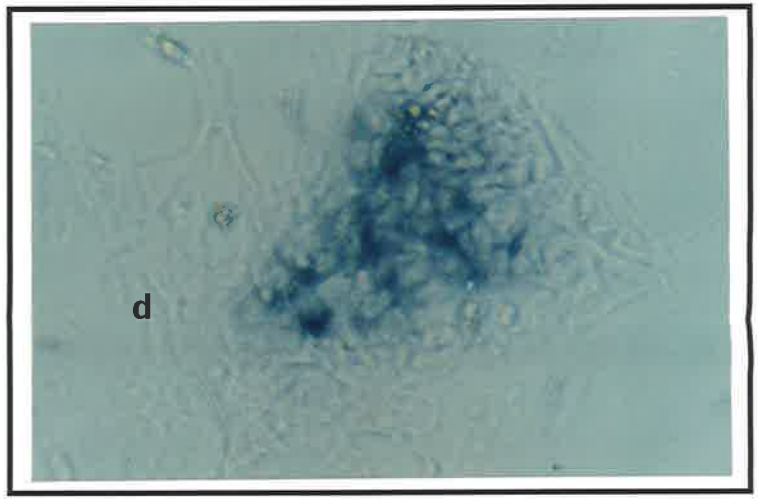
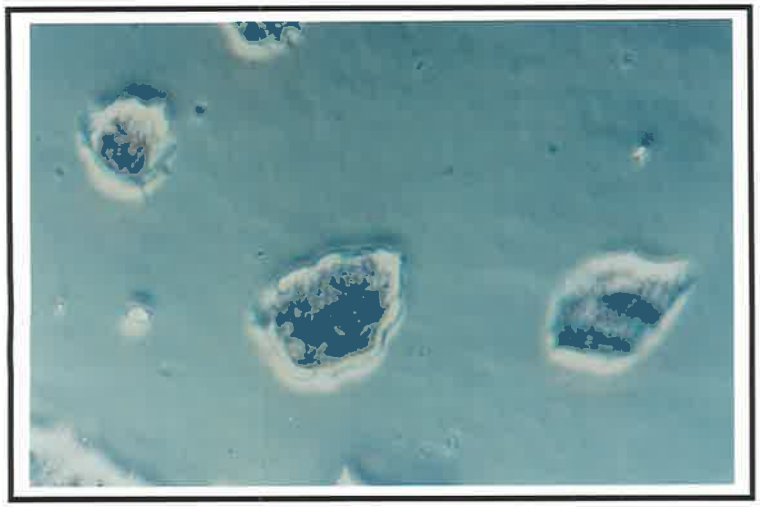


FIGURE 4.5 *In situ* analysis of *Rex-1* expression in ES and Xp0- cells.

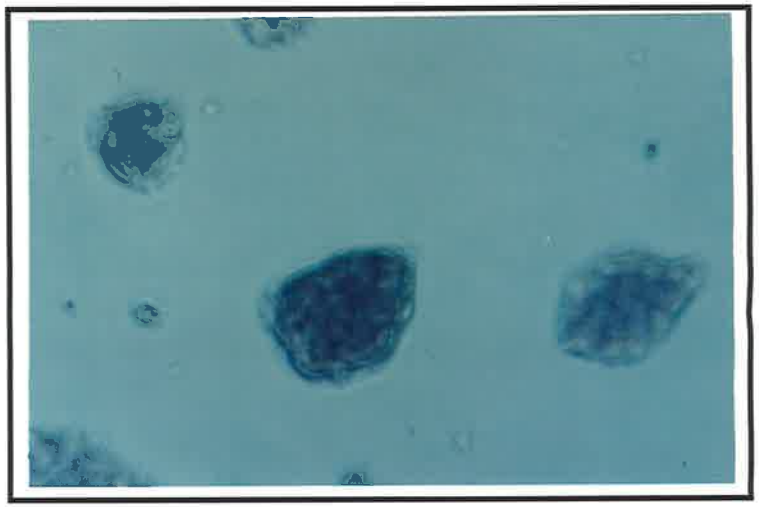
In situ analysis of *Rex-1* expression in ES (A, B) and Xp0- (C, D) cultures (2.5.19) *Rex-1* expression was detected using a 848bp DIG labelled antisense riboprobe (2.5.17). Plates were developed for 10 hours before the colour reaction was stopped. Cells expressing *Rex-1* were detected by purple staining. Staining was not detected in differentiated cells (d).

Photographs were taken under phase contrast (A, C) and bright field optics (B, D) at a magnification of 50x.

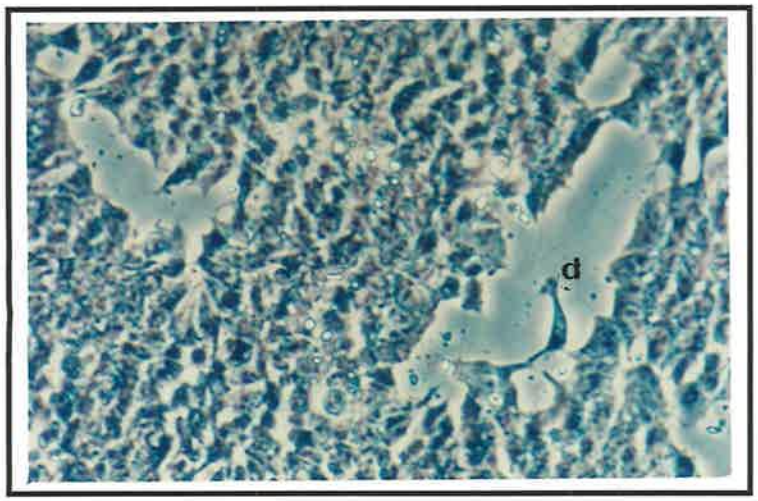
A



B



C



D

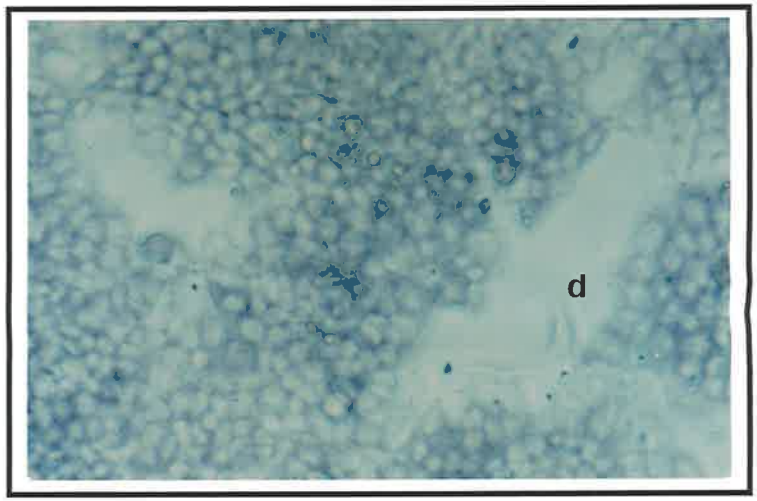
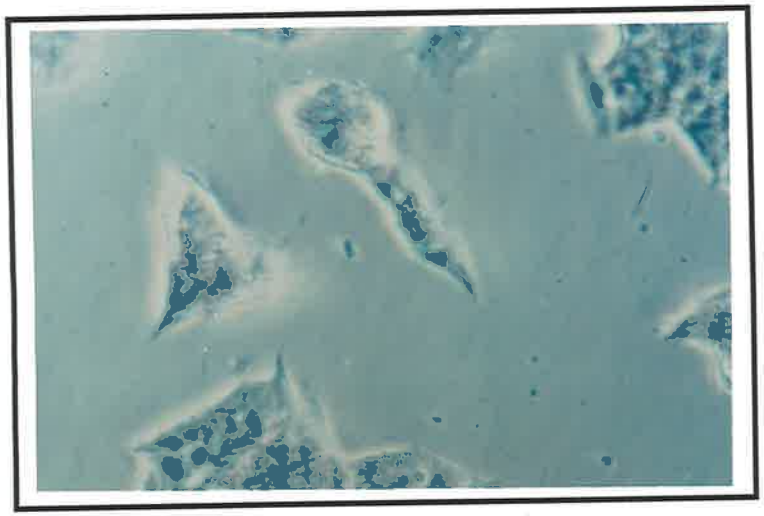


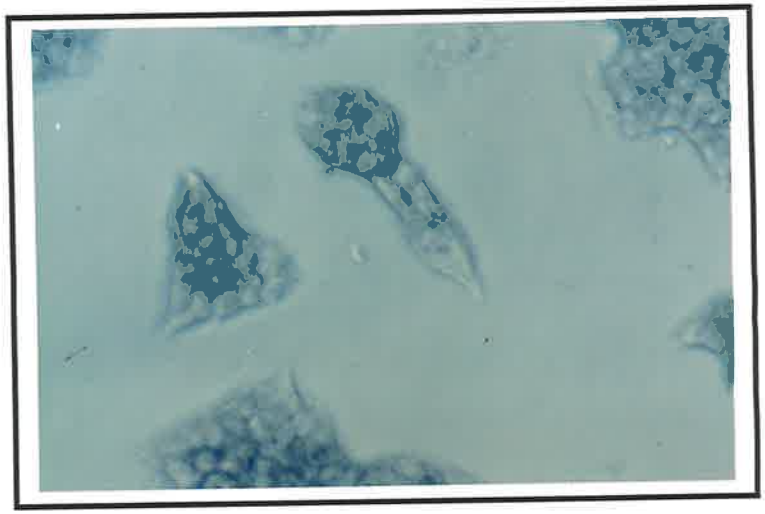
FIGURE 4.6 *In situ* analysis of *Gbx-2* expression in ES and Xp0- cells. *In situ* analysis of *Gbx-2* expression in ES (A, B) and Xp0- (C, D) cultures (2.5.19). *Gbx-2* expression was detected using a 440bp DIG labelled antisense riboprobe (2.5.17). Plates were developed for 24 hours before the colour reaction was stopped. Cells expressing *Gbx-2* were detected by purple staining. Staining was not detected in differentiated cells (d).

Photographs were taken under phase contrast (A, C) and bright field optics (B, D) at a magnification of 50x.

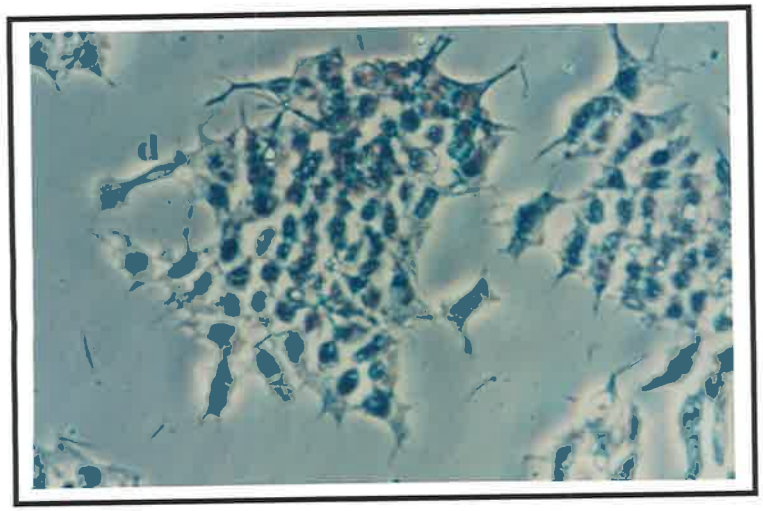
A



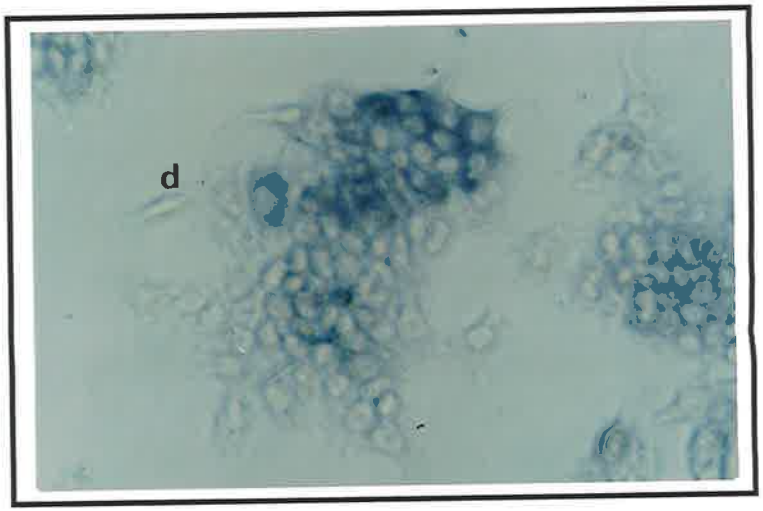
B



C



D



The results of these experiments demonstrated that *FGF-5*, *Gbx-2* and *Rex-1* expression in RNA derived from X cell cultures was confined to X cells rather then differentiated cells. Therefore alterations in gene expression during X cell culture are attributable to X cells within the population rather than alterations in the level of differentiated cells. This examination verifies the gene expression associated with X cells which suggests that X cells represent the *in vitro* equivalent of early primitive ectoderm.

4.3 Comparison of X Cell Cultures and Spontaneously Differentiated ES Cell Cultures

When ES cells are cultured in the absence of LIF they differentiate spontaneously into multiple differentiated cell types over a 6 day period (Smith *et al.* 1988, Williams *et al.* 1988). Conover *et al.* 1993 has shown that depriving ES cells of LIF results in the induction of *FGF-5* expression within 18 to 48 hours, well before obvious morphological differentiation occurs. This observation was thought to represent the differentiation of ES cells into cells of the primitive ectoderm prior to overt differentiation. *Rex-1* expression also decreases as ES cells are differentiated by culture in the absence of LIF (Rogers *et al.* 1991). Thus spontaneous differentiation of ES cells appears to proceed *via* an intermediate cell population with a gene expression similar to that exhibited by X cells (4.2.1). The transient gene expression observed in spontaneously differentiated ES cell cultures may reflect a transient appearance of X cells within these cultures. This would suggest that X cells represent a natural differentiation intermediate of ES cells and further support their proposed relationship with the primitive ectoderm.

4.3.1 The Morphology of Spontaneously Differentiated ES Cell Cultures

ES cells were cultured and passaged at cell densities routinely used for X cell formation and maintenance in incomplete ES medium without added LIF (2.3.15). When ES cells were seeded into incomplete medium at high cell density, after two days the culture (SPp0) consisted of colonies with morphologies intermediate between ES cell colonies and X cell colonies (Figure 4.7A). The morphology of the cells within this culture was clearly different from the morphology of X cells in Xp0+ and Xp0- cultures (Figure 3.9). When SPp0 cultures were re-passaged at high cell density (SPp1) after two

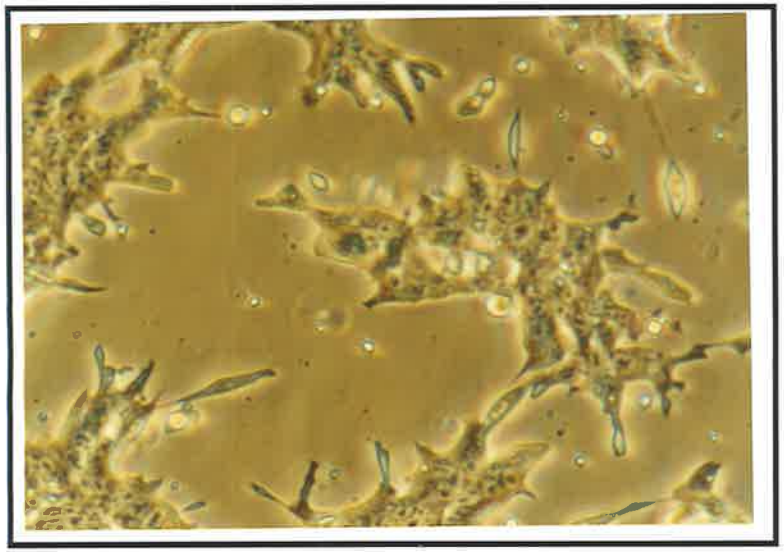
FIGURE 4.7 The culture morphology of spontaneously differentiated ES cells.

Spontaneously differentiated ES cells were formed and passaged as described in 2.3.15. Briefly, ES cells were cultured at high cell density in the absence of LIF for 2 days (SPp0). These cells were passaged at high cell density in the absence of LIF for a further 2 days (SPp1). Subsequently, upon further passage the plating efficiency decreased substantially cultures were therefore grown for 7 days (SPp2). Photographs representative of cells present in SPp0 (A), SPp1(B), SPp2 (C, D) cultures are shown.

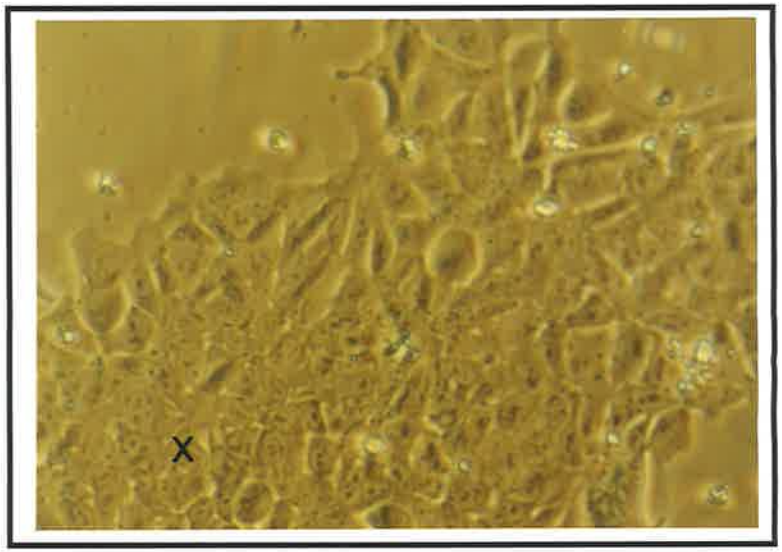
ES = nest of stem cells, PE = cells with the morphology of parietal endoderm (Strickland *et al.* 1980), F = cells with the morphology of fibroblasts and X = cells with the morphology of X cells.

Photographs were taken using phase contrast optics at a magnification of 50x.

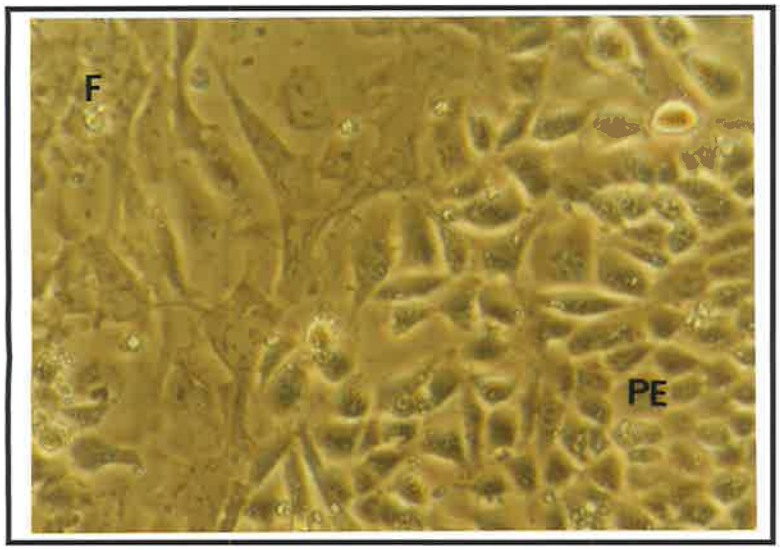
A



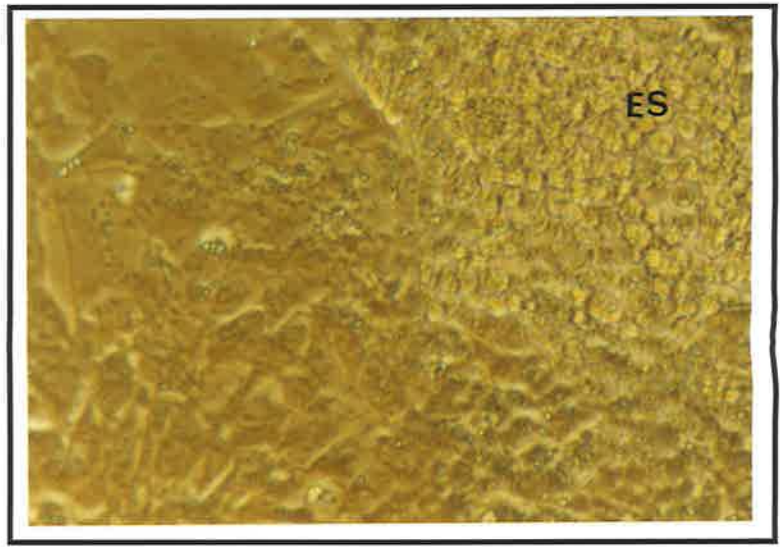
B



C



D



days, a decrease in plating efficiency was evident by the sparse distribution of relatively differentiated colonies within cultures. Cells with a morphology similar to X cells were observed within a relatively high proportion of colonies in SPp1 cultures, however the colonies were highly differentiated (Fig. 4.7B). When SPp1 cells were passaged further (SPp2) and observed after two days the plating efficiency had decreased substantially such that very few colonies could be detected. SPp2 cultures were therefore cultured for a total of seven days before sufficient cells were present for RNA preparation. At this time cultures were characterised by the sparse distribution of relatively large colonies which contained high levels of terminally differentiated cells, including fibroblasts and cells with the morphology of parietal endoderm (Figure 4.7C). Nests of stem cells with an ES cell morphology were also observed (Figure 4.7D). These cells are known to be pluripotent and are thought to be maintained by LIF which is expressed by terminally differentiated cells (Rathjen *et al.* 1990). The extremely differentiated state and sparse distribution of colonies within SPp2 cultures at this time prevented further passage.

Culture of ES cells in MED2 induced the rapid and uniform formation of X cells. After two days X cells existed as a relatively confluent homogeneous cell population, ES cell colonies were not observed and the level of differentiated cells was low. Upon further passage, X cells proliferated rapidly and could be indefinitely maintained by passage in the presence of MED2 as a relatively homogeneous pluripotential cell population (3.6). This contrasts with the behaviour of ES cells when differentiated by culture in the absence of LIF. Such differentiation generated a relatively heterogeneous and differentiated cell population which could not be maintained indefinitely. While cells with a morphology similar to X cells could be observed within these cultures, their appearance was transient. These cells were not formed immediately and were present at a relatively low, non-uniform level. Furthermore, these cells could not be maintained in culture beyond two passages due to extensive differentiation.

ES cells are considered analogues of the inner cell mass, differentiation into embryonic cell types occurs *via* a primitive ectoderm intermediate *in vivo*. A similar progression has been proposed to occur during the spontaneous differentiation of ES cells *in vitro* (Conover *et al.* 1993). The transient appearance of cells with the morphology of X cells during spontaneous differentiation of ES cells suggests that they constitute a

natural cell intermediate in this process. This observation, coupled with X cell gene expression therefore supports their proposed relationship to the primitive ectoderm.

4.3.2 The Gene Expression of Spontaneously Differentiated ES Cell Cultures

RNA was harvested from SPp0, SPp1 and SPp2 cultures and the expression of *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* were determined by Northern analysis of total RNA (Figure 4.8). Gene expression levels were quantitated against a *mGAP* loading control expressed as a percentage of ES cell expression. The results of this analysis are presented in Figure 4.9.

ES cells cultured in the absence of LIF at high cell density exhibited increased levels of *FGF-5* expression and decreased levels of *Rex-1* expression. This is consistent with the expression of these genes during spontaneous differentiation reported by others (Conover *et al.* 1993, Rogers *et al.* 1991). In SPp1 the level of *Oct-4* expression was 64%, the level of *FGF-5* expression 210% and *Rex-1* expression 4%. *FGF-5* and *Rex-1* expression are confined to stem cell populations (4.2.2). Therefore, pluripotential cells within the SPp1 population had a gene expression characterised by high *FGF-5* and low *Rex-1*. This gene expression is consistent with the presence of morphologically recognisable X cells in this population (4.3.1). Passage of SPp1 cultures resulted in a substantial decrease in plating efficiency as assessed by the relatively sparse distribution of colonies after two days. RNA could not be harvested from these cultures at this time due to insufficient cell numbers therefore the gene expression alterations during this time could not be followed. RNA was harvested from cultures after seven days (SPp2). In these cultures the level of *Oct-4* expression was 29%, *FGF-5* 46% and *Rex-1* 20%. The expression of *Oct-4* in these cultures is indicative of the presence of pluripotential cells within the culture. The pluripotential cell population in this culture was characterised by a lower *FGF-5* expression and a higher *Rex-1* expression than those in SPp1 cultures. Thus gene expression was reminiscent of ES cells. Nests of cells with an ES cell morphology were observed within these cultures at this time (4.3.1). Therefore the gene expression associated with SPp2 cultures is consistent with the observation of ES cells within these cultures.

FIGURE 4.8 Northern blot analysis of *FGF-5*, *Rex-1* and *Oct-4* expression in spontaneously differentiated ES cells.

RNA isolated from ES, SPp0, SPp1 and SPp2 cells was analysed *via* Northern blot. *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* expression was determined as previously described (Fig. 4.1). The exposure time for *FGF-5* was 24 hours, *Rex-1* 22 hours, *Oct-4* 20 hours and *mGAP* 20 hours.

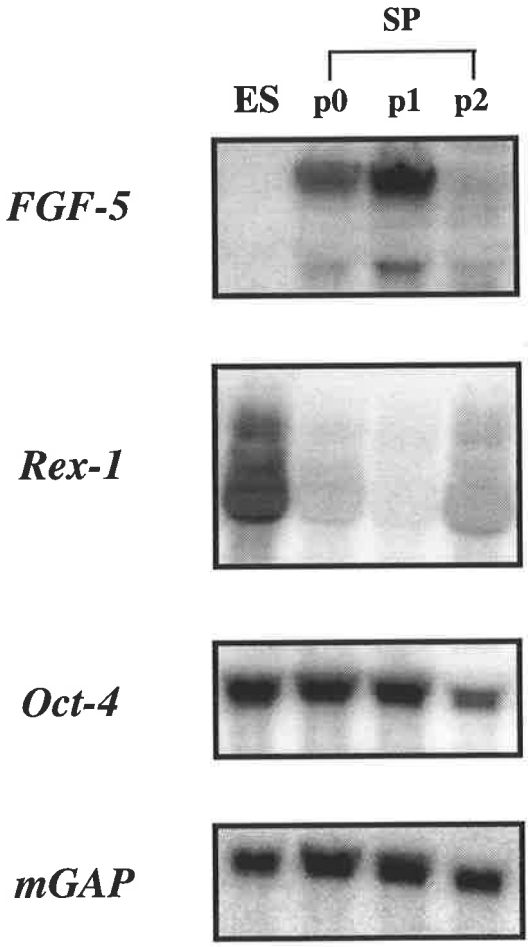
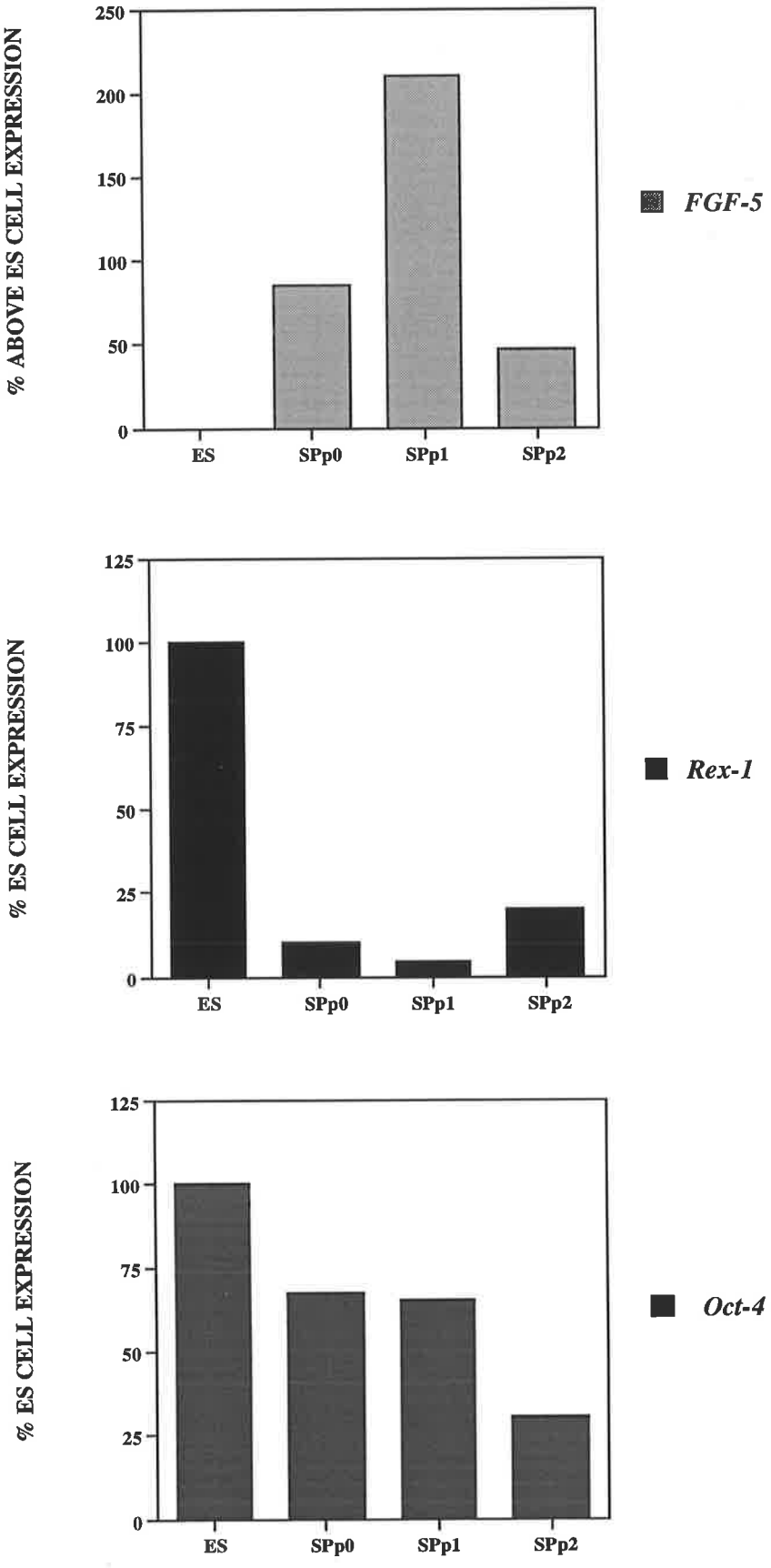

SP
ES
p0
p1
p2
FGF-5
Rex-1
Oct-4
mGAP

FIGURE 4.9 Comparison of *FGF-5*, *Rex-1* and *Oct-4* expression in spontaneously differentiated ES cells.

The level of *FGF-5*, *Rex-1* and *Oct-4* in ES cells and spontaneously differentiated cultures (Fig. 4.8) compared to *mGAP* was quantitated by volume integration (2.5.20) and is represented as previously described (Fig. 4.2).


% ABOVE ES CELL EXPRESSION
250
200
150
100
50
0
ES
SPp0
SPp1
SPp2
FGF-5
% ES CELL EXPRESSION
125
100
75
50
25
0
ES
SPp0
SPp1
SPp2
Rex-1
% ES CELL EXPRESSION
125
100
75
50
25
0
ES
SPp0
SPp1
SPp2
Oct-4

The observation that the appearance of X cells during spontaneous ES cell differentiation is transient, and can be followed by morphology and alteration in gene expression to high *FGF-5*, low *Rex-1*, suggests that X cells represent a natural intermediate in ES cell differentiation. This further supports the proposal that this stem cell population is likely to be the *in vitro* equivalent of early primitive ectoderm.

4.4 Discussion

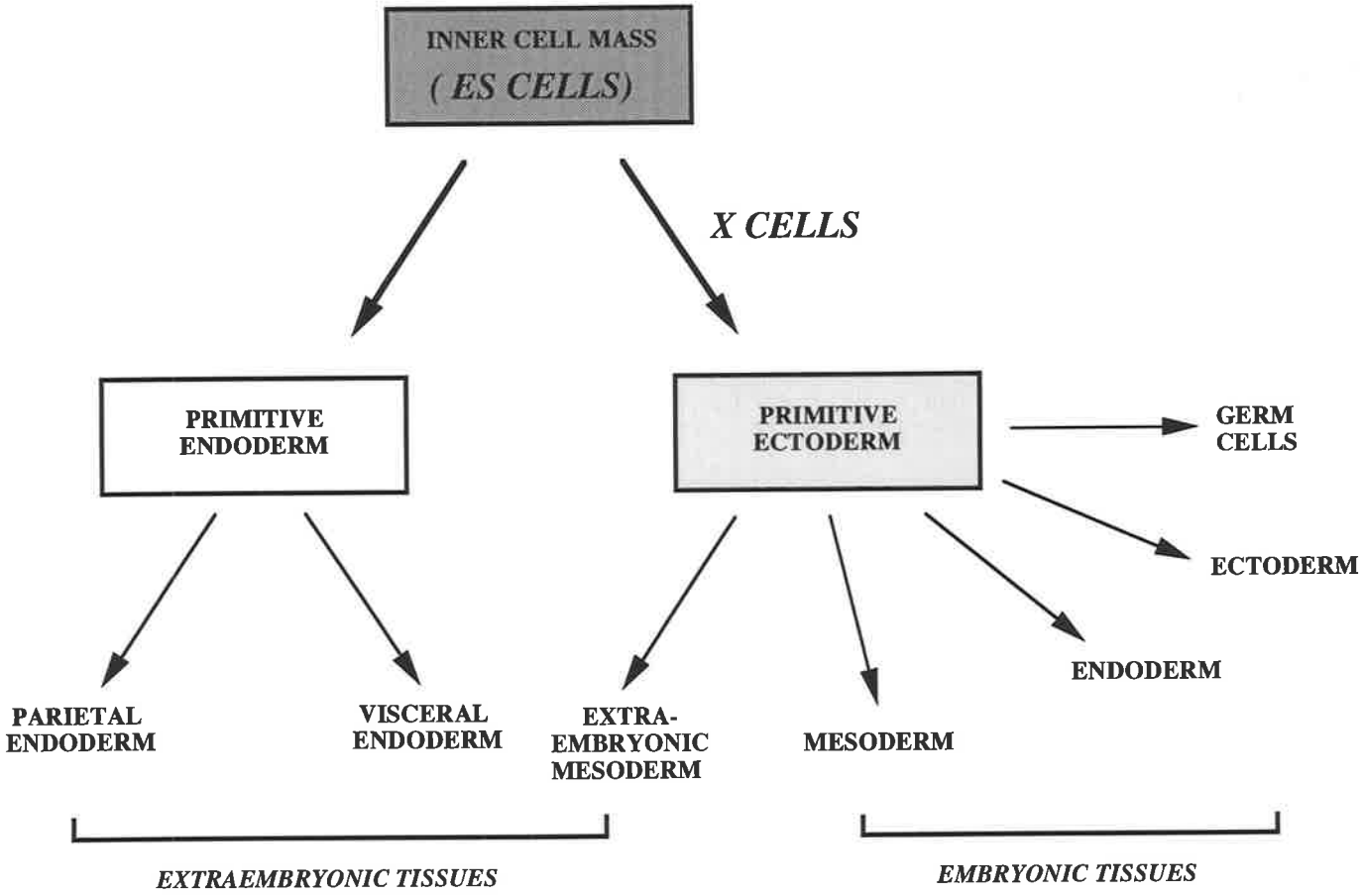
Work carried out in collaboration with M. Bettess, (Bettess 1993) investigating the expression of marker genes in X cells restricted their biological equivalent to the inner cell mass, primitive ectoderm or an unrecognised pluripotential cell population. To resolve these possibilities the expression of pluripotential marker genes which discriminate between the inner cell mass and primitive ectoderm were analysed in X cells and ES cells. The expression of such markers suggested that X cells represent the *in vitro* equivalent of early primitive ectoderm. X cells expressed increasing levels of *FGF-5* during culture in MED2 and decreasing levels of *Rex-1* and *Gbx-2*. *FGF-5* is expressed in the cells of the primitive ectoderm at day 5.25. *Rex-1* and *Gbx-2* are expressed in the ICM and are down regulated upon primitive ectoderm formation *in vivo*. These genes are not expressed in the primitive ectoderm at day 6.0. This gene expression therefore suggested that X cells were equivalent to primitive ectoderm prior to day 6.0. Based on their gene expression profile the proposed position of X cells in embryonic development is schematically represented in Figure 4.10.

A more precise identification of X cells is limited by our knowledge of pluripotential cell marker gene expression *in vivo*. The exact developmental stage at which *FGF-5* expression commences and *Rex-1* and *Gbx-2* expression are down regulated *in vivo* are unknown. Definitive identification will ultimately require comprehensive analysis of the expression of these genes in the inner cell mass at day 4.0 through to the late primitive ectoderm at day 6.5 by whole mount *in situ* hybridisation.

Gene expression alterations during the transition of the inner cell mass to primitive ectoderm have not been well characterised, due largely to the difficulties associated with the context, size and fragility of early murine embryos. Differential display PCR has been used successfully for the isolation of genes specifically expressed in ES cells and X cells

FIGURE 4.10 The embryonic equivalent of X cells based on gene expression analysis.

The proposed position of X cells in embryonic development is schematically represented. Based on marker gene expression X cells can not be considered equivalent to early mesodermal, primitive endodermal derivatives or germ cells. The expression of pluripotential cell marker genes, which discriminate between the inner cell mass and primitive ectoderm, suggests that X cells represent the *in vitro* equivalent of a pluripotential cell population downstream of the inner cell mass toward the primitive ectoderm, or the early primitive ectoderm.


INNER CELL MASS
(ES CELLS)
X CELLS
PRIMITIVE ENDODERM
PRIMITIVE ECTODERM
GERM CELLS
ECTODERM
ENDODERM
MESODERM
EXTRA-EMBRYONIC MESODERM
VISCERAL ENDODERM
PARIETAL ENDODERM
EXTRAEMBRYONIC TISSUES
EMBRYONIC TISSUES

(T. Shultz, S. Sharma, unpublished observations). These genes have not yet been fully characterised *in vivo* but may represent markers which can be used for definitive identification of the biological equivalent of X cells.

Shen and Leder (1992) cultured ES cells as embryoid bodies in the presence and absence of LIF. The addition of LIF at 1000U/ml had no effect on the ability of embryoid bodies to form primitive endoderm and its derivatives as assessed by the expression of *H19*, alpha fetoprotein, collagen and laminin. However, the addition of LIF to embryoid body culture inhibited the ability of these embryoid bodies to form late stage primitive ectoderm as assessed by the absence of *FGF-5* expression and the persistence of *Rex-1* expression. This result is consistent with results described here. Although the addition of LIF to X cell cultures did not completely inhibit *FGF-5* expression, increases in expression were lower and observed later when compared to X cells cultured in MED2 in the absence of added LIF.

Analysis of cells present within spontaneously differentiated ES cell cultures and the gene expression profiles associated with these cultures suggested that X cells represent a natural intermediate between ES and their differentiated derivatives. X cells were clearly discernible in spontaneously differentiated ES cell cultures at times when pluripotential gene expression profiles exhibited high *FGF-5* and very low *Rex-1* expression. The appearance of these X cells was transient and they could not be maintained in the absence of MED2.

The results of this chapter therefore suggest that X cells represent a natural pluripotential cell intermediate of ES cell differentiation and that this intermediate is the *in vitro* equivalent of the early primitive ectoderm.

4.4.1 The Isolation of XB Cells from Embryoid Bodies

During embryoid body formation ES cells differentiate in a sequential and relatively organised manner giving rise to cell types similar to those found in the early murine embryo (1.5.4). By day 6 of culture embryoid bodies have differentiated into organised cellular structures which consist of an outer layer of primitive endoderm and an inner layer of primitive ectoderm (Doetschman *et al.* 1985, Robertson 1987). During the course of this work a number of clonal X cell lines were isolated at high frequency from

day 6.0 embryoid bodies in the presence of MED2 and LIF. These cells were termed XB cells (J. Rathjen and J. Washington, unpublished observations). ES cells were not isolated from the same embryoid bodies in LIF alone. This suggested that XB cells did not arise from undifferentiated ES cells within the embryoid body. XB cells constitute clonal cell lines with a morphology identical to X cells. Gene expression analyses of XB cells indicated that these cells have a gene expression profile similar to X cells derived from ES cells (data not shown). The isolation of X cell lines which express *Oct-4*, high levels of *FGF-5* and low levels of *Rex-1* and *Gbx-2*, from embryoid bodies which contain primitive ectoderm, supports the proposal that X cells represent the *in vitro* equivalent of primitive ectoderm.

CHAPTER FIVE:

X CELL GENE EXPRESSION

DEFINES THREE DISTINCT PLURIPOTENTIAL CELL STATES

CHAPTER FIVE: X CELL GENE EXPRESSION DEFINES THREE DISTINCT PLURIPOTENTIAL CELL STATES

5.1 Introduction

When ES cells are cultured in MED2 they convert to X cells. X cells constitute a morphologically distinct pluripotential cell population. While uniform conversion of ES cells to X cells occurred within 24 hours, gene expression alterations occurred gradually as X cells were passaged, and were impeded in the presence of added LIF (4.2.1). As X cells were cultured in MED2 the gene expression profile altered from one which was characterised by low *FGF-5*, high *Rex-1* and high *Gbx-2* (Xp0), to one characterised by higher *FGF-5*, lower *Rex-1* and lower *Gbx-2* (Xp2). Therefore while X cells within different X cell cultures were morphologically identical they appeared to possess distinct gene expression profiles.

X cells have the capacity to revert at high frequency to ES cells when cultured in the presence of LIF, in the absence of MED2 (3.7). This has also been demonstrated for clonal X cell lines (J. Rathjen, unpublished observations) and therefore appears to be a general property of X cells. Thus X cells can exist in at least three distinct pluripotential cell states depending on the presence of MED2 and/or LIF.

The interconvertibility of stem cell populations is not unprecedented. If murine primordial germ cells are cultured in the presence of Steel factor, LIF and bFGF they convert to ES-like cells, termed EG cells (Matsui *et al.* 1992, Resnick *et al.* 1992). EG cells contribute extensively to murine tissues including the germ line following blastocyst injection (Stewart *et al.* 1994). The finding that ES cells could be derived from primordial germ cells led to the prediction that all pluripotential cell populations, including the primitive ectoderm, might be convertible to a common ES like-cell (Rossant *et al.* 1993). X cells appear to represent the *in vitro* equivalent of early primitive ectoderm and are able to revert to ES cells, a finding which is consistent with this prediction.

The early murine embryo exhibits considerable developmental lability. It is possible to destroy a large proportion of the cells present in the blastocyst or egg cylinder stage embryo and a normal embryo will still form (Snow and Tam 1979, Smith 1992). This developmental lability must involve reprogramming of pluripotential cells within the

murine embryo at the level of gene expression. The interconvertibility of pluripotential stem cell populations *in vitro* may explain the developmental flexibility associated with these cells *in vivo*.

Gene expression alterations as ES cells form X cells and X cells are passaged in MED2 are reminiscent of gene expression alterations which occur as the ICM forms the primitive ectoderm *in vivo*. X cells appear to exist in at least two states based on gene expression, and are able to revert to ES cells. Although a phenotypic reversion of X cells to ES cells has been shown (J. Rathjen, unpublished observations, 3.7), the gene expression associated with the reverted phenotype has not been assessed.

This chapter describes the gene expression associated with passaged X cells and their reverted derivatives and indicates that the phenotypic reversion to ES cells is reflected by a reversion in gene expression. Analysis of gene expression associated with X cells cultured for extended periods suggests that X cells can exist in two morphologically identical states which are distinguished by gene expression. A correlation between the state of X cells and capacity for reversion and differentiation is proposed.

5.2 Gene Expression in Reverted X Cells

ES cells were cultured in the presence of MED2 with and without LIF. X cells were harvested at Xp0, Xp1 and Xp2 for RNA preparation. At each passage X cells were seeded into medium containing LIF in the absence of MED2. Reversion of X cells to ES cells was observed within 36 hours. Reverted X cells were cultured in the presence of LIF for two passages and harvested for RNA isolation. The phenotypes of reverted X cells were comparable to the phenotype of an ES cell culture and are shown in Figure 5.1. The expression of *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* in X cells and their reverted (R) derivatives was determined by Northern analysis of total RNA and quantitated as previously described. The results of these analyses are presented in Figure 5.2 and Figure 5.3 respectively.

The gene expression profile of X cells passaged in MED2 with and without LIF was consistent with gene expression profiles shown in section 4.2.1. Reverted X cells exhibited low *FGF-5* expression, high *Rex-1* expression which was comparable to the

FIGURE 5.1 The morphology of reverted X cells.

X cells were reverted to an ES cell phenotype by culture in the presence of LIF and the absence of MED2 (2.3.10). Reverted X cell lines were cultured in LIF for two passages. The photograph shown was taken of reverted X cells derived from Xp2- cultures (Xp2-R) and is representative of all reverted X cell populations analysed.

The photograph was taken at a magnification of 50x using phase contrast microscopy.

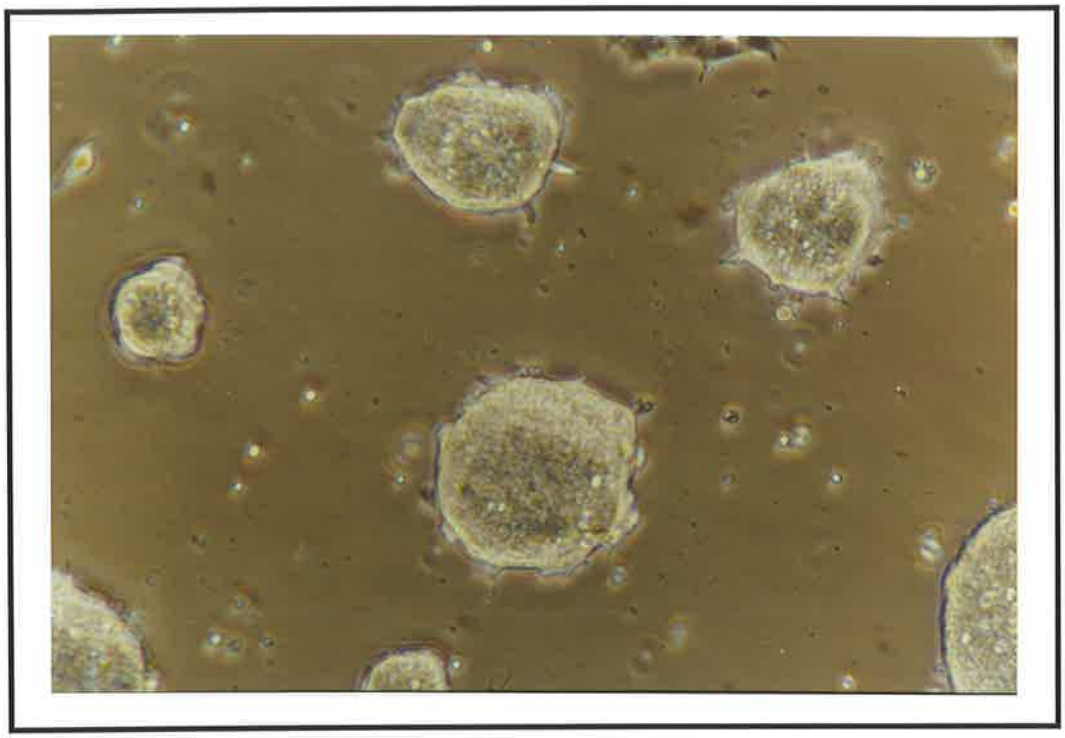


FIGURE 5.2 Northern blot analysis of *FGF-5*, *Rex-1* and *Oct-4* expression in reverted X cells.

X cells were passaged every two days in the presence of MED2 with and without added LIF (2.3.9). At each passage, X cells were reverted to an ES cell phenotype by culture in the presence of LIF, and absence of MED2 (2.3.10). RNA was isolated from cultures of ES cells and Xp0+/- cells to Xp2+/- cells and reverted derivatives of these cultures, denoted Xp0+/-R to Xp2+/-R (2.5.10). 30μg of total RNA was analysed *via* Northern blot (2.5.12). *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* expression was determined as previously described (Fig. 4.1). The exposure time for *FGF-5* was 24 hours, *Rex-1* 22 hours, *Oct-4* 20 hours and *mGAP* 20 hours.

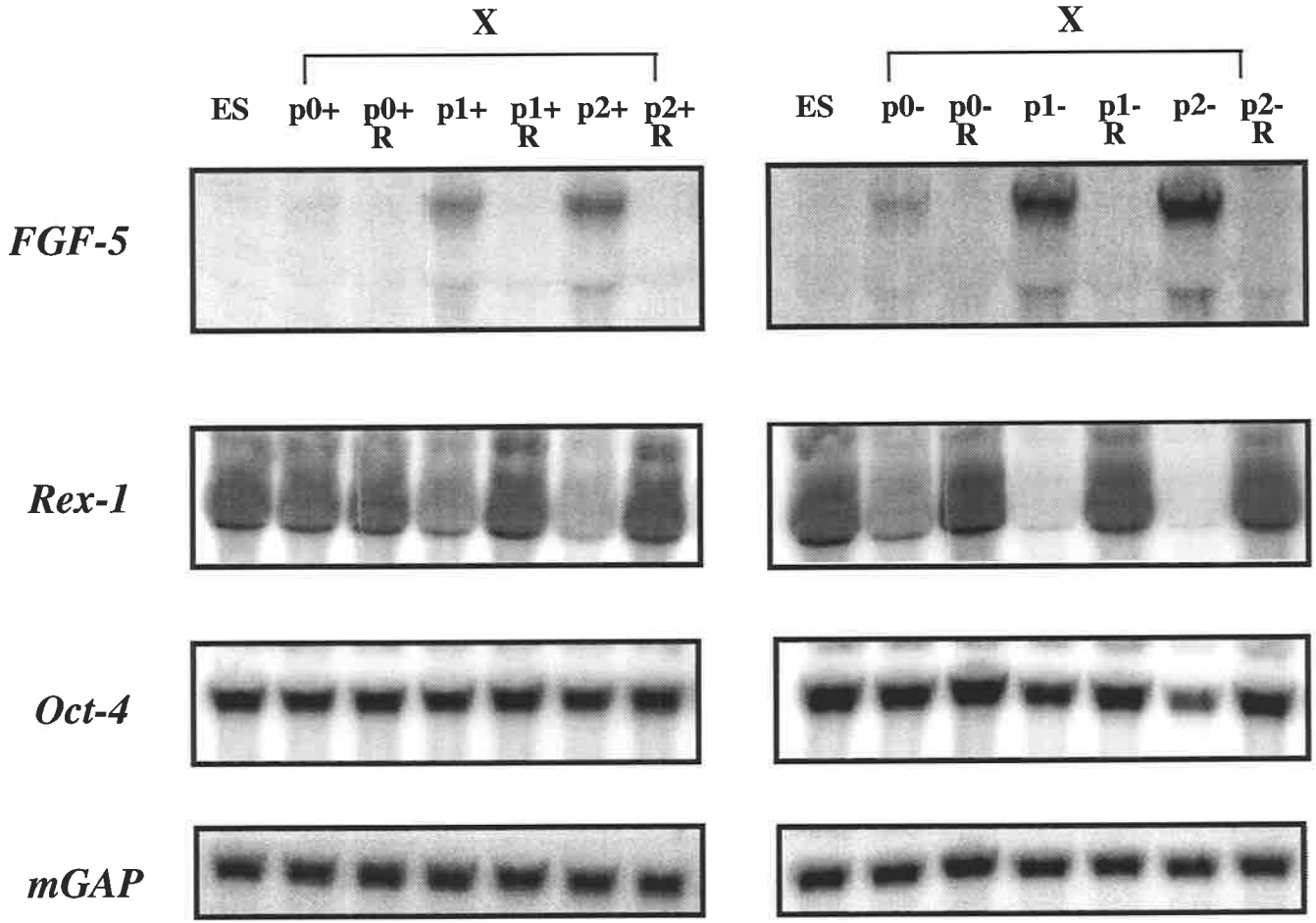
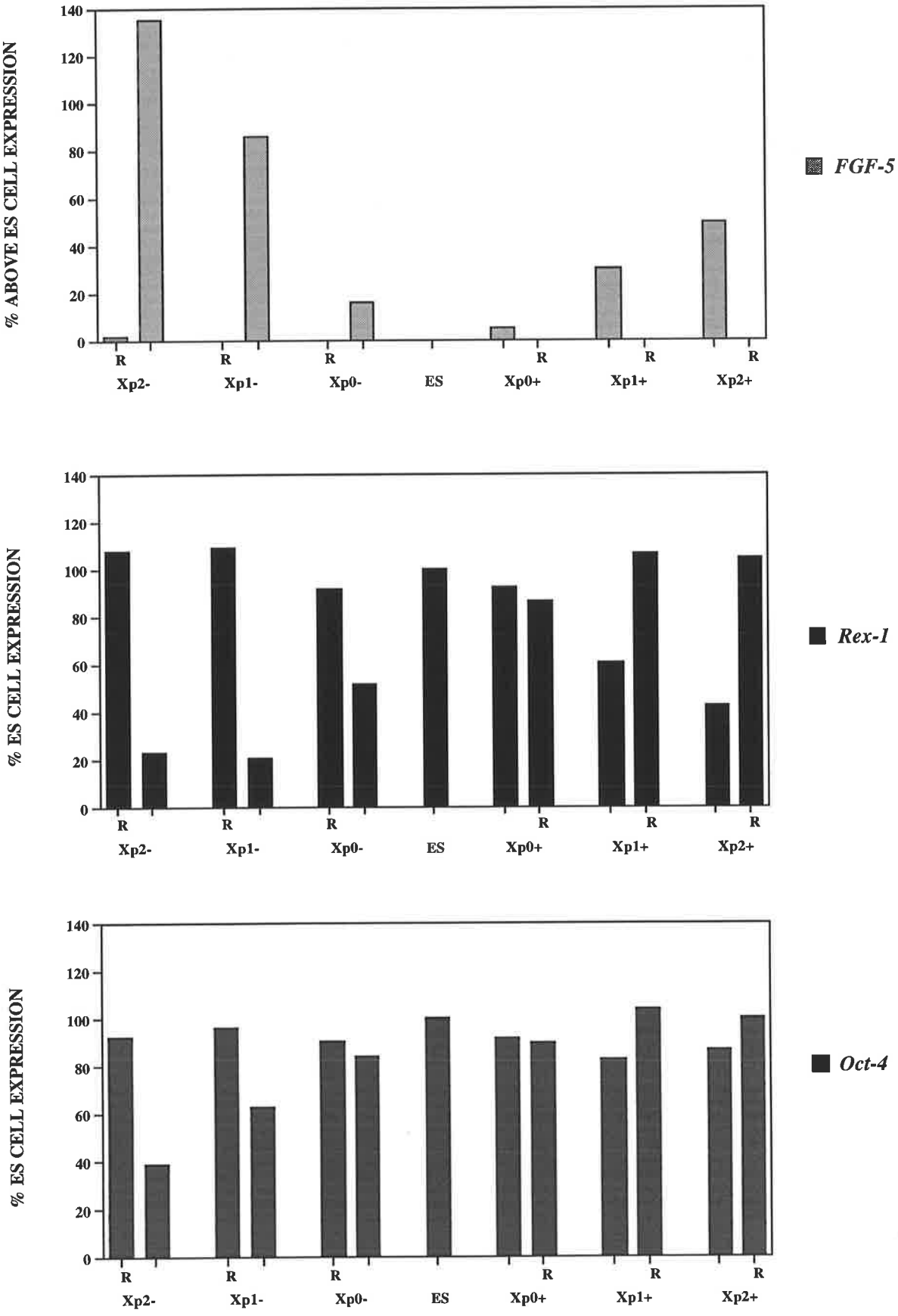

X
X
ES
p0+
p0+ R
p1+
p1+ R
p2+
p2+ R
ES
p0-
p0- R
p1-
p1- R
p2-
p2- R
FGF-5
Rex-1
Oct-4
mGAP

FIGURE 5.3 Comparison of *FGF-5*, *Rex-1* and *Oct-4* expression in reverted X cells.

The level of *FGF-5*, *Rex-1* and *Oct-4* expression in ES cells, X cells and reverted X cells (Fig. 5.2), compared to *mGAP* was quantitated by volume integration (2.5.20). The levels of expression are represented as previously described (Fig. 4.2).


% ABOVE ES CELL EXPRESSION
140
120
100
80
60
40
20
0
R
Xp2-
Xp1-
Xp0-
ES
Xp0+
Xp1+
Xp2+
FGF-5
% ES CELL EXPRESSION
Rex-1
% ES CELL EXPRESSION
Oct-4

gene expression observed in ES cells. *FGF-5* expression was detected at very low levels in reverted Xp2- cultures (Xp2-R). This may represent an inherent variability between ES cell cultures or may reflect incomplete conversion of X cells to ES cells. The gene expression of reverted X cells derived from Xp3+/-, Xp4+/-, Xp6+/- and Xp8+/- cells was also consistent with a conversion to ES cells (data not shown).

The expression of *Gbx-2* was examined by RNAase protection of RNA derived from ES, Xp3+, Xp3+R, Xp3- and Xp3-R. The levels of *Gbx-2* expression were quantified against a *mGAP* loading control by volume integration and expressed as a percentage of the ES cell expression level. The results of this analysis are presented in Figure 5.4. Reversion of both Xp3+ and Xp3- cells was accompanied by an increase in *Gbx-2* expression to levels comparable to ES cell expression levels.

XB cells (4.4.1) are clonal X cell lines which also possess the capacity to revert to ES cells when cultured in the presence of LIF, in the absence of MED2 (J. Washington, J. Rathjen, unpublished observations). The analysis of *FGF-5*, *Rex-1* and *Gbx-2* in reverted XB17 and XB15 cells indicated that reversion in XB phenotype was also accompanied by reversion in gene expression (data not shown).

The results presented in this section indicate that X cells and XB cells can be reverted to ES cells at any stage of culture, independent of the gene expression profile of the X cell population. The gene expression of every reverted X cell population was comparable to the gene expression determined for ES cell populations.

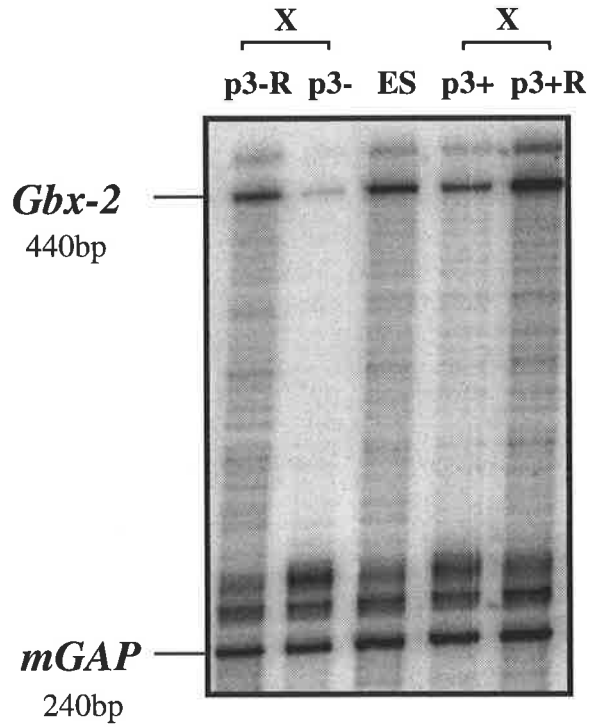
If reverted X cells behave as ES cells they should retain the capacity to re-differentiate into X cells when cultured in the presence of MED2. The ability of reverted X cells to differentiate into X cells was investigated. XB17 cells are routinely cultured in MED2 with added LIF. At passage 23, XB17 cells were reverted to ES cells by culture in the presence of LIF and absence of MED2. Reverted XB17 cells were passaged as ES cells for seven passages (XB17Rp7) and then cultured in the presence of MED2 with and without LIF. When XB17R cells were cultured in MED2 they converted to X cells indistinguishable from those shown in Figure 3.9. X cells were harvested at stages equivalent to Xp0- and Xp1- and RNA was isolated. The expression of *FGF-5*, *Rex-1* and *Oct-4* was determined by Northern analysis of total RNA. Results of this analysis are presented in Figure 5.5.

FIGURE 5.4 RNAase protection analysis of *Gbx-2* expression in reverted X cells.

A) RNA was isolated from ES, Xp3+/- and Xp3+/-R cultures (2.5.10). RNAase protection analysis was carried out on 20μg of total RNA, using a *Gbx-2* antisense riboprobe and a *mGAP* loading control (2.5.13). RNAase protection products were separated on a 6% polyacrylamide gel (2.5.2) and exposed in a phosphorimager cassette (2.5.20) for 12 hours.

B) The level of *Gbx-2* expression in A compared to *mGAP* was quantitated by volume integration (2.5.20) and is represented as previously described (Fig. 4.3B).

A


X
X
p3-R p3- ES p3+ p3+R
Gbx-2
440bp
mGAP
240bp

B

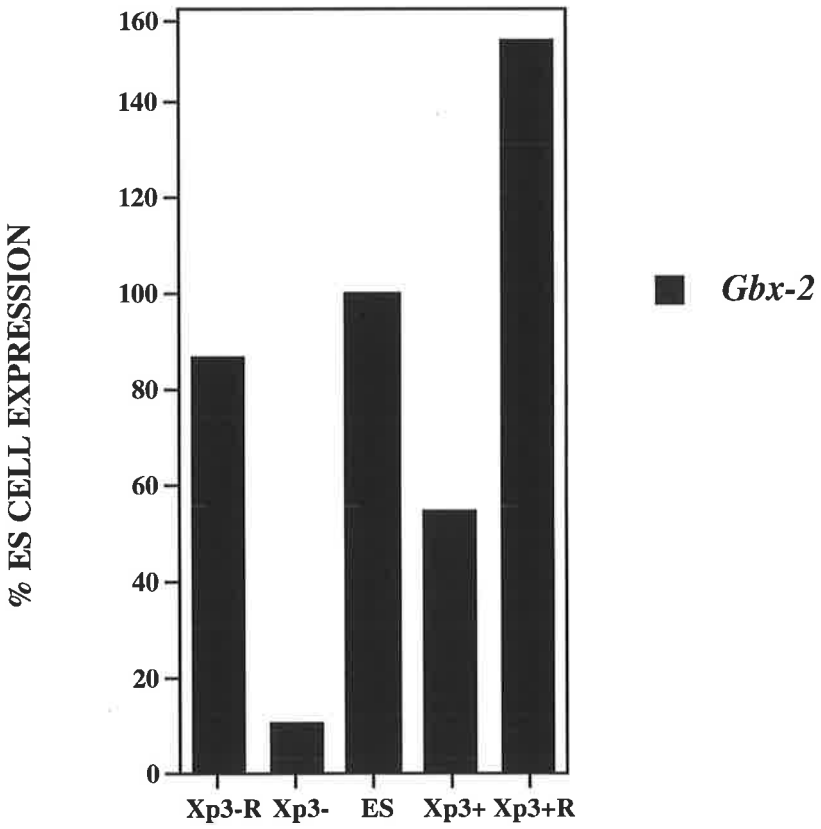
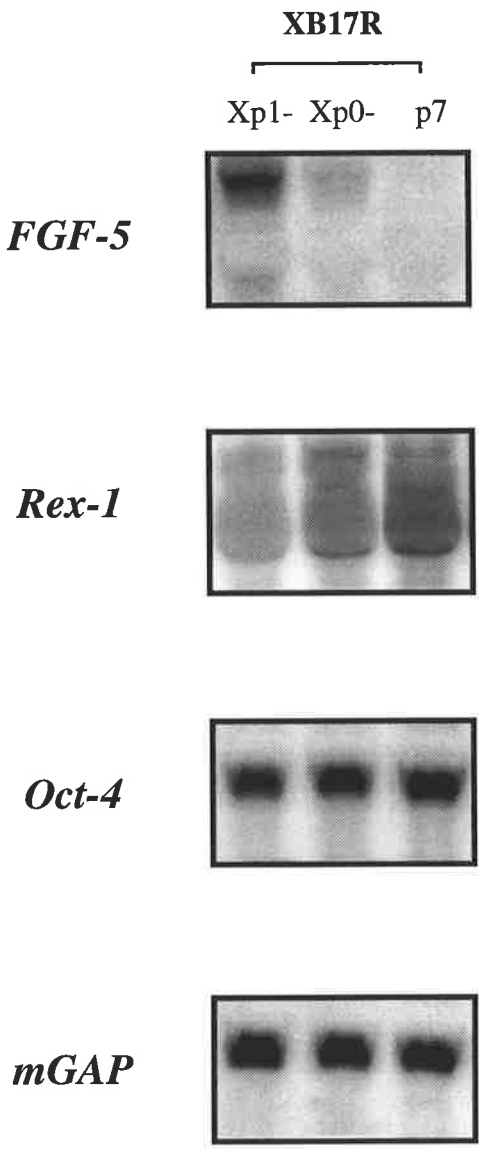

% ES CELL EXPRESSION
160
140
120
100
80
60
40
20
0
Xp3-R Xp3- ES Xp3+ Xp3+R
Gbx-2

FIGURE 5.5 Northern blot analysis of *FGF-5*, *Rex-1* and *Oct-4* expression in X cells derived from XB17 reverted cells.

Reverted XB17 cells passage seven, (XB17Rp7) were cultured in the presence of MED2 with and without added LIF as described for ES cells (2.3.9). RNA was isolated from XB17Rp7, XB17RXp0- and XB17RXp1- cultures (2.5.10). 30μg of total RNA was analysed *via* Northern blot (2.5.12). *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* expression were determined as previously described (Fig.4.1). The exposure time for *FGF-5* was 40 hours, *Rex-1* 22 hours, *Oct-4* 20 hours and *mGAP* 20 hours.


XB17R
Xp1-
Xp0-
p7
FGF-5
Rex-1
Oct-4
mGAP

The gene expression changes were similar to those seen in passaged X cells derived directly from ES cells. Thus it can be concluded that reverted XB cells regain the capacity to differentiate into X cells in the presence of MED2. Therefore phenotypic reversion of X cells to ES cells is accompanied by a reversion in both gene expression and differentiation capability.

5.3 The Cyclic Nature of X Cell Gene Expression During Long Term Culture

It appears that X cells can exist in two distinct, morphologically identical states based on the expression of *FGF-5*, *Rex-1* and *Gbx-2* (5.1). Therefore the gene expression associated with X cells during long term cultured could not be assumed. The expression of these genes in X cells passaged for extended periods was therefore examined.

RNA was harvested from ES cells, Xp0+ to Xp8+ and Xp0- to Xp8- cells and the expression of *FGF-5*, *Rex-1* and *Oct-4* was ascertained and compared to a *mGAP* loading control. Results of this analysis are shown in Figure 5.6. The expression of *FGF-5*, *Rex-1* and *Oct-4* was quantitated as previously described and the expression of these genes is represented relative to ES cell expression in Figure 5.7.

As reported previously (4.2.1) *FGF-5* expression increased as X cells were cultured in MED2, and *Rex-1* expression decreased steadily relative to *Oct-4* expression until day 6 or Xp2. The increase in *FGF-5* expression and decrease in *Rex-1* expression was more pronounced in X cells passaged in MED2 in the absence of added LIF than in X cells passaged in the presence of LIF.

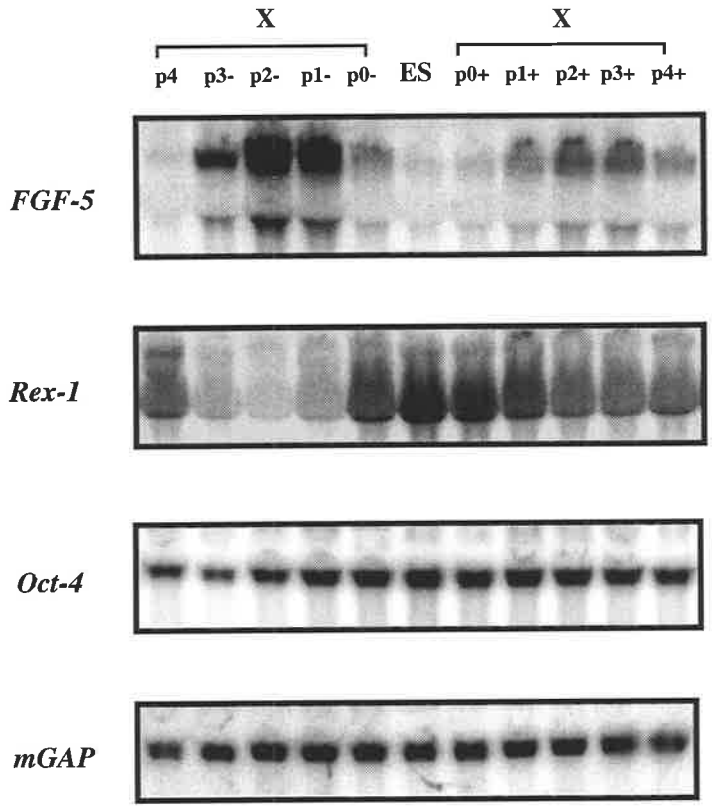
From Xp3- to Xp4- *FGF-5* levels decreased and *Rex-1* levels increased such that the gene expression levels were approaching levels seen in ES cells. From Xp5- to Xp6- *FGF-5* expression increased again while *Rex-1* expression decreased in a manner reminiscent of X cell formation from ES cells. The levels of *FGF-5* and *Rex-1* expression were then maintained to passage 8, although *Rex-1* had again increased slightly. Thus gene expression profiles of X cells beyond passage 2 altered cyclically from high *FGF-5*, low *Rex-1* expression to low *FGF-5*, high *Rex-1* expression. Similar cyclic gene expression profiles were seen in X cells cultured in the presence of LIF

FIGURE 5.6 Northern blot analysis of *FGF-5*, *Rex-1* and *Oct-4* expression in X cells passaged in the presence and absence of added LIF.

X cells were passaged every two days in the presence of MED2 with and without added LIF (2.3.9). RNA was isolated from cultures of ES cells and Xp0+/- through to Xp8+/- (2.5.10) and 30µg of total RNA was analysed *via* Northern blot (2.5.12). *FGF-5*, *Rex-1*, *Oct-4* and *mGAP* expression was determined as previously described (Fig.4.1). The exposure time for *FGF-5* was 24 hours, *Rex-1* 4 hours, *Oct-4* 19 hours and *mGAP* 20 hours.

(A) depicts expression in Xp0+/- to Xp4+/- cultures compared to ES cells. Expression in Xp5+/- to Xp8+/- cultures compared with ES cells is shown in (B).

A


X
X
p4
p3-
p2-
p1-
p0-
ES
p0+
p1+
p2+
p3+
p4+
FGF-5
Rex-1
Oct-4
mGAP

B

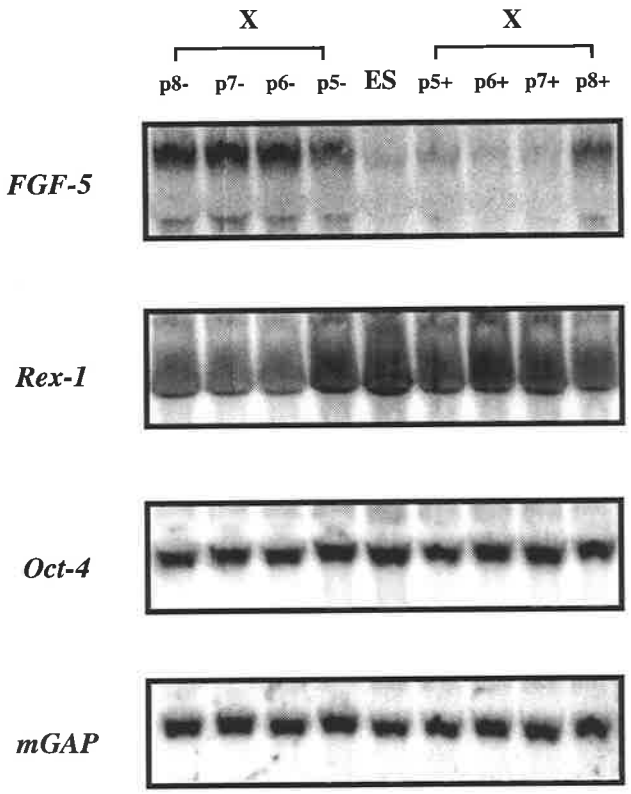
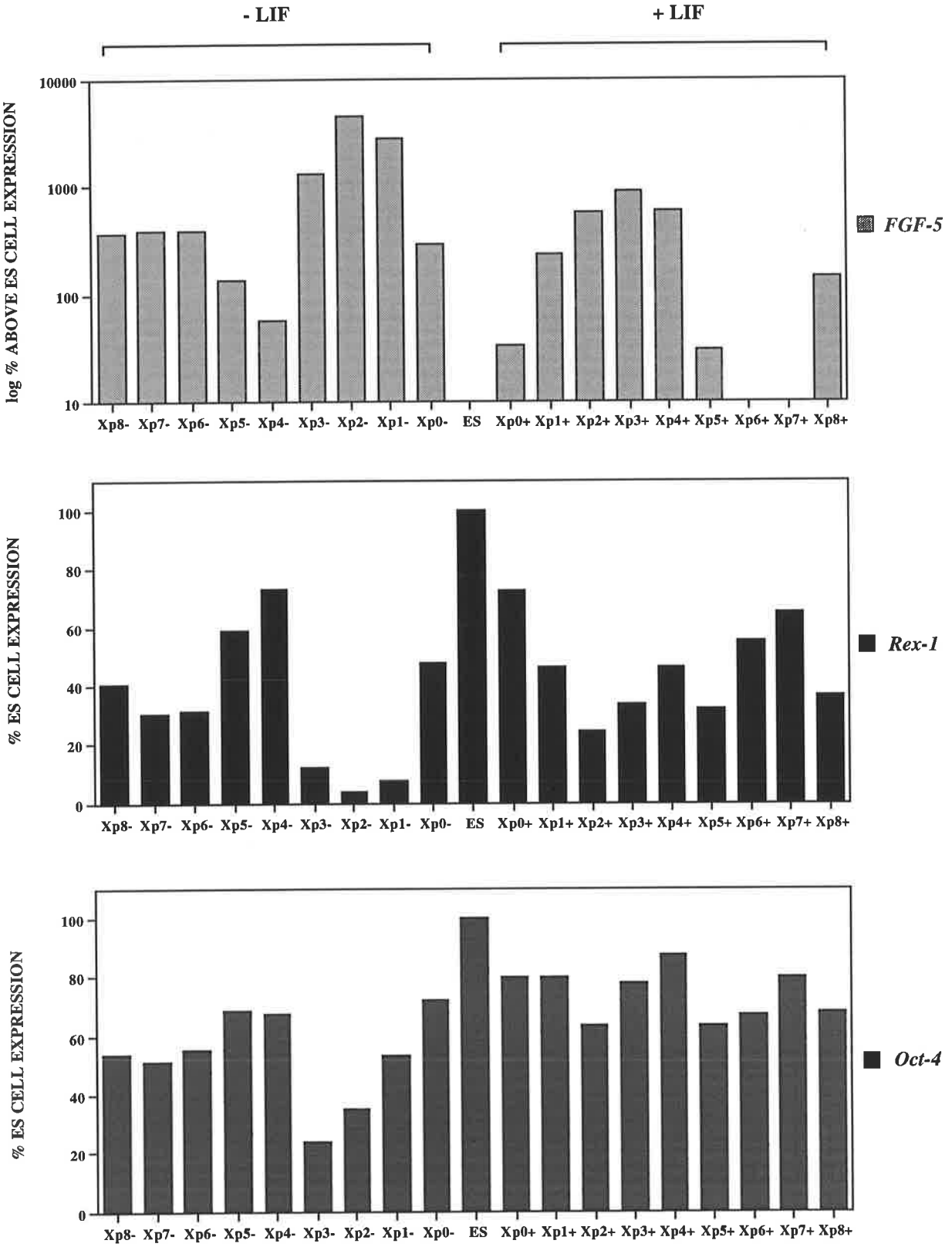

X
X
p8-
p7-
p6-
p5-
ES
p5+
p6+
p7+
p8+
FGF-5
Rex-1
Oct-4
mGAP

FIGURE 5.7 Comparison of *FGF-5*, *Rex-1* and *Oct-4* expression in X cells passaged in MED2 with and without added LIF.

The level of *FGF-5*, *Rex-1* and *Oct-4* expression compared to *mGAP* in ES cells and passaged X cells cultured in the presence of MED2 with and without added LIF (Fig. 5.6) was quantitated by volume integration (2.5.20). The levels of expression are represented as previously described (Fig. 4.2).


- LIF
+ LIF
log % ABOVE ES CELL EXPRESSION
10000
1000
100
10
FGF-5
Xp8- Xp7- Xp6- Xp5- Xp4- Xp3- Xp2- Xp1- Xp0- ES Xp0+ Xp1+ Xp2+ Xp3+ Xp4+ Xp5+ Xp6+ Xp7+ Xp8+
% ES CELL EXPRESSION
100
80
60
40
20
0
Rex-1
Xp8- Xp7- Xp6- Xp5- Xp4- Xp3- Xp2- Xp1- Xp0- ES Xp0+ Xp1+ Xp2+ Xp3+ Xp4+ Xp5+ Xp6+ Xp7+ Xp8+
% ES CELL EXPRESSION
100
80
60
40
20
0
Oct-4
Xp8- Xp7- Xp6- Xp5- Xp4- Xp3- Xp2- Xp1- Xp0- ES Xp0+ Xp1+ Xp2+ Xp3+ Xp4+ Xp5+ Xp6+ Xp7+ Xp8+

beyond passage 2 (Figure 5.7). However, alterations in gene expression were delayed compared to those observed in X cells cultured in the absence of added LIF.

After passage 3, X cells cultured in the presence and absence of added LIF could not be distinguished morphologically. Photographs of Xp6+/- cultures are shown in Figure 5.8 and are representative of all X cell cultures after passage three. Although ES cells may be present within cultures at low levels, colonies with the morphology of ES cells were not detected in X cell cultures at these stages. In particular, nests of stem cells with ES morphology, which are often seen in spontaneously differentiated ES cell cultures (4.3.1), were never observed at any time in any X cell culture. Therefore the cyclic alterations in X cell gene expression were not reflected in a morphological alteration. Further evidence for cyclic alterations in X cell gene expression was obtained from the gene expression profiles of XB cell lines during long term culture (data not shown).

5.4 The Behaviour of X Cell Cultures at Clonal Cell Density

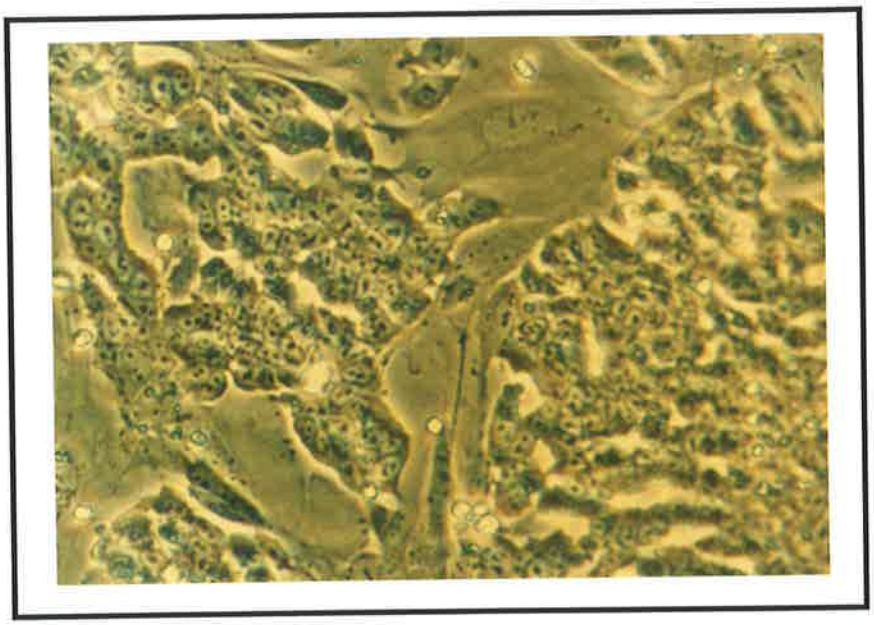
FGF-5 is expressed in the primitive ectoderm prior to and during gastrulation (Haub and Goldfarb 1991). It has been hypothesised that FGF-5 acts as a competence factor which renders cells of the primitive ectoderm competent to respond to other inductive signals during gastrulation (Hebert *et al.* 1991). FGF is expressed in the marginal zone of the *Xenopus* embryo and has been proposed to act as a competence factor which permits these cells to form mesoderm in response to an activin-type signal (Cornell *et al.* 1995). High levels of *FGF-5* may render X cells competent to respond to additional factors, present in MED2 or serum, by differentiation. In agreement with this, as X cells were cultured in MED2, *FGF-5* expression increased and the level of differentiated cells also increased (5.3, 3.6). Culture of X cells in MED2 in the presence of added or higher levels of LIF impeded increases in *FGF-5* expression and the level of differentiated cells was lower. Thus there is a correlation between the level of differentiation observed in high density X cell cultures and their *FGF-5* expression profile.

The level of *FGF-5* expression distinguishes two morphologically identical X cell states. The proposal that *FGF-5* is a competence factor for X cell differentiation suggests

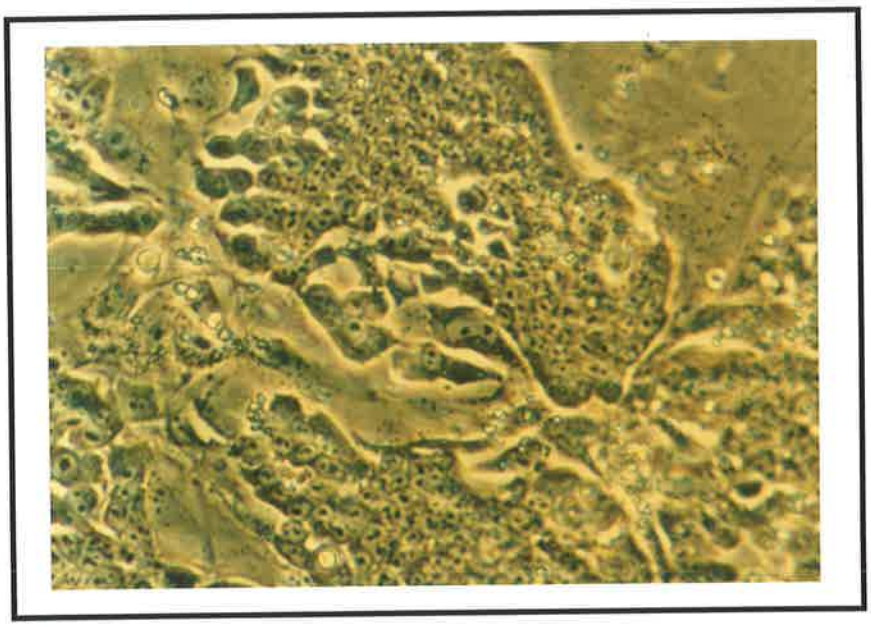
FIGURE 5.8 The morphology associated with passaged X cells.

The photographs shown are of Xp6- (A) and Xp6+ (B) cultures and are representative of X cell cultures beyond passage three.

A



B



that the two X cell states should also be distinguished by their respective abilities to differentiate and perhaps revert to ES cells.

Xp0+ cells and Xp0- cells while morphologically identical, exhibit distinct gene expression profiles. Although Xp0+ and Xp0- cultures exhibit similar *Oct-4* expression levels and therefore contain similar levels of X cells, Xp0- cells consistently exhibit higher levels of *FGF-5* and lower levels of *Rex-1* than Xp0+ cells (4.2.1, 5.2 and 5.3). The differentiation behaviour of these cells at clonal cell density in the presence of MED2 and/or LIF was investigated. ES, Xp0+ and Xp0- cells were cultured in the presence of incomplete ES medium containing 1) LIF alone, 2) No addition, 3) MED2 + LIF and 4) MED2 alone. The assays were stained at 5 days for alkaline phosphatase activity and colony analysis was carried out as previously described (Figure 3.4). The results of this analysis are presented in Figure 5.9.

The general behaviour of X cells as described in section 3.7 applied. X cells reverted to ES cells when cultured in the presence of LIF in the absence of MED2. If LIF and MED2 were omitted from the culture conditions X cells differentiated spontaneously. X cells were maintained in MED2 supplemented conditions. The level of X cell maintenance was lower in MED2 in the absence of added LIF.

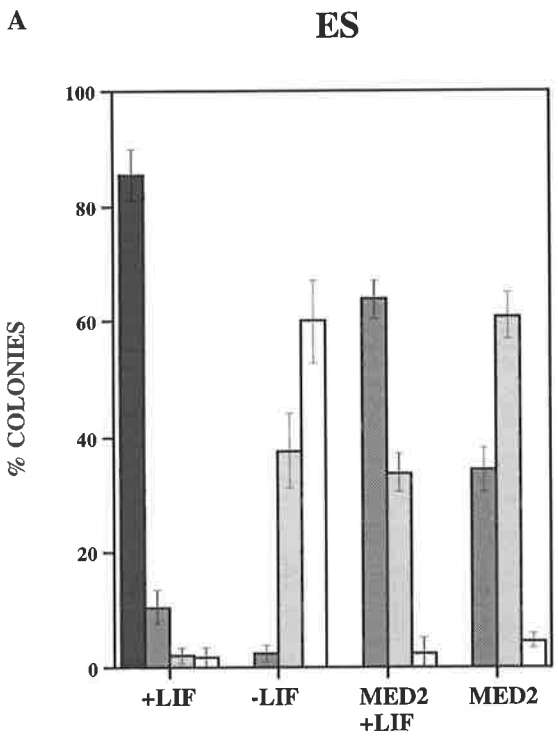
However, the relative stability of X cells derived from ES, Xp0+ and Xp0- cultures differed. While 64% of colonies were maintained as undifferentiated X cell colonies when ES cells were cultured in MED2 and LIF, a lower level (40%) were maintained under these conditions with Xp0+ cells. This level decreased further (20%) when Xp0- cells were cultured in these conditions. Similar decreases in stability were observed in all described conditions. Thus the level of X cell stability at clonal cell density correlated with the initial level of *FGF-5* expression in ES, Xp0+ and Xp0- cells. Xp0- cells exhibited the highest level of *FGF-5* and also exhibited the highest level of differentiation.

X cell reversion to ES cells appears to occur relatively early during culture in the presence of LIF in the absence of MED2 (3.7, 5.2). Culture of ES cells in the presence of LIF resulted in maintenance of ES cell colonies at a level of 84%. When Xp0+ cells were cultured in the presence of LIF and absence of MED2 they reverted at high frequency to ES cells. 80% of colonies were undifferentiated ES cell colonies. By contrast when Xp0- cells were cultured in these conditions the reversion frequency

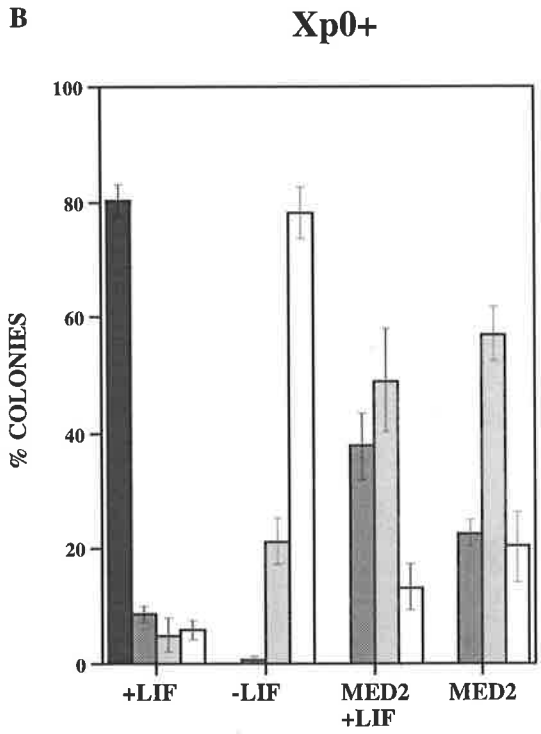
FIGURE 5.9 The behaviour of passaged X cells at clonal cell density in MED2 and LIF supplemented conditions.

ES, Xp0+ and Xp0- cells were cultured in duplicate wells containing incomplete ES cell medium and/or MED2, and/or LIF (2.3.13ii, 2.3.14i). The assays were stained for alkaline phosphatase activity at day five (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD), and differentiated colonies (D) present in each well was determined as previously described (Fig.3.4). The mean percentage and standard deviation for each variable was derived from colony analysis of four duplicate wells and are represented graphically. Results for ES, Xp0+ and Xp0- cells are presented in A, B and C respectively. The mean plating efficiency for each culture is tabulated in D.

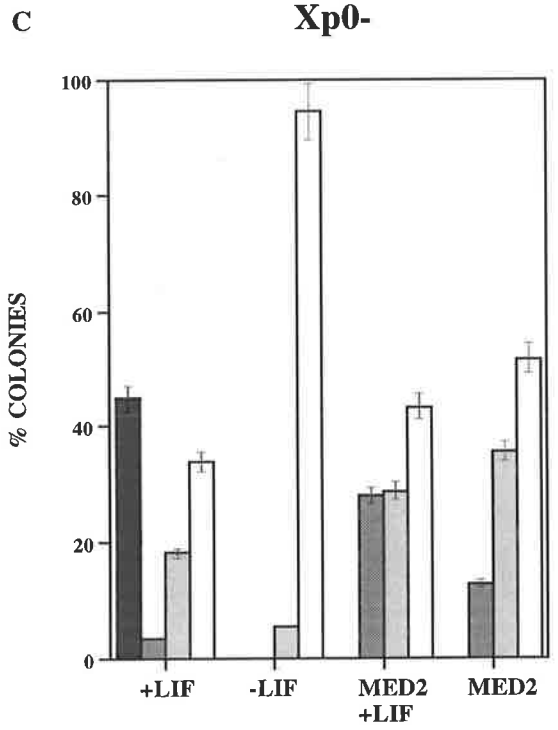
A

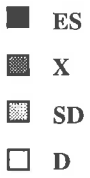


B



C





D

	% PLATING EFFICIENCY			
	+LIF	-LIF	MED2+LIF	MED2
ES	42.6 +/- 6.7	43.3 +/- 7.5	47.6 +/- 0.4	45.9 +/- 1.7
Xp0+	32.4 +/-7.4	41.4 +/- 6.6	41.9 +/- 5.0	40.5 +/- 5.9
Xp0-	49.1 +/- 9.1	25.9 +/- 1.7	41.6 +/- 0.74	46.8 +/- 4.8

decreased, only 45% of colonies were ES cell colonies. Thus Xp0- cells were distinguished from Xp0+ cells by a lower reversion frequency (Figure 5.9).

Thus while Xp0+ and Xp0- cultures are morphologically identical and contain similar levels of pluripotent cells, Xp0- cultures could be distinguished from Xp0+ cultures by their lower capacity for reversion and higher capacity for differentiation. This suggests that X cell populations expressing higher levels of *FGF-5* and lower levels of *Rex-1* have a decreased ability to revert and an increased ability to differentiate, compared to X cell populations exhibiting lower levels of *FGF-5* and higher levels of *Rex-1*. This is supported by the behaviour of other X cell cultures in MED2 and/or LIF at clonal cell density (data not shown) and is consistent with the proposal that *FGF-5* acts as a competence factor for X cell differentiation.

5.5 Discussion

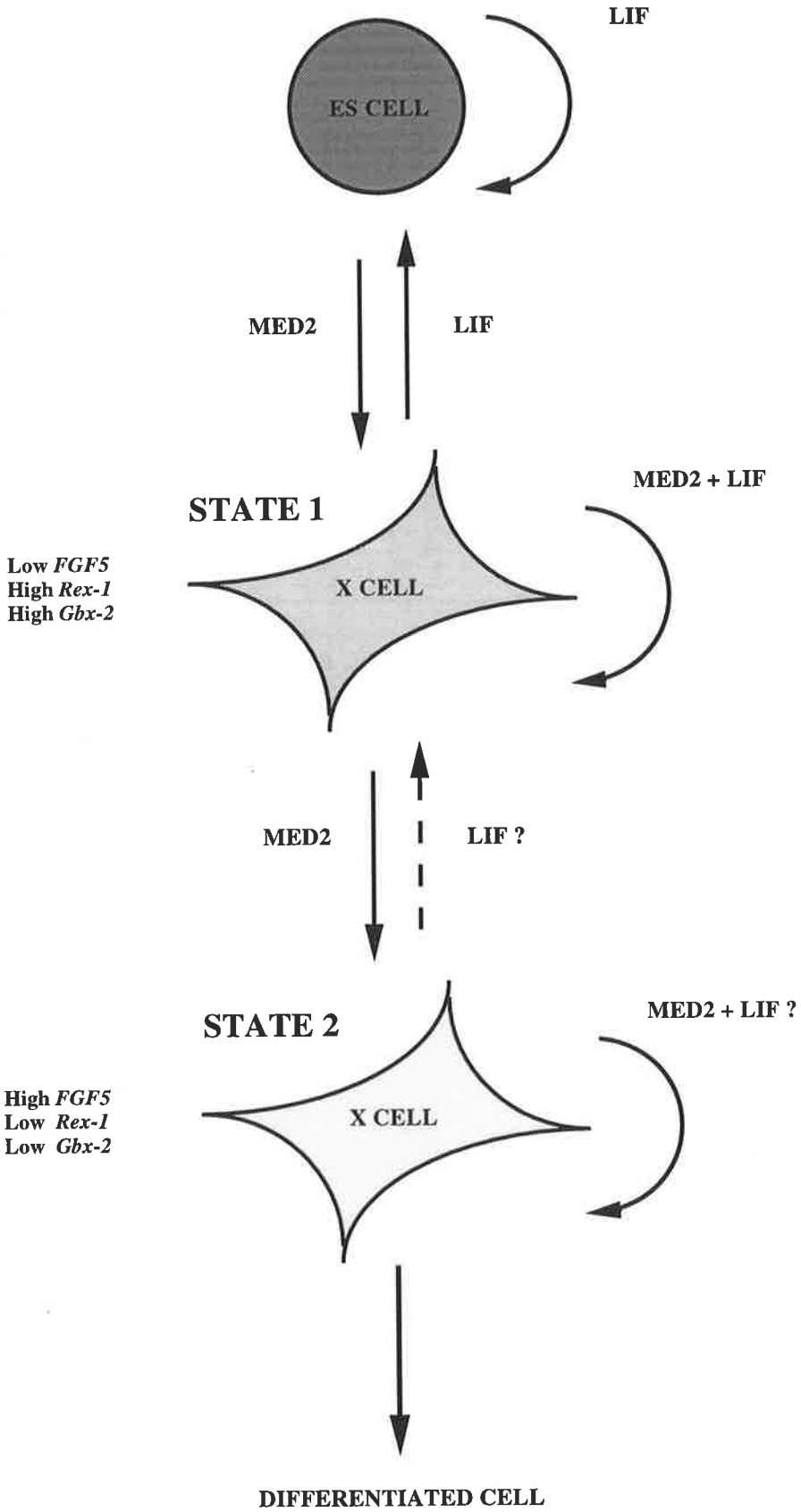
5.5.1 A Model for X Cell Formation, Maintenance and Differentiation

Based on the experimental observations described in this chapter a revised model for X cell formation, maintenance and differentiation is presented in Figure 5.10. This model proposes that X cells within X cell populations can exist in two morphologically identical states which can be distinguished by gene expression and differentiation capacity. X cells in state 1 express lower levels of *FGF-5* and higher levels of *Rex-1* and *Gbx-2*, while X cells in state 2 express higher levels of *FGF-5* and lower levels of *Rex-1* and *Gbx-2*. The proportion of cells in state 1 and state 2 in any given X cell population is dependent on the culture time spent in MED2 and the concentration of LIF present. X cells in state 1 have a higher capacity for reversion while cells in state 2 have a higher capacity for differentiation. The level of differentiation and frequency of reversion exhibited by X cell cultures is therefore dependent on the proportion of X cells in state 1 and state 2.

Thus the behaviour of Xp0+ and Xp0- cells at clonal cell density can be explained by the proportion of X cells in state 1 and state 2 within these cultures. Since Xp0+ cells were formed in MED2 in the presence of high concentrations of LIF, a high proportion of X cells will be in state 1 compared to state 2. Thus Xp0+ cells revert at high frequency in

FIGURE 5.10 A model for X cell formation, maintenance and differentiation.

Modifications of the model depicted in Figure 3.18 were made based on the gene expression profile observed in passaged X cells and their behaviour at clonal cell density in MED2 and/or LIF culture conditions. See text for complete description (5.5.1). Briefly the model proposes that X cells can exist in two morphologically identical states. State 1 is characterised by a lower *FGF-5*, higher *Rex-1* and *Gbx-2* expression than cells in state 2. Cells in state 1 exhibit a high capacity for reversion while cells in state 2 exhibit a high capacity to differentiate. The proportion of X cells in each state is dependent on the presence of added LIF and culture time in MED2.


LIF
ES CELL
MED2
LIF
STATE 1
MED2 + LIF
Low FGF5
High Rex-1
High Gbx-2
X CELL
MED2
LIF ?
STATE 2
MED2 + LIF ?
High FGF5
Low Rex-1
Low Gbx-2
X CELL
DIFFERENTIATED CELL

the presence of LIF in the absence of MED2. Since Xp0- cells were formed in the presence of low concentrations of LIF a higher proportion of X cells will be in state 2. Therefore Xp0- cells exhibit a lower capacity for reversion and are more prone to differentiation.

This model might explain the cyclic alterations in gene expression of X cell populations described in this chapter. Culture in MED2 at high cell density results in a decrease in the proportion of X cells in state 1 and an increase in the proportion of X cells in state 2 (Xp0 to Xp2). High concentrations of LIF slow this transition. A high proportion of cells within Xp2- cultures exist in state 2 and therefore differentiate. Differentiated cells within the population may possess a lower ability to reseed and/or proliferate in subsequent cultures. This may therefore result in a population of X cells largely consisting of cells in state 1 (Xp3-, Xp4-). As these cells are cultured in MED2 they again progress to state 2 (Xp5-, Xp6-). Similarly cells in state 2 in Xp2+ cultures may differentiate leaving behind a population of cells in state 1(Xp3+ to Xp5+). Since transition to state 2 occurs more slowly in the presence of high concentrations of LIF it is possible that the population may exhibit low *FGF-5*, high *Rex-1* expression for an extended period (Xp6+, Xp7+) before increases in *FGF-5* expression and decreases in *Rex-1* are detected.

In situ hybridisation analysis of gene expression in X cell cultures indicated that X cells express *FGF-5*, *Rex-1* and *Gbx-2*. Cells expressing high levels of *Gbx-2* in Xp0- cultures were detected (Figure 4.6). While differences in *FGF-5* and *Rex-1* expression levels between X cells were not obvious, slight differences between cells were seen (Figure 4.4 and 4.5). This supports the model that X cells exist in two morphologically identical states distinguished by gene expression

Investigation of this model will ultimately require the development of a highly sensitive, quantitative *in situ* hybridisation technique and rigorous examination of gene expression and differentiation capabilities of different X cell populations.

5.5.2 Implications for Pluripotential Cell Isolation from Mammalian Embryos

Results presented in this chapter demonstrated that X cells can be phenotypically reverted to ES cells when cultured in LIF in the absence of MED2, at any stage of culture. Reverted X cells had a gene expression profile characteristic of ES cells.

At present embryonic stem cell isolation is a relatively species and strain specific exercise. Embryonic stem cells can only be routinely isolated from strain 129 mice. In addition, stem cells have only been obtained at low frequency from murine blastocysts at specific developmental stages. Thus pluripotential stem cell isolation is only possible during a relatively small developmental time period using existing approaches. The ability of MED2 to aid the isolation of XB cell lines from embryoid bodies supports the proposal that X cells are present *in vivo* and may represent early primitive ectoderm. In addition it implies that a similar method may enable the isolation and maintenance of primitive ectoderm from the murine embryo. Preliminary experiments using MED2 to aid the isolation of pluripotential stem cells from the primitive ectoderm have met with some success. Cells expressing pluripotential stem cell markers have been cultured for up to 3 weeks (J. Rathjen, unpublished observations). The development of any alternative method of stem cell isolation might be of importance for the isolation of pluripotential stem cells from species that have proved refractory to current approaches. The ability of a primitive ectoderm cell line to revert to ES cells might be important for the ultimate success of any transgenic technique.

The observation that X and XB cell lines reverted to ES cells, which were capable of reconversion to X cells, may signify that the primitive ectoderm within the murine embryo is capable of such reversion. This may explain the considerable developmental lability associated with the early murine embryo. As previously described it is possible to destroy a large proportion of the cells present in the egg cylinder stage embryo and a normal embryo will still form (Snow and Tam 1979, Smith 1992). If primitive ectoderm cells present within the embryo are capable of reversion to ICM-type cells then normal embryogenesis may well proceed in such circumstances.

As ES cells form X cells and X cells are maintained in culture, pluripotential cells proliferate rapidly, *FGF-5* expression increases and cells acquire the ability to

differentiate. Similarly, as the inner cell mass forms the primitive ectoderm, cells proliferate rapidly (Snow 1977), *FGF-5* expression increases (Haub and Goldfarb 1991) and cells may become competent to respond to signals which induce differentiation (Hebert *et al.* 1991). Gene expression alterations occurring during X cell culture appear progressive and heterogeneous, cells capable of reversion to ES cells are maintained. Similar alterations in gene expression as the inner cell mass forms the primitive ectoderm may constitute a system for ensuring the maintenance of a population of cells capable of reversion well into gastrulation. In a reverted, non competent state pluripotential cells may proliferate and again acquire competence to differentiate, thus ensuring the progression of development when the established system fails.

CHAPTER SIX:

THE DIFFERENTIATION POTENTIAL OF X CELLS

CHAPTER SIX: THE DIFFERENTIATION POTENTIAL OF X CELLS

6.1 Introduction

ES cells are representative of cells constituting the inner cell mass (ICM) in the early murine embryo (1.6). Cells of the ICM form the primitive endoderm and the primitive ectoderm. Subsequently the primitive endoderm differentiates into parietal and visceral endodermal cell types, which gives rise to part of the extraembryonic tissues of the embryo. The primitive ectoderm during the process of gastrulation forms the mesoderm, ectoderm, endoderm, germ cells and remaining extraembryonic tissue of the embryo. The gene expression associated with X cells is consistent with their existence downstream of the ICM toward the primitive ectoderm or equivalent to early stage primitive ectoderm (Figure 4.10). Investigation of the developmental potential of X cells would further define their position in this developmental progression.

ES cells can be induced to differentiate *in vitro* by culture in the presence of chemical inducers of differentiation, or *via* embryoid body formation (1.5.4). When ES cells are aggregated by culture in suspension in the absence of LIF they form structures termed embryoid bodies. Embryoid bodies differentiate in a sequential and relatively organised manner, giving rise to tissues which are present in the early murine embryo such as primitive endoderm and primitive ectoderm, and ultimately form a variety of cell types, including muscle, cartilage, neurons, blood and pigmented epithelium (Evans and Kaufman 1981, Martin 1981, Doetschmann *et al.* 1985, Robertson 1987).

6.2. X Cells can Differentiate as Embryoid Bodies.

X cells grow as monolayer colonies in which individual cells are clearly discernible. ES cells form compact, dome-shaped colonies in which individual cells can not be seen (Figure 1.8). The difference in the colony morphology of ES and X cells is likely to reflect differences in cell adhesion. When ES cells are grown in suspension, by culture in non-adhesive bacteriological dishes, cells adhere to one another forming aggregates. Since X cells appear to have different cell adhesion properties from ES cells, the ability of X cells to aggregate in a similar manner to ES cells was investigated.

Xp0+ cells were trypsinised and cultured at a cell density of 1 x 10^5 cells/ml in bacteriological petri dishes containing incomplete ES cell medium (2.3.16b). After 2 days aggregated cells were observed. These continued to grow and by four days a small proportion (10%) of aggregates had formed a discernible outer layer of cells, reminiscent of primitive endoderm. By day 6, cavitation had also occurred in a low proportion of aggregates (8%), and an inner layer of columnar cells reminiscent of primitive ectoderm was observed in a low percentage of aggregates (4%). Overt layer formation was not apparent in the majority of aggregates (90%).

Embryoid bodies, once formed, can be treated in two ways (Robertson 1987). Free floating embryoid bodies are continually cultured in suspension. Seeded embryoid bodies are cultured in gelatinised tissue culture plates after 4-6 days. Embryoid bodies treated in this manner attach to the surface of plates and outgrowths containing a multitude of differentiated cell types are formed.

Differentiation is more clearly visualised in seeded embryoid bodies than in free floating embryoid bodies, therefore X cell aggregates were seeded into tissue culture dishes at day 6 of development. In the majority of these aggregates a multitude of cell types and structures could be distinguished based on morphology including parietal endoderm, cardiac muscle, haemopoietic cells, vasculature, neuronal filaments and pigmented epithelium. Despite differences between the cell adhesion properties of X cells and ES cells, X cells were therefore capable of forming and differentiating as embryoid bodies. This ability demonstrates that X cells are pluripotent, and allows the differentiation potential associated with ES and X cells during this complex differentiation regime to be compared directly.

The cells chosen for this analysis were Xp0+ cells and Xp0- cells. *Oct-4* expression levels indicate that Xp0+ and Xp0- cells constitute relatively homogeneous X cell populations and contain a similar level of cells expressing *Oct-4* (3.6). However Xp0- cultures consistently exhibit higher levels of *FGF-5* expression and lower levels of *Rex-1* and *Gbx-2* expression than Xp0+ cells. Xp0+ cells exhibit a gene expression profile, intermediate to that exhibited by ES and Xp0- cells (4.2.1, 5.2, 5.3).

6.3 Comparison of ES, Xp0+ and Xp0- Cell Differentiation as Free Floating Embryoid Bodies

D3 ES cells were seeded at $7.5x10^3$ cells/cm^2 into medium containing LIF alone to generate ES cells and at $2.5x10^3$cell/cm^2 into MED2 with and without LIF to form Xp0+ and Xp0- cultures respectively (2.3.9). A lower seeding density was used for X cell formation to compensate for the higher proliferation rate of X cells in culture. After three days, cultures were partially trypsinised and the cellular aggregates were cultured in bacteriological petri dishes in incomplete ES cell medium (2.3.16a). Partial trypsinisation results in the formation of cellular aggregates rather than single cells. It was thought that this method would overcome any differential cell adhesion between cells within cultures. While this method was routinely employed, alternative methods of embryoid body formation (2.3.16) produced similar results.

After four days a phenotypic distinction could be made between the different cultures. Photographs representing the differences observed between ES, Xp0+ and Xp0- embryoid bodies are shown in Figure 6.1. ES cell cultures were defined by the presence of round, relatively smooth aggregates which contained a discernible outer layer of cells (A). In Xp0+ and Xp0- cultures the majority of embryoid bodies appeared globular, relatively disorganised and did not contain a distinct outer layer of cells (B). A low proportion of the embryoid bodies observed in Xp0+ cultures exhibited morphologies characteristic of ES cell embryoid bodies (Table C).

The first differentiation event to occur during embryoid body development is the formation of an outer layer of primitive endoderm at day 4 (Doetschman *et al.* 1985). Embryoid bodies derived from ES cells exhibited a clearly discernible outer layer of cells at day 4. A similar layer was not detected in embryoid bodies derived from X cells. This suggested that ES and X cells may be distinguished by their ability to give rise to primitive endoderm during embryoid body differentiation. This proposal is examined in Section 6.7.3.

A striking difference in the level of cardiac muscle in ES, Xp0+ and Xp0- embryoid body cultures at day 6 was also observed. Cardiac muscle was identified as cellular regions within the embryoid body which had the ability to contract rhythmically. In Xp0+ and Xp0- cultures, embryoid bodies which contained large areas of beating

FIGURE 6.1 The morphology of ES, Xp0+ and Xp0- embryoid bodies.

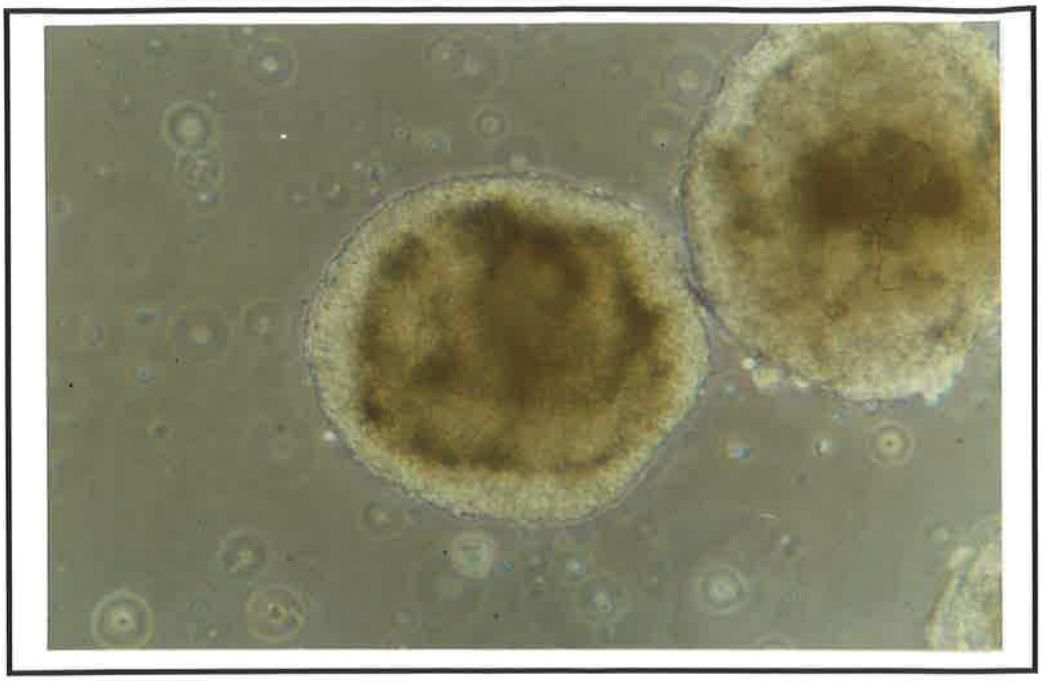
The layered morphology exhibited by the majority of embryoid bodies derived from ES cells is shown in A.

The morphology exhibited by the majority of embryoid bodies derived from Xp0+ and Xp0- cells is shown in B.

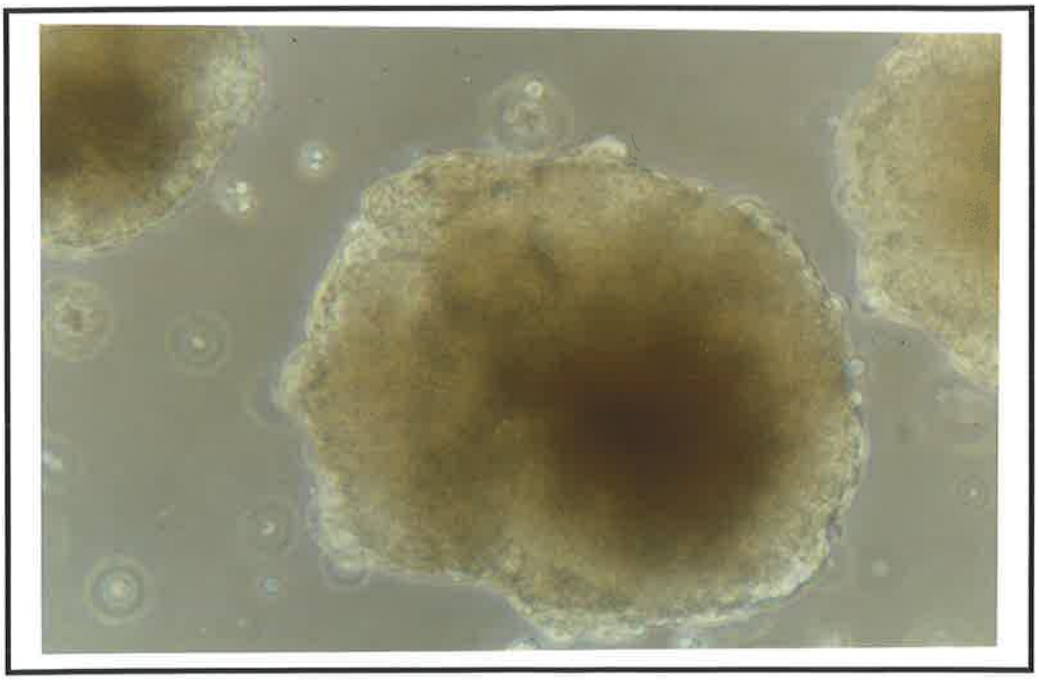
At day 4 the number of embryoid bodies exhibiting morphology shown in A and B was determined for ES, Xp0+ and Xp0- cultures. Percentages were obtained from the analysis of 100 embryoid bodies in each culture. The results of this analysis are tabulated in C.

Photographs were taken at a magnification of 50x using phase contrast microscopy.

A



B



C

	% OF EMBRYOID BODIES		
	ES	*Xp0+*	*Xp0-*
A	*98*	*22*	*0*
B	*2*	*78*	*100*

muscle were first detected at day 6 of development. It was not until day 8 of embryoid body development that beating was detected in ES cell cultures. X cells therefore appeared to form cardiac muscle, a mesodermal derivative, earlier than ES cells when differentiated as embryoid bodies.

6.3.1 The Formation of Cardiac Muscle in ES, Xp0+ and Xp0- Embryoid Bodies

The profile of beating muscle formation in embryoid bodies derived from ES, Xp0+ and Xp0- cells was determined by monitoring the development of individual embryoid bodies. These profiles were compared with the expression of the cardiac specific marker gene *Nkx 2.5* (Lints *et al.* 1993).

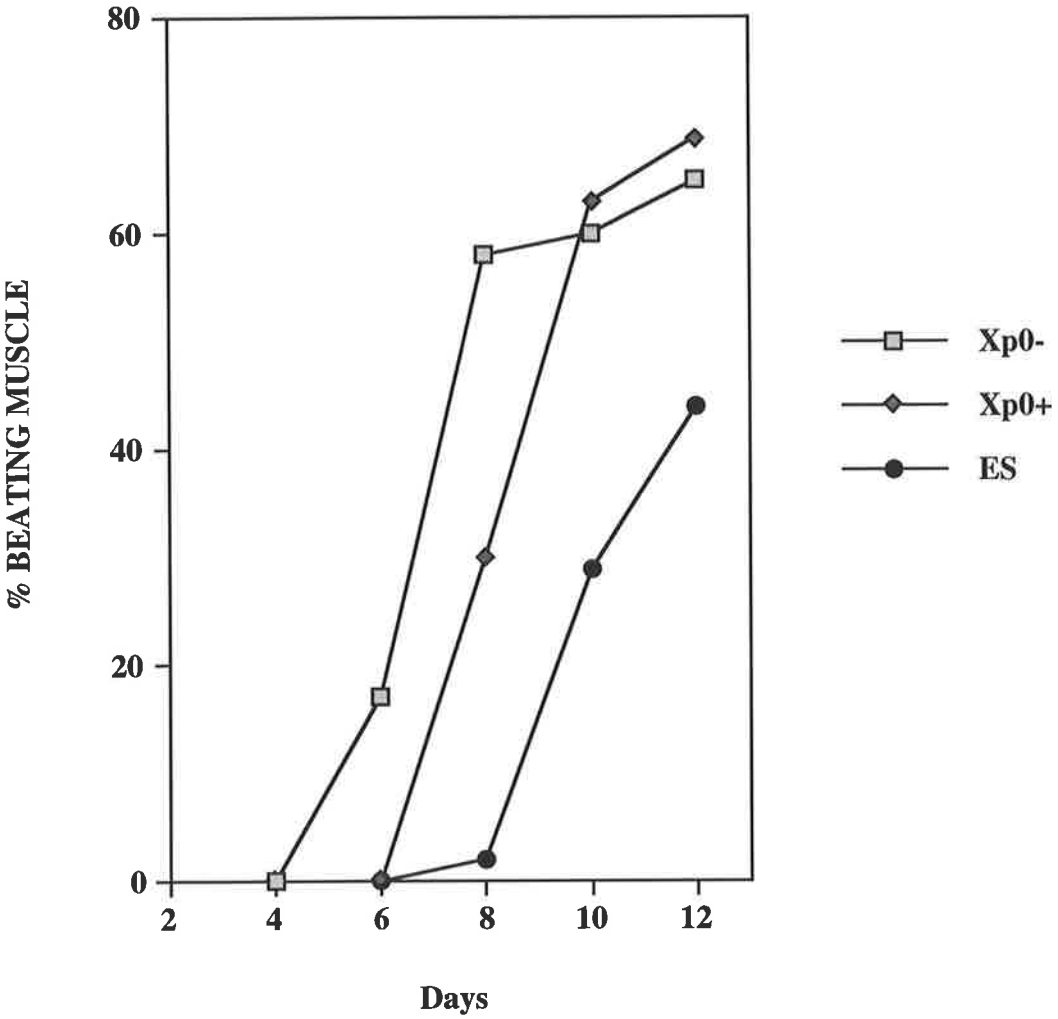
Embryoid bodies were formed from ES, Xp0+ and Xp0- cells as previously described (6.3). At day 4 embryoid bodies were placed individually into agarose coated wells (2.3.17). Coating of wells with agarose inhibits the attachment of embryoid bodies, which therefore remain in suspension. Individual embryoid bodies were examined for the presence of beating muscle on days 4, 6, 8, 10 and 12. The profile of beating muscle formation in ES, Xp0+ and Xp0- embryoid bodies is depicted in Figure 6.2.

At day 6, 18% of Xp0- embryoid bodies were observed to contain beating muscle, this level rose to 58% at day 8 and stabilised thereafter. In Xp0+ embryoid bodies 30% of embryoid bodies contained beating muscle by day 8 and by day 10 the level was comparable to the level in Xp0- cultures. By contrast only 2% of embryoid bodies derived from ES cells contained beating muscle at day 8. This level increased to 42% at day 12, however, this level was below the percentage observed in Xp0- and Xp0+ cultures. The regions of beating muscle observed in X cell embryoid bodies were considerably larger than regions observed in ES cell embryoid bodies, but could not be quantitated reliably.

Consistent with previous observations (6.3) embryoid bodies derived from X cells formed beating muscle earlier and to a greater extent than ES cell embryoid bodies. This capacity for beating muscle formation was higher in embryoid bodies derived from Xp0- cells than Xp0+ cells which in turn was higher than ES cells.

FIGURE 6.2 The formation of beating muscle in ES, Xp0+ and Xp0- free floating embryoid bodies.

Embryoid bodies derived from ES, Xp0+ and Xp0- cells (2.3.16) were placed individually into agarose coated wells at day 4 of development (2.3.17). Embryoid bodies were cultured until day 12 and inspected at day 6, 7, 8, 10 and 12 for the presence of beating muscle. The percentage of embryoid bodies containing beating muscle was derived from the analysis of 48 embryoid bodies for each variable The percentage of embryoid bodies, derived from ES, Xp0+ and Xp0-, containing beating muscle is represented graphically.


80
60
40
20
0
% BEATING MUSCLE
2
4
6
8
10
12
Days
Xp0-
Xp0+
ES

The homeobox gene *Nkx 2.5* is first detected in the murine embryo at day 7.5 in myocardiogenic progenitor cells and expression is largely confined to regions of the developing heart (Lints *et al.* 1993). *Nkx 2.5* is expressed prior to observable contraction both *in vivo*, during murine embryogenesis, and *in vitro*, during embryoid body formation (Lints *et al.* 1993, Lyons *et al.*, 1995, Kaufman 1992). Targeted disruption of *Nkx 2.5* results in abnormal heart morphogenesis (Lyons *et al.* 1995). Thus *Nkx 2.5* expression serves a marker for cardiac tissue.

Embryoid bodies derived from ES, Xp0+ and Xp0- cells were harvested at day 4, 6, 8, 10 and 12 of development. The expression of *Nkx 2.5* compared to a *mGAP* loading control was determined by Northern analysis of total RNA. The results of this analysis are presented in Figure 6.3. The levels of *Nkx 2.5* and *mGAP* expression were determined by volume integration and the ratio of *Nkx 2.5/mGAP* expression is represented graphically in Figure 6.4.

Expression of *Nkx 2.5* in ES, Xp0+ and Xp0- embryoid bodies was consistent with the profile of beating muscle formation observed in these cultures (Figure 6.2). At day 6 high levels of expression were detected in Xp0- and Xp0+ cultures. By contrast, significant *Nkx 2.5* expression was not detected in ES cell cultures. The level of *Nkx 2.5* expression increased in the order ES, Xp0+, Xp0-. This correlated with the percentages of beating muscle observed in these cultures at day 8 which also increased in the order ES, Xp0+, Xp0- (Figure 6.2). Thus X cells could be distinguished from ES cells by their capacity to form cardiac muscle earlier and at higher levels when differentiated as embryoid bodies.

In vivo, the ICM gives rise to the primitive ectoderm which subsequently through the process of gastrulation gives rise to mesoderm, including cardiac muscle. A similar progression occurs during embryoid body development *in vitro* (Doetschman *et al.* 1985). ES cells are analogous to the ICM (1.6), therefore differentiation into cardiac muscle should progress *via* a primitive ectoderm intermediate prior to mesodermal differentiation. X cells possess a gene expression consistent with the cells of the early primitive ectoderm. It therefore follows that differentiation into mesodermal derivatives, such as cardiac muscle, during embryoid body differentiation should occur earlier in X cells than ES cells. The intermediate nature of cardiac muscle formation in Xp0+ cells compared to

FIGURE 6.3 The expression of the cardiac specific marker *Nkx 2.5* in ES, Xp0+ and Xp0- free floating embryoid bodies.

RNA was prepared from ES, Xp0+ and Xp0- embryoid body cultures harvested at day 0, 4, 6, 8, 10 and 12 of development (2.3.19, 2.5.11). The expression of *Nkx 2.5* was determined by Northern analysis of total RNA (2.5.12) using an antisense riboprobe derived from *Nkx 2.5* cDNA (2.5.15). Filters were washed in 2xSSC/0.1%SDS and then in 0.2xSSC/0.1% SDS at 50°C for 15 minutes. This was followed by washes in 0.2xSSC/0.1%SDS and subsequently 0.1xSSC/0.1%SDS at 75°C for 45 minutes. Filters were exposed to a phosphorimager screen for 24 hours (2.5.20). A differentially expressed band corresponding to the 1.7 kb transcript of *Nkx 2.5* was detected in RNA derived from embryoid bodies. Filters were stripped and reprobed with a *mGAP* loading control (2.5.14, 2.5.16), exposure time 21 hours.

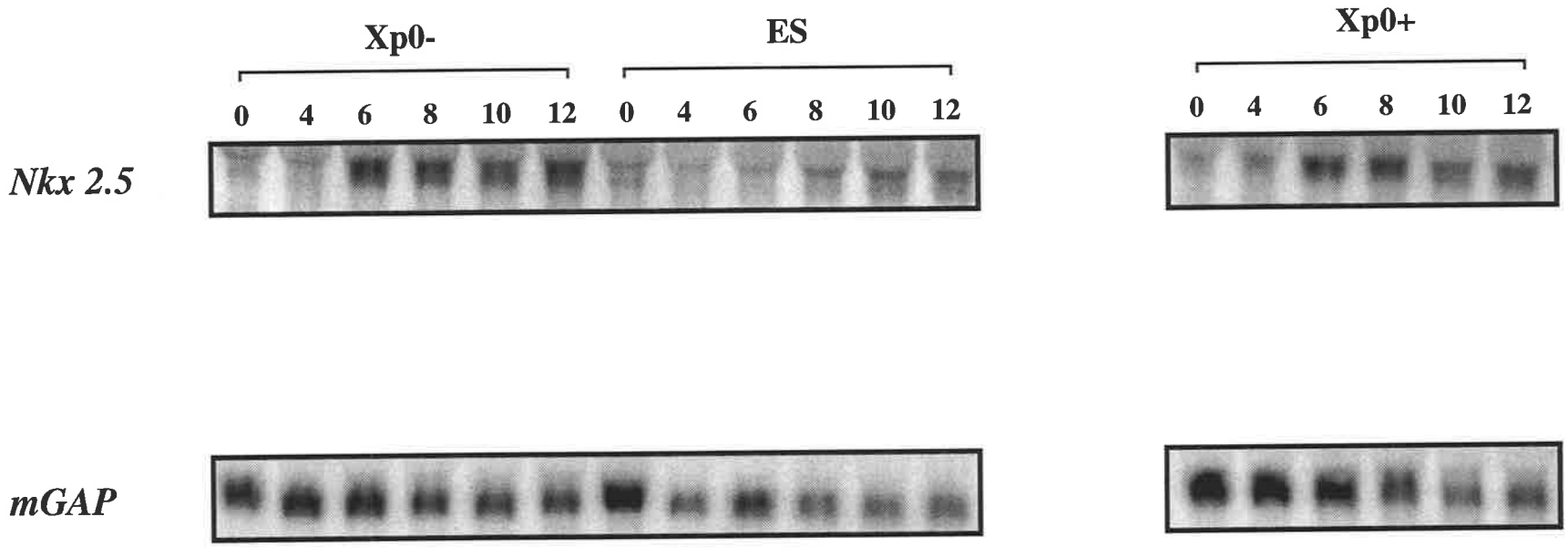
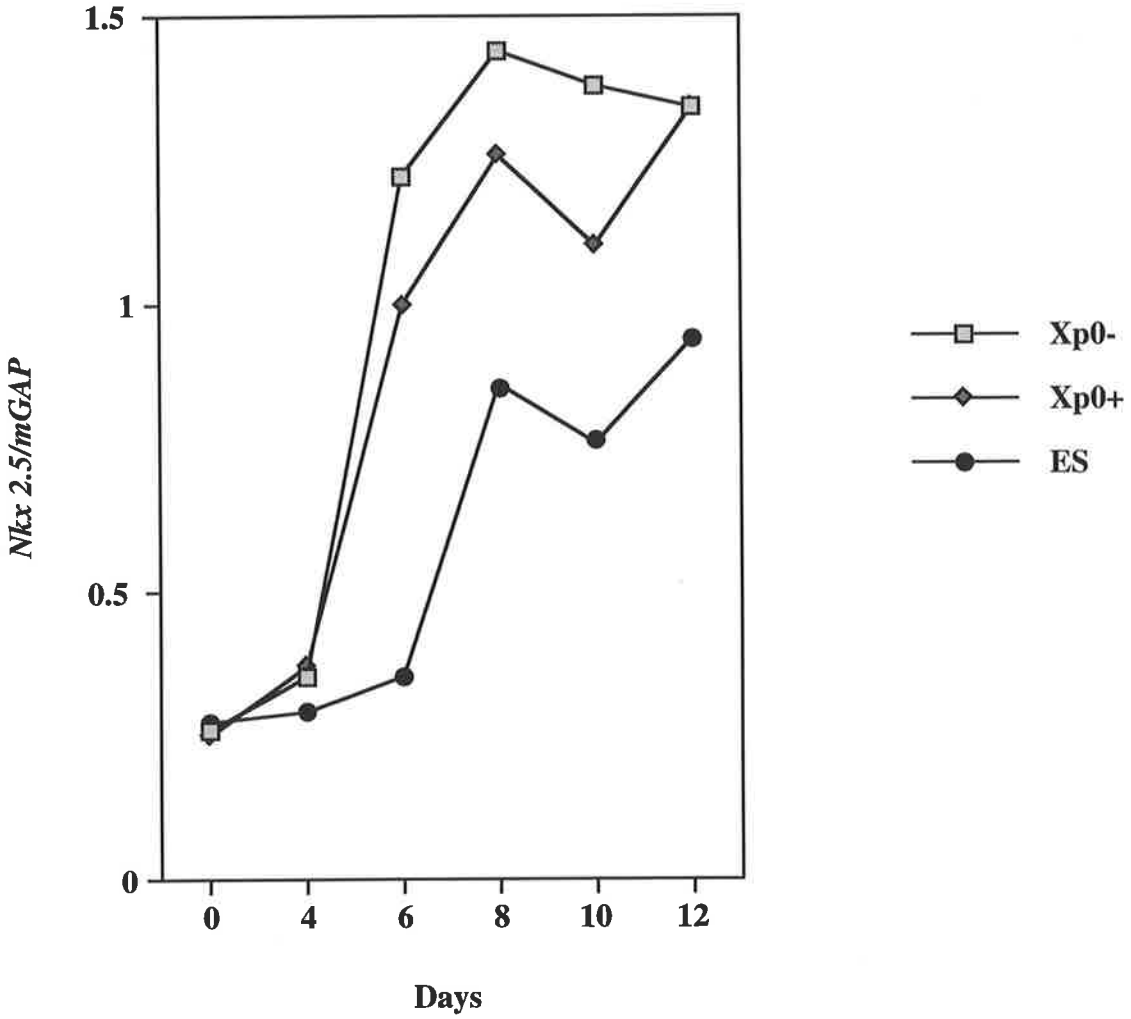

Xp0-
ES
Xp0+
0 4 6 8 10 12 0 4 6 8 10 12
0 4 6 8 10 12
Nkx 2.5
mGAP

FIGURE 6.4 Comparison of the expression of *Nkx 2.5* in ES, Xp0+ and Xp0- free floating embryoid bodies.

The level of *Nkx 2.5* and *mGAP* expression in embryoid bodies derived from ES, Xp0+ and Xp0- at day 0, 4, 6, 8, 10 and 12 (Fig. 6.3) was quantitated by volume integration (2.5.20). The expression of *Nkx 2.5* normalised to the *mGAP* loading control is represented graphically.


1.5
1
0.5
0
Nkx 2.5/mGAP
0
4
6
8
10
12
Days
Xp0-
Xp0+
ES

ES and Xp0- cells is consistent with this hypothesis. ES and Xp0- cells possess a gene expression consistent with the ICM and early primitive ectoderm, respectively. Since Xp0+ cells exhibit a gene expression intermediate to that observed in ES and Xp0- cells formation of a mesodermal derivative such as cardiac muscle might be delayed compared to Xp0- cells, but earlier compared to ES cells. Thus the onset of cardiac muscle formation in Xp0-, Xp0+ and ES cell cultures is therefore consistent with the proposed relationship of X cells to the primitive ectoderm as indicated by gene expression.

As with murine embryogenesis, differentiated cell types produced during embryoid body differentiation are dependent on multiple and sequential interactions between different cell populations. Therefore while the onset of cardiac muscle formation can be correlated with the stage of primitive ectoderm to which X cells correspond, the extent of cardiac muscle formation is likely to be dependent on other differentiation events which give rise to a cellular environment conducive to cardiac muscle formation.

6.4 Comparison of ES, Xp0+ and Xp0- Cell Differentiation as Seeded Embryoid Bodies

ES, Xp0+ and Xp0- embryoid bodies were prepared as described previously (6.3) and at day 6 were seeded into gelatinised tissue culture dishes (2.3.16). By day 7 embryoid bodies had attached to tissue culture surfaces and outgrowths of cells surrounding a central embryoid body core were observed. At this stage obvious differences could be seen in the proportion of beating muscle in ES cell and X cell cultures. Consistent with previous observations (6.3), beating muscle could be detected at significant levels in Xp0- cultures. By day 8 beating muscle was observed in Xp0+ cell cultures, however it was not until day 10 that the presence of beating muscle was detected in ES cell cultures.

At day 8 a morphological difference in the cells surrounding embryoid body cores was observed in ES, Xp0+ and Xp0- cultures. While cells with the morphology of parietal endoderm were observed in approximately 15% of embryoid bodies in all cultures, ES cell cultures were discernible by the presence of a flat non refractile layer of cells in which individual cell boundaries could not be deciphered (Figure 6.5 A). This cell type was observed in 65% of ES cell embryoid bodies compared to 10% of X cell

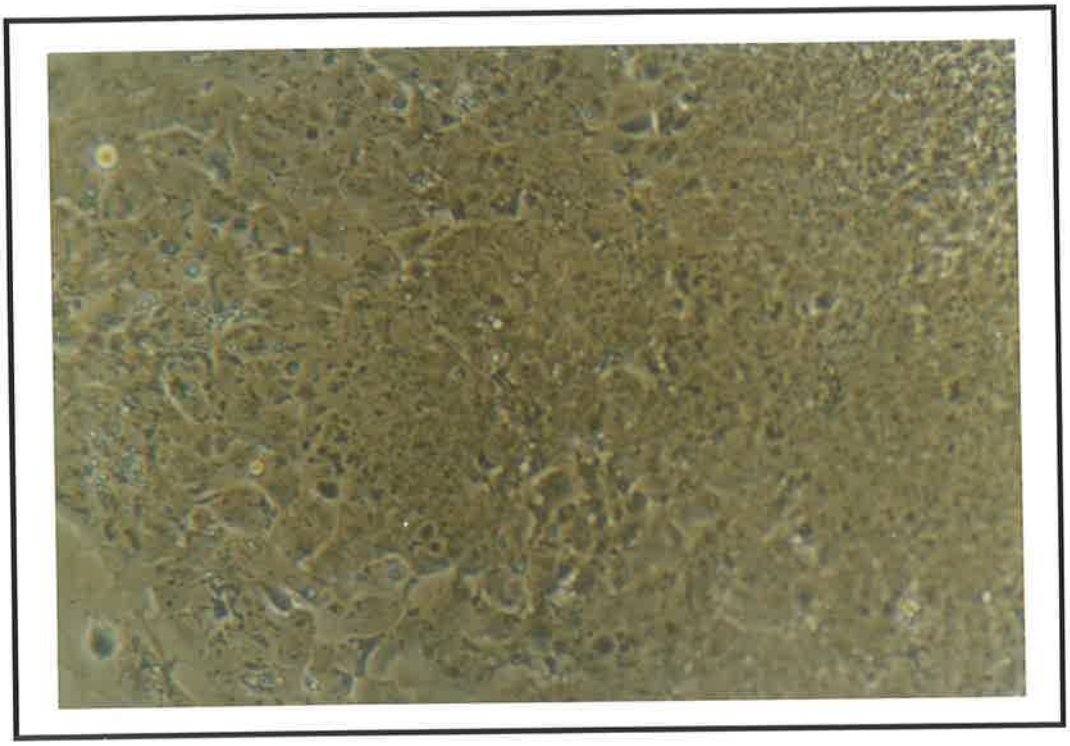
FIGURE 6.5 Cell types present in ES cell seeded embryoid bodies.

At day 8 seeded embryoid bodies derived from ES cells were distinguished from those derived from Xp0+ and Xp0- cultures by the presence of the cell type shown in A. This cell type had a morphology consistent with visceral endoderm.

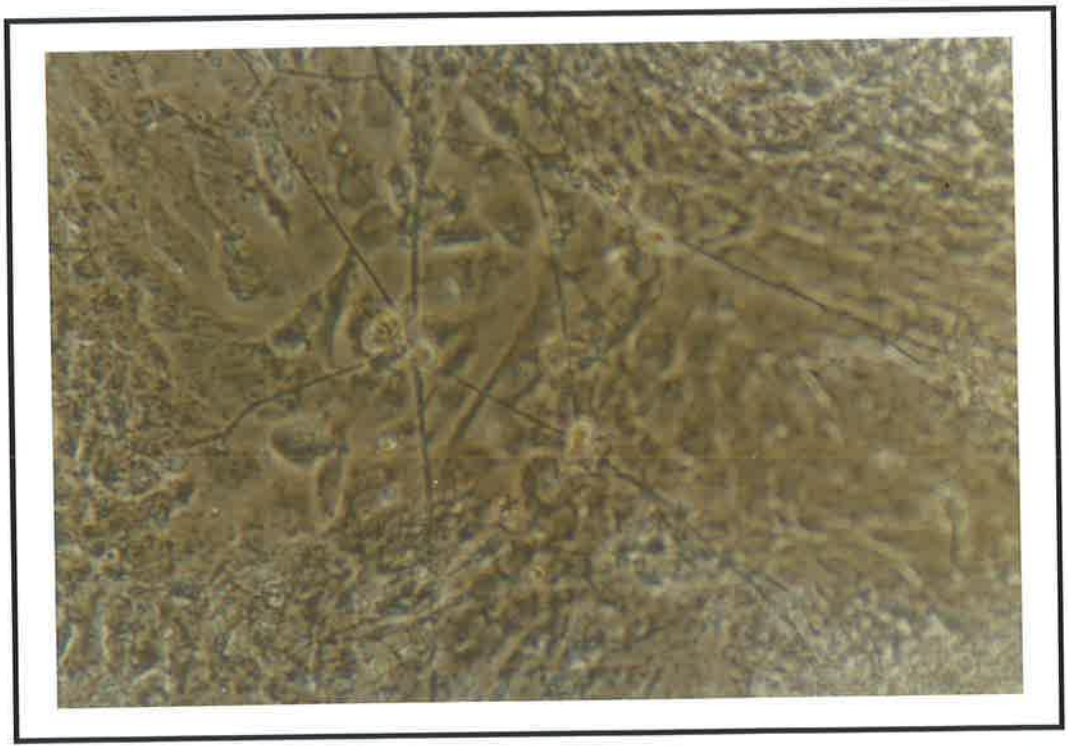
At day 10 seeded embryoid bodies derived from ES cell embryoid bodies could also be distinguished from X cell embryoid bodies by the presence of extensive neuronal networks (B).

Photographs were taken at a magnification of 50x using phase contrast microscopy.

A



B



embryoid bodies and had a distinctive morphology reminiscent of visceral endodermal cells derived from EC cells (Van den Eijen-van Raaij *et al.* 1991). The majority of X cell embryoid bodies (80%) were surrounded by cells with a fibroblastic appearance similar to that shown in Figure 6.9 B. As embryoid bodies were cultured for longer periods these differences were masked by further differentiation.

At day 10 an obvious difference in the level of neuron-like cells in ES and X cell embryoid bodies became apparent. Seeded embryoid body cultures derived from ES cells were distinguished from both Xp0+ and Xp0- cultures by the formation of neurons which were present as interconnecting cell networks (Figure 6.5B). Neuron formation constitutes a clear and quantifiable biological difference in the differentiation potential of ES and X cells.

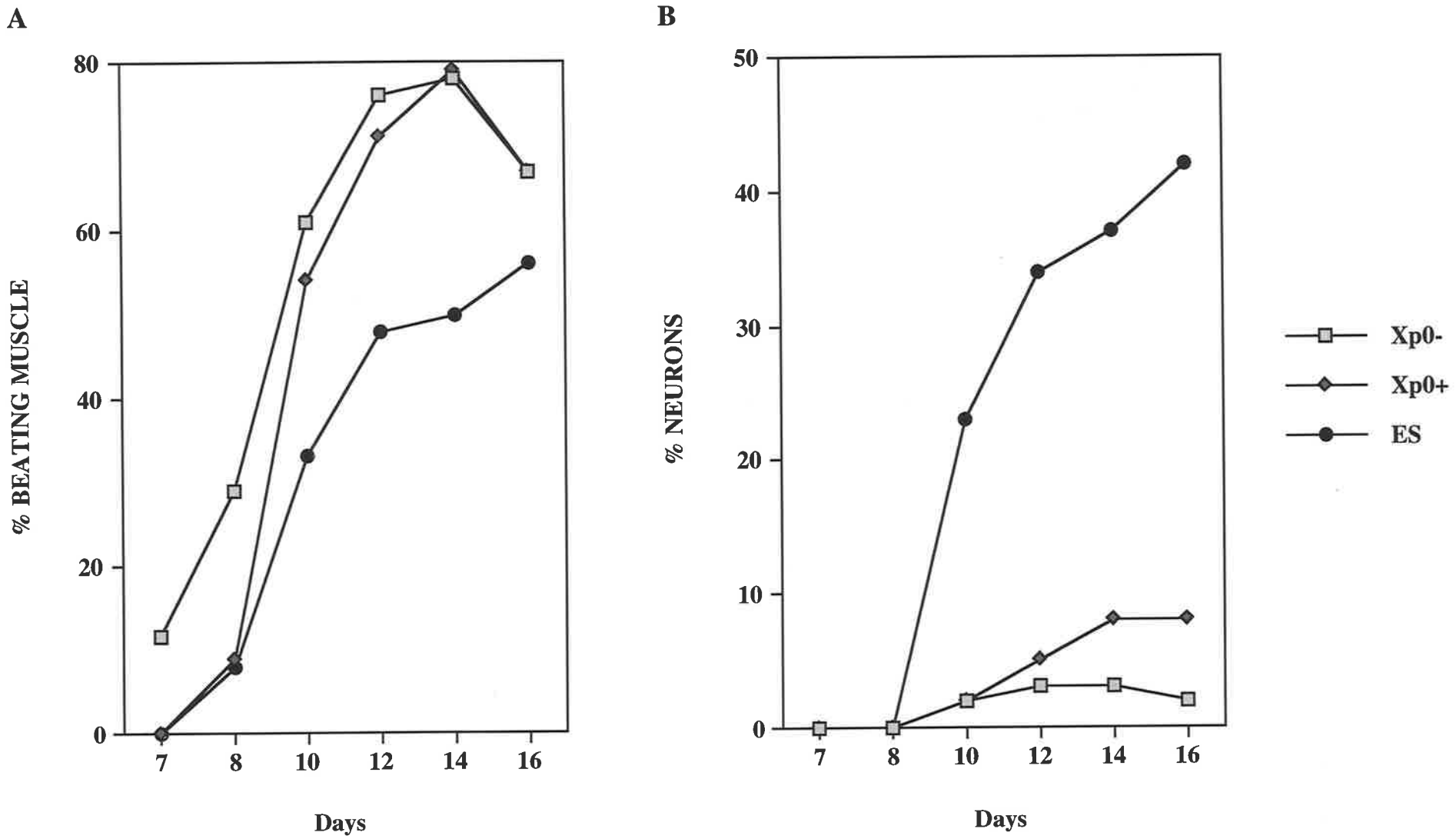
6.4.1 The Formation of Beating Muscle and Neuronal Networks in Seeded Embryoid Bodies

The profiles of beating muscle and neuron formation in ES, Xp0+ and Xp0- embryoid bodies were determined. At day 6 individual embryoid bodies were seeded into gelatinised tissue culture wells containing incomplete ES cell medium. Wells were inspected for the presence of beating muscle and/or neurons at day 7, 8, 10, 12, 14 and 16. The percentages of embryoid bodies containing beating muscle and neurons are presented graphically in Figure 6.6.

The level of beating muscle in seeded embryoid bodies derived from ES, Xp0+ and Xp0- cells was consistent with results obtained with free floating embryoid bodies (6.3). Beating muscle formation was observed at day 7 in embryoid bodies derived from Xp0- cells. It was not until day 8 that beating muscle was detected in embryoid bodies derived from ES and Xp0+ cells. At day 10 the percentage of embryoid bodies containing beating muscle was higher in Xp0- cultures (62%) than in Xp0+ cultures (54%) which in turn exhibited higher levels than ES cell cultures (32%). The percentage of beating muscle increased in all cultures thereafter. However, while Xp0+ cultures reached levels similar to Xp0- cultures at Day 14 (78%), the level observed in ES cell cultures was significantly lower (49%). The expression profile of the cardiac specific marker *Nkx 2.5* correlated with the profiles of beating muscle in these embryoid bodies (data not shown).

FIGURE 6.6 The formation of beating muscle and neurons in ES, Xp0+ and Xp0- seeded embryoid bodies.

At day 6 of development embryoid bodies derived from ES, Xp0+ and Xp0- cells were individually seeded into gelatinised tissue culture wells containing incomplete ES cell medium. The presence of beating muscle and/or neurons in individually seeded embryoid bodies was assessed by observation of each well at day 7, 8, 10, 12, 14 and 16 (2.3.17, 2.3.18). 48 embryoid bodies were analysed for each variable. The percentage of ES, Xp0+ and Xp0- embryoid bodies containing beating muscle and neurons is represented graphically in A and B respectively.


A
% BEATING MUSCLE
80
60
40
20
0
7
8
10
12
14
16
Days
B
% NEURONS
50
40
30
20
10
0
7
8
10
12
14
16
Days
Xp0-
Xp0+
ES

Neurons were not detected in cultures until day 10. At this stage 25% of the ES cell embryoid bodies contained obvious neuronal networks. This rose to 42% by day 16. Neurons were detected in embryoid bodies derived from Xp0+ (8%) and to a lesser extent Xp0- (3%) cells, however the levels were significantly lower than the levels observed in ES cell embryoid bodies.

To confirm the identity of neurons, cultures were stained for the presence of neurofilaments. ES cell and Xp0+ embryoid bodies were seeded on to glass slides at day 6 of development. Slides were stained for the presence of neurofilaments at day 12 using immunofluorescent antibody staining (2.3.25). Examples of anti-neuronal filament staining were observed in ES cell embryoid bodies (Figure 6.7) but were not observed at a significant level in X cell embryoid bodies.

The results of these experiments demonstrated that ES and X cell differentiation within embryoid bodies can be distinguished by their respective abilities to form neurons and beating muscle. The earlier onset and increased formation of cardiac muscle during differentiation of X cells as seeded embryoid bodies is consistent with the proposed relationship of X cells to the primitive ectoderm, as previously described (6.3.1). X cells were also distinguished from ES cells by their lower capacity to form neurons when cultured as embryoid bodies. Xp0+ cells again possessed differentiation capacities intermediate between those of ES and Xp0- cells.

6.5 Reverted X Cells and Embryoid Body Formation

Embryoid bodies were formed from ES, Xp0+, Xp0+R, Xp0- and Xp0-R cells as previously described (6.3). Embryoid bodies derived from reverted X cells were identical in morphology to those derived from ES cells. At day 6 embryoid bodies were seeded individually into wells as previously described (6.4.1). The number of embryoid bodies containing beating muscle and/or neurons were determined by inspection of cultures at day 7 through to 10 and subsequently at day 12. A total of 32 aggregates were analysed for each variable and the formation of neurons and beating muscle is presented in Figure 6.8.

Embryoid bodies derived from ES, Xp0+ and Xp0- cells exhibited neuron and beating muscle differentiation potentials as previously described (6.4.1). In contrast to X

FIGURE 6.7 The identification of neurons in seeded embryoid bodies.

At day 6, ES and Xp0+ embryoid bodies were seeded on to glass slides. At day 10 slides were stained for the presence of neuronal filaments, using immunofluorescent antibody staining (2.3.25). An example of neuronal staining observed in embryoid bodies derived from ES cells is shown.

The photograph were taken at a magnification of 40x under fluorescence.

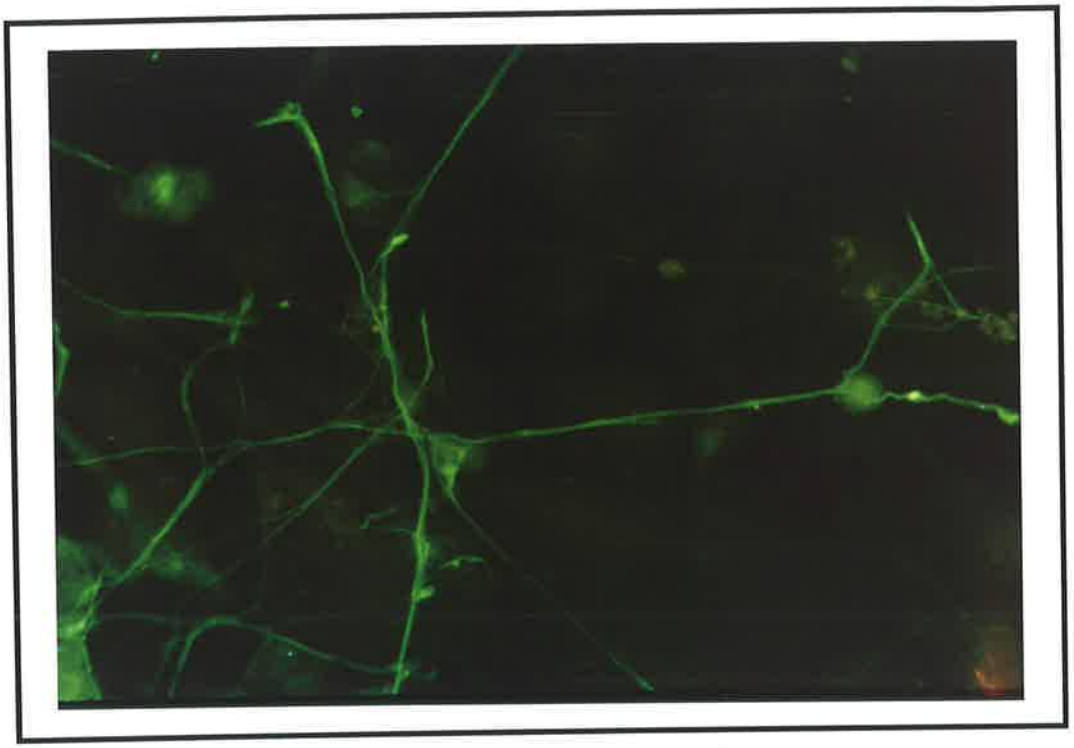
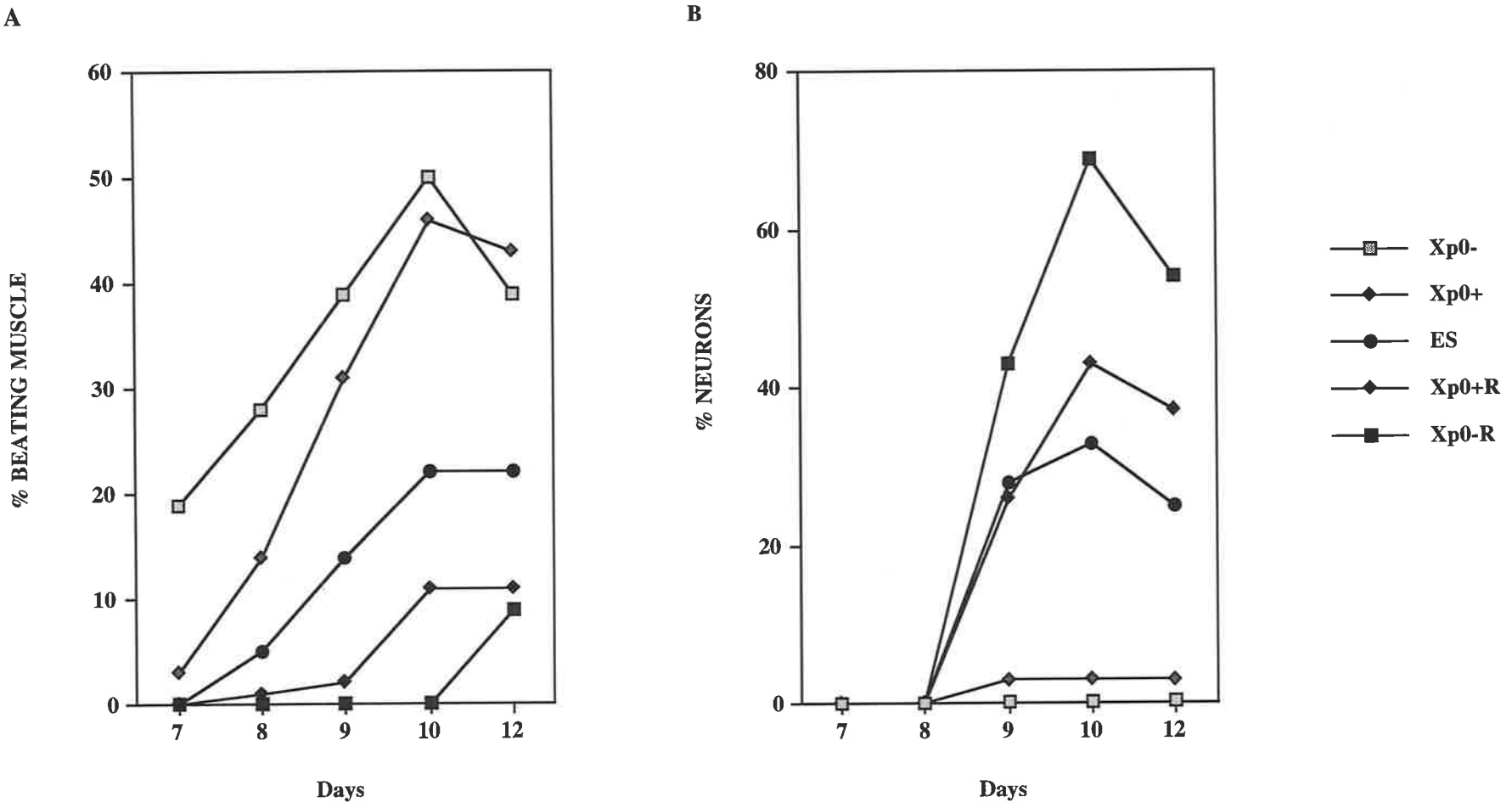


FIGURE 6.8 The formation of beating muscle and neurons in reverted Xp0+ and Xp0- cells.

At day 6 of development embryoid bodies derived from ES, Xp0+, Xp0+R, Xp0- and Xp0+R cells were individually seeded into gelatinised tissue culture wells containing incomplete ES cell medium. The number of embryoid bodies containing beating muscle and neurons were determined on day 7, 8, 10 and 12 (2.3.17, 2.3.18). The percentage of embryoid bodies containing beating muscle and neurons were determined from the analysis of 32 wells for each variable. The percentage of beating muscle is shown in graph A and the percentage of neurons observed in these cultures is shown in graph B.


A
% BEATING MUSCLE
60
50
40
30
20
10
0
7
8
9
10
12
Days
B
% NEURONS
80
60
40
20
0
7
8
9
10
12
Days
Xp0-
Xp0+
ES
Xp0+R
Xp0-R

cells, reverted X cells exhibited a lower potential for beating muscle formation and a higher potential for neuron formation. Reverted X cells therefore differentiated along differentiation pathways analogous to ES cells when cultured as embryoid bodies. This complements the gene expression profile of reverted X cells which is comparable to the gene expression profile of ES cells and indicates that X cell reversion constitutes a genuine event with respect to differentiation potential.

6.6 X Cells form Neuronal Networks at High Frequency when Treated with Retinoic Acid

The differentiation of murine embryonal carcinoma (EC) cells with chemical inducers such as retinoic acid (RA) and DMSO, has been examined by a number of investigators (Strickland and Mahdavi 1978, Strickland *et al.* 1980, Hogan *et al.* 1981, McBurney *et al.* 1982). P19 EC cells are widely regarded as similar to the primitive ectoderm (Rogers *et al.* 1991). Consistent with this is the observation that P19 EC cells, unlike other EC/ES cell lines, do not express *Rex-1* (Hosler *et al.* 1989, Rogers *et al.* 1991). X cells express lower levels of *Rex-1* than ES cells and have a morphology which is very similar to P19 EC cells. When P19 EC cells are aggregated in the presence of retinoic acid (RA) they differentiate predominantly into neuroectodermal lineages, including neurons and astroglial cells, and a poorly defined fibroblastic mesodermal cell type (McBurney *et al.* 1982, Jones-Villeneuve *et al.* 1982). X cells have a low capacity to form neurons when differentiated as embryoid bodies (6.4.1). Given the similarities between X cells and P19 EC cells it is possible that differentiation with RA could allow the differentiation potential of X cells with regard to neuron formation to be assessed.

ES, Xp0+ and Xp0- cells were trypsinised and resuspended in bacteriological petri dishes at a cell density of 10^5 cells/ml in incomplete ES cell medium containing 10^{-6} M RA (2.3.20). After four days aggregates were cultured for 2 days in the absence of RA, then seeded into gelatinised tissue culture dishes containing incomplete ES cell medium. After a further 2 days, aggregates were inspected for the presence of differentiated cell types. RA-treated aggregates derived from ES, Xp0+ and Xp0- cells differentiated into four principal cell types, neurons, fibroblasts and cells with morphologies reminiscent of

visceral and parietal endoderm (Van den Eijen-van Raaij *et al.* 1991, Strickland *et al.* 1980). Photographs of these four cell types are shown in Figure 6.9.

The proportion of cell types present in ES, Xp0+ and Xp0- RA-treated aggregates was determined by inspection of individually seeded aggregates. ES, Xp0+ and Xp0- cells were aggregated in the presence of RA as previously described. At day 6 aggregates were seeded individually into gelatinised wells containing incomplete medium and scored for the presence of differentiated cell types after 2 days. 36 aggregates were analysed for each variable (ES, Xp0+ and Xp0-) and the results of this analysis are presented in Figure 6.10.

RA-treated aggregates derived from ES, Xp0+ and Xp0- exhibited similar capacities for neuronal formation of 65%, 62% and 70%, respectively. Differences were seen between ES, Xp0+ and Xp0- in the formation of cells classed as parietal endoderm, visceral endoderm and fibroblast cells. RA-treated aggregates derived from ES cells were characterised by a high level of parietal endoderm (61%), a lower level of visceral endoderm (36%) and a very low level of fibroblasts (3%). Xp0+ RA-treated aggregates were distinguished from ES cell cultures by the presence of a higher level of fibroblasts (14%) and a lower level of visceral endoderm (23%). The level of parietal endoderm in Xp0+ cultures was similar to that observed in ES cell cultures (63%). Xp0- cultures were distinguished from both ES and Xp0+ cultures by a higher level of fibroblasts (46%) and lower levels of visceral (9%) and parietal endoderm (45%).

SPARC is expressed in the parietal endoderm of the murine embryo (Holland *et al.* 1987) and therefore can be used to identify cells classed as parietal endoderm. *In situ* hybridisation was carried out on RA-treated aggregates derived from ES cells using *SPARC* DIG labelled antisense riboprobes. *SPARC* expression was observed in cells classed as parietal endoderm based on morphology (Figure 6.11).

SPARC expression levels in RA-treated aggregates derived from ES, Xp0+ and Xp0- were determined by Northern blot analysis of total RNA. The expression of *SPARC* compared with a *mGAP* loading control is shown in Figure 6.12. As expected from observation of individually seeded aggregates, ES and Xp0+ RA-treated aggregates expressed higher levels of *SPARC* than Xp0- aggregates.

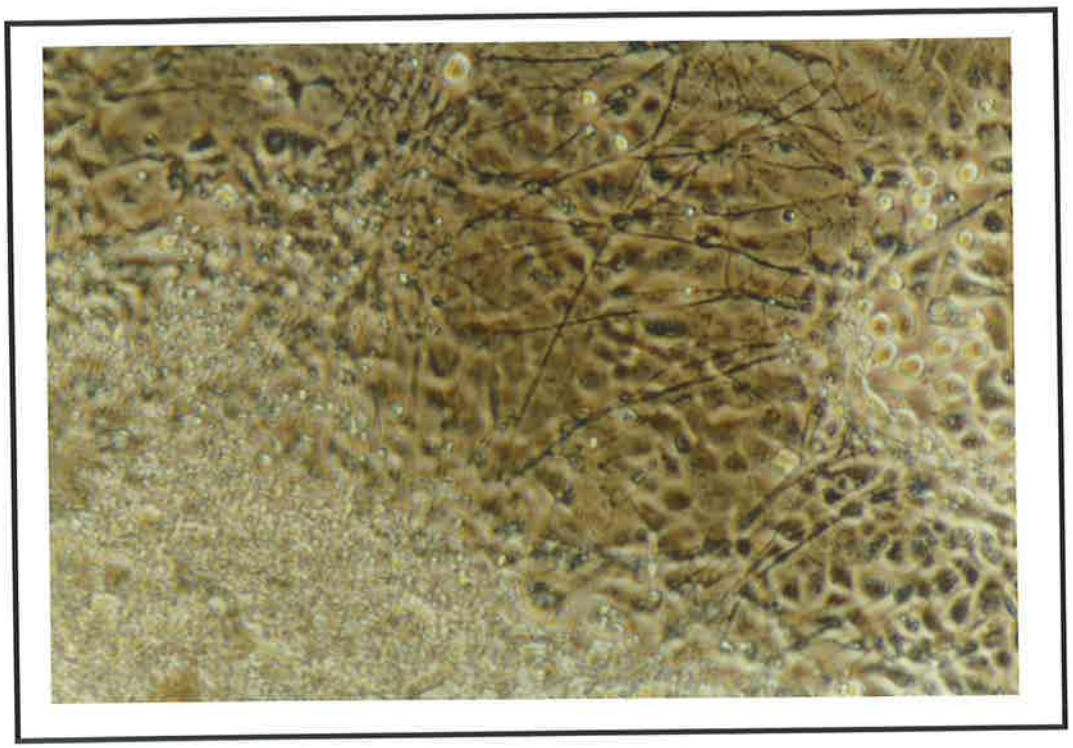
FIGURE 6.9 The morphology of differentiated cell types observed in RA-treated aggregates derived from ES, Xp0+ and Xp0- cells.

When ES, Xp0+ and Xp0- cells were differentiated by aggregation in the presence of RA (2.3.20), four distinct cells types were observed, neurons (A), fibroblasts (B), parietal endoderm (C) and visceral endoderm (D).

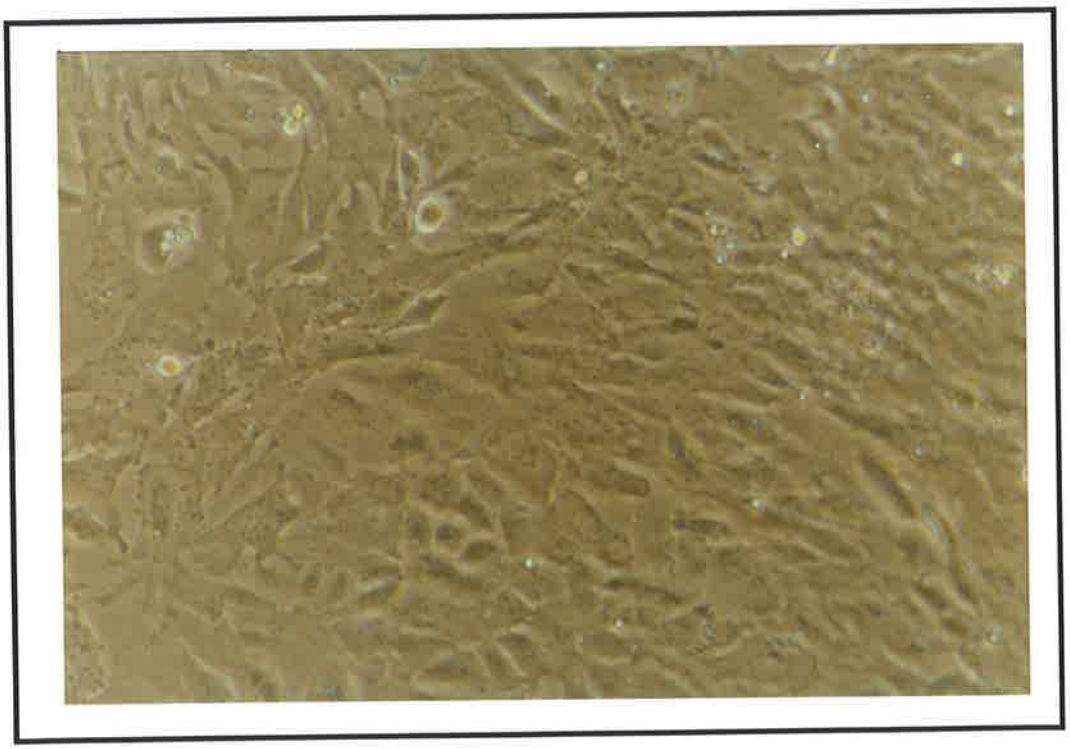
Photographs were taken at a magnification of 50x using phase contrast optics.

Photographs A, B and C are of X cells treated with RA.
Photograph D is from ES cells treated with RA

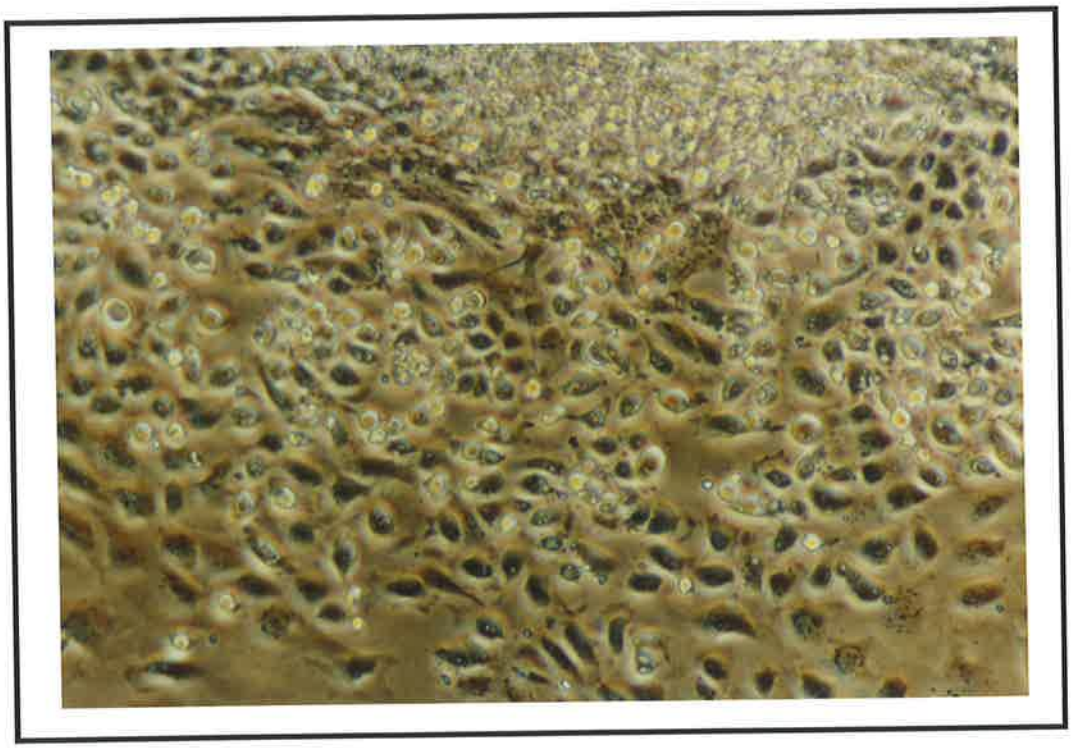
A



B



C



D

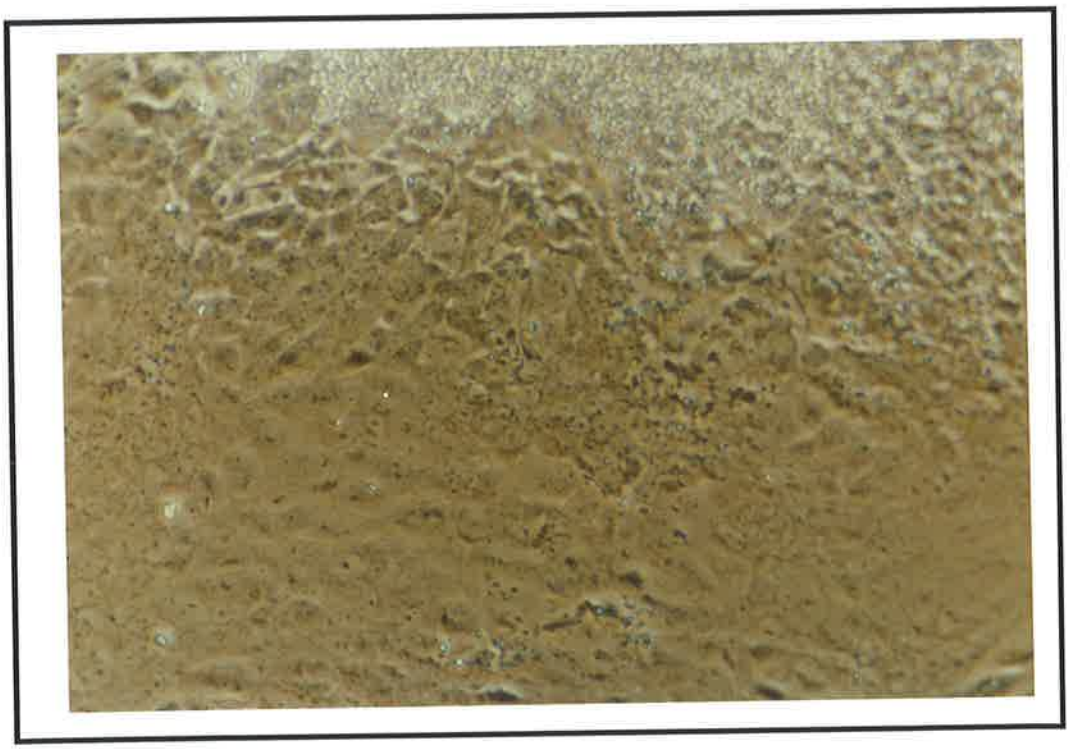
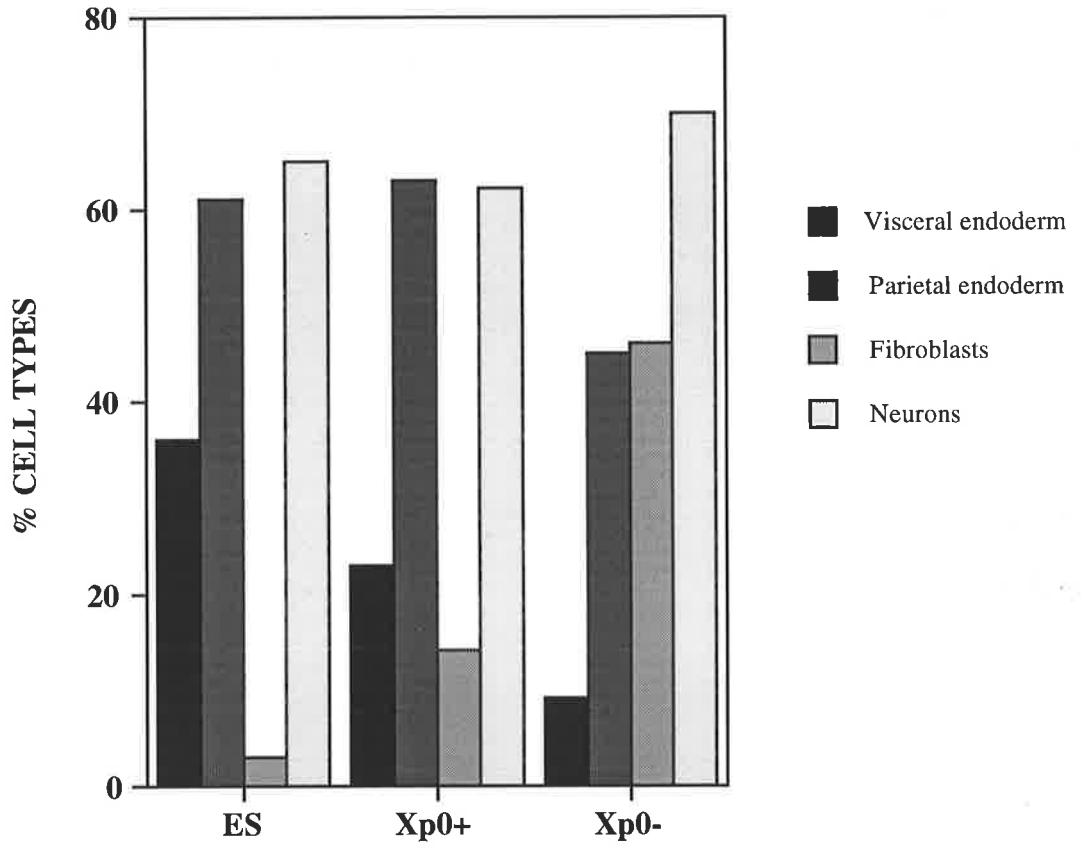


FIGURE 6.10 The proportion of differentiated cell types in ES, Xp0+ and Xp0- RA-treated aggregates.

RA-treated aggregates derived from ES, Xp0+ and Xp0- cells were individually seeded into tissue culture wells containing incomplete ES cell medium (2.3.20). After 2 days individually seeded aggregates were scored for the presence of the cell types outlined in Figure 6.9. 36 aggregates were analysed for each variable (ES, Xp0+ and Xp0-). The presence of neurons was established by examination of each well. The percentage of neurons was determined by dividing the number of wells scored as positive for neurons with the total number of wells examined. Since aggregates often contained more than one of the other cell types, the percentage of each cell type within the well was determined. The percentage of each cell type present in each well was totalled and divided by the number of wells examined. Results of this analysis are presented graphically in A and tabulated in B.

A



B

	% CELL TYPES			
	Visceral endoderm	*Parietal endoderm*	*Fibroblasts*	*Neurons*
ES	*36*	*61*	*3*	*65*
Xp0+	*23*	*63*	*14*	*62*
Xp0-	*9*	*45*	*46*	*70*

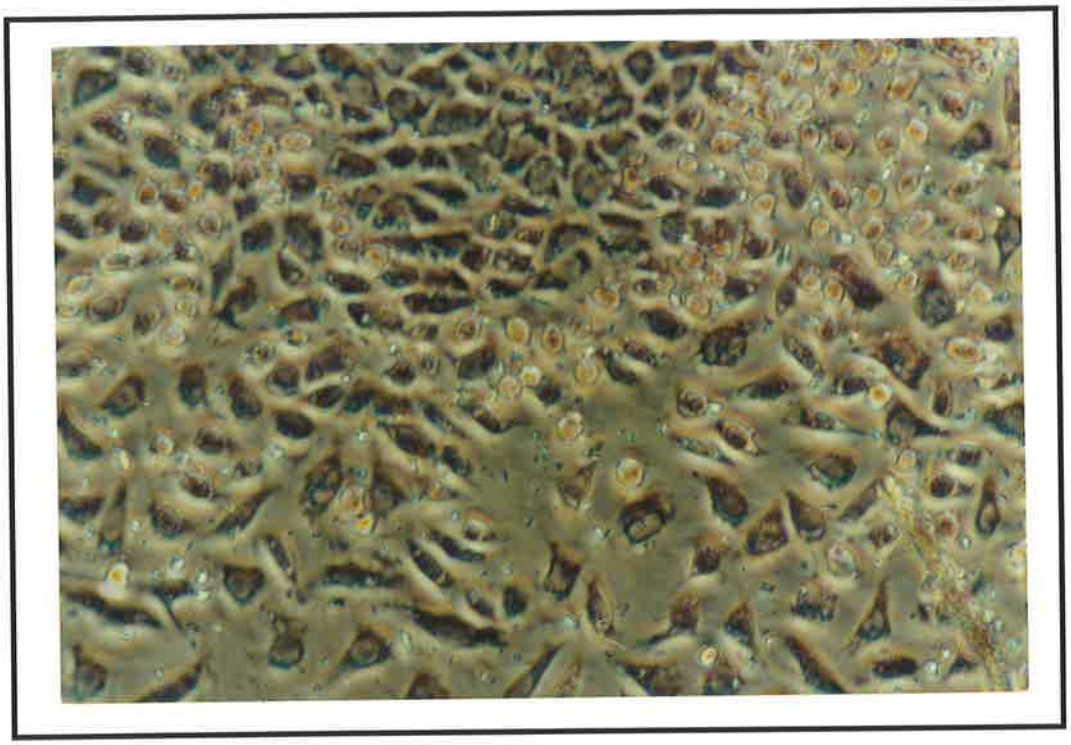
FIGURE 6.11 Identification of parietal endoderm by *in situ* analysis of *SPARC* expression.

SPARC expression was detected by *in situ* hybridisation, using a 1.5kb DIG labelled antisense riboprobe (2.5.17), on RA-treated aggregates derived from ES, Xp0+ and Xp0- cells (2.3.20). Plates were developed for 5 hours. Cells expressing *SPARC* were detected by purple staining. Expression was confined to cells with the morphology of parietal endoderm.

Photographs were taken under phase contrast (A) and bright field optics (B) at a magnification of 50x.

Photographs A & B are examples of SPARC expression in RA-treated aggregates derived from ES cells.

A



B

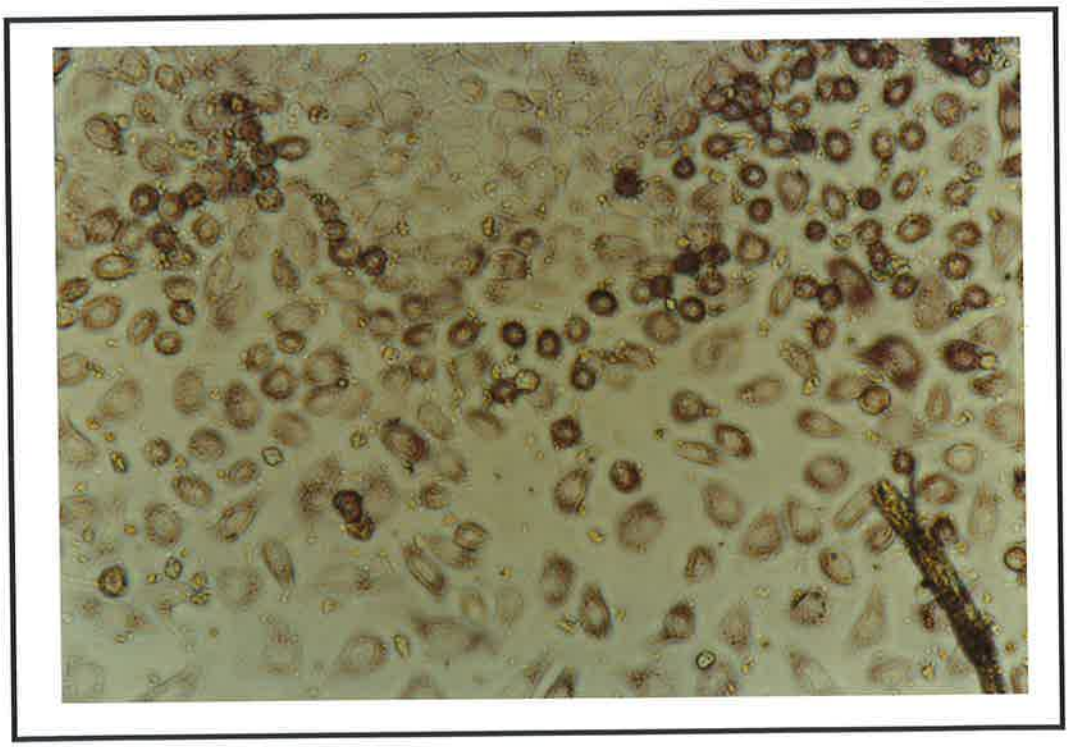
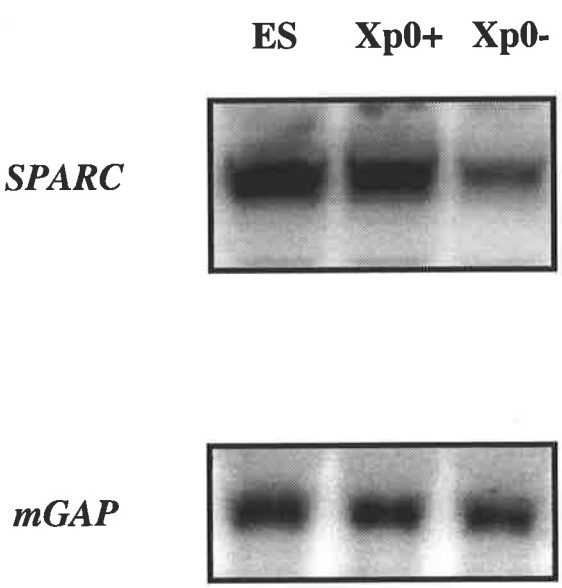


FIGURE 6.12 The expression of *SPARC* in RA-treated aggregates derived from ES, Xp0+ and Xp0- cells.

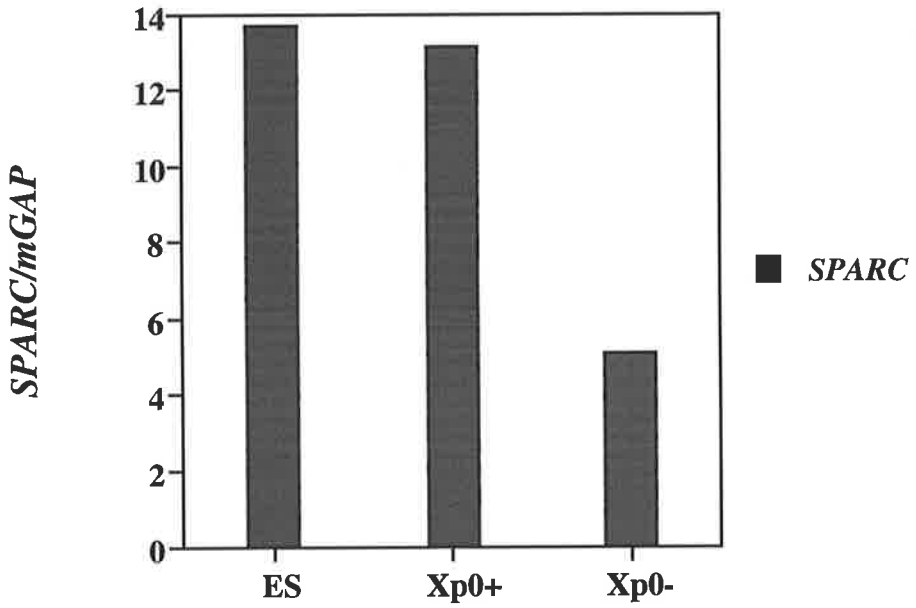
RNA was prepared from RA-treated aggregates derived from ES, Xp0+ and Xp0- cell (2.5.11). The expression of *SPARC* was determined by Northern analysis of total RNA (2.3.12) using a *SPARC* DNA probe (2.5.14). The filter was washed in 2xSSC/0.1%SDS at 42°C for 20 minutes, 0.2xSSC/0.1%SDS at 42°C for 20 minutes, 0.2xSSC/0.1%SDS at 65°C for 30 minutes and finally in 0.1xSSC/0.1%SDS 65°C for 60 minutes. The filter was exposed to a phosphorimager screen for 24 hours (2.5.20). A band corresponding to the 2.2kb *SPARC* transcript could be detected in RNA derived from RA-treated aggregates. The filter was stripped and reprobed with a *mGAP* loading control (2.5.14, 2.5.16), exposure time 21 hours. The results of this analysis are presented in A.

The level of *SPARC* and *mGAP* expression in ES, Xp0+ and Xp0- RA-treated aggregates was quantitated by volume integration (2.5.20). The level of *SPARC* expression normalised to *mGAP* is represented graphically in B.

A


ES
Xp0+
Xp0-
SPARC
mGAP

B


SPARC/mGAP
14
12
10
8
6
4
2
0
ES
Xp0+
Xp0-
SPARC

These results firstly confirm the ability of X cells to differentiate into neurons as would be predicted for primitive ectoderm cells. This differs from the observation during embryoid body differentiation which indicates that X cells have a reduced capacity to form neurons compared to ES cells. The reason for this apparent discrepancy probably lies in the nature of the differentiation environments. Embryoid body differentiation is more complex than differentiation with RA. Embryoid bodies give rise to different tissues in a sequential manner and these tissues in turn produce factors which influence the differentiation of other tissues. The differentiation of neural tissue is a relatively late step in development both *in vitro* and *in vivo* (Kaufman 1992, work described here). Therefore the inability of X cells to form neurons when differentiated within embryoid bodies may be due to the absence of a neural inducer or conversely the presence of a neural repressor at this particular stage of development. Clearly it does not reflect an innate restriction of X cell developmental potential.

Secondly, the distribution of cell types formed upon differentiation in the presence of RA defines further differences between the differentiation potential of ES and X cells. ES cell RA-treated aggregates were characterised by the presence of a high proportion of primitive endodermal cell types and a low proportion of fibroblasts, while Xp0- aggregates were characterised by a lower proportion of primitive endodermal cell types and a higher level of fibroblasts. Aggregates derived from Xp0+ cells exhibited cell types at a level intermediate between those observed in ES and Xp0- cultures. This is consistent with the behaviour of Xp0+ cells during embryoid body differentiation and appears to be related to their initial gene expression profile (6.3.1).

Cells with a fibroblastic morphology are thought to have a mesodermal origin. It can therefore be concluded that when X cells are differentiated by aggregation in the presence of RA they have a higher capacity to form mesoderm and a lower capacity to form primitive endodermal cell types. This is consistent with their differentiation as embryoid bodies (6.3, 6.4) and supports a proposed relationship between X cells and the cells of the primitive ectoderm.

6.7 Gene Expression During Embryoid Body Differentiation

Differentiation events occurring during embryoid body development parallel differentiation events occurring during murine embryogenesis. The inner cell mass gives rise to primitive endoderm and then subsequently primitive ectoderm. The primitive ectoderm through the process of gastrulation gives rise to the mesoderm, endoderm and ectoderm of the embryo. A number of genes which are expressed stage and tissue specifically during murine embryogenesis have been identified.

The expression of genes specific for the inner cell mass, primitive ectoderm, primitive endoderm and early mesoderm were examined during the differentiation of ES, Xp0+ and Xp0- embryoid bodies.

6.7.1 Formation of Primitive Ectoderm in Embryoid Bodies

The formation of primitive ectoderm in embryoid bodies derived from ES, Xp0+ and Xp0- embryoid bodies was examined by analysing the expression of *Rex-1*, *FGF-5* and *Oct-4* during embryoid body differentiation. *Rex-1* is highly expressed in the ICM of the preimplantation murine blastocyst and expression decreases as the ICM forms the primitive ectoderm. *Rex-1* expression is not detected in the primitive ectoderm at day 6.0 of development (Rogers *et al.* 1991). The expression of *FGF-5* during early development is confined to the primitive ectoderm prior to and during gastrulation, from day 5.25 to day 7.75. *FGF-5* is not expressed in the ICM of preimplantation embryos at day 3.25 (Haub and Goldfarb 1991). *Oct-4* is expressed in the inner cell mass and primitive ectoderm (Scholer *et al.* 1990b, Rosner *et al.* 1991).

ES, Xp0+ and Xp0- embryoid bodies were harvested for RNA preparation at days 1, 2, 3 and 4. The expression of *Rex-1*, *FGF-5* and *Oct-4* was assessed by Northern analyses of total RNA and compared to a *mGAP* loading control. The results of these analysis are presented in Figure 6.13. The expression of each gene was quantified by volume integration. The ratio of the expression of each gene, normalised against a *mGAP* loading control is represented graphically in Figure 6.14.

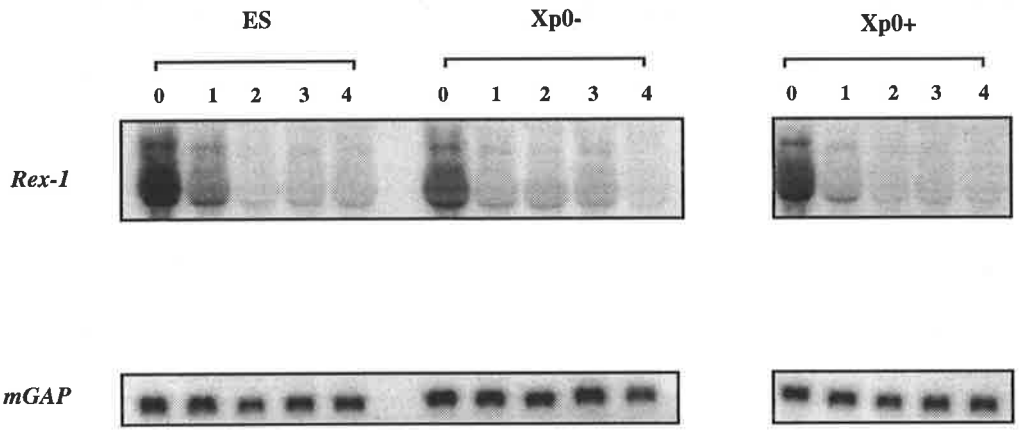
Rex-1 expression decreased as ES, Xp0+ and Xp0- cells were differentiated as embryoid bodies. One day after embryoid body formation, *Rex-1* expression had declined substantially in Xp0- and Xp0+ cultures. It was not until day 2 that *Rex-1*

FIGURE 6.13 Northern blot analysis of *Rex-1*, *FGF-5* and *Oct-4* expression in ES, Xp0+ and Xp0- embryoid bodies.

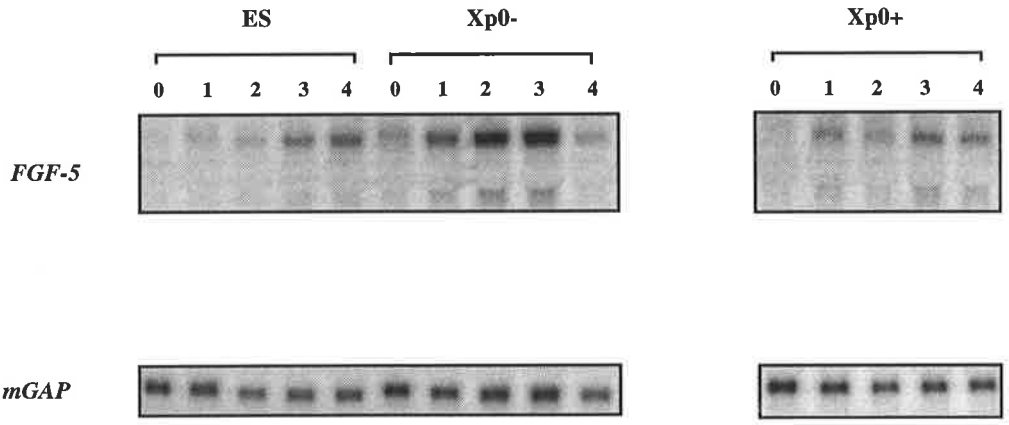
RNA was prepared from ES, Xp0+ and Xp0- cells harvested at day 0 (2.5.10) and at day 1,2,3 and 4 of embryoid body development (2.5.11). The expression of *Rex-1*, *FGF-5* and *Oct-4* compared to a *mGAP* loading control was determined by Northern blot analysis of total RNA (2.5.12). DNA probes were used in all analyses (2.5.14). Filters were washed in 2xSSC/0.1%SDS at 42°C, 0.2xSSC/0.1%SDS at 42°C and finally in 0.2xSSC/0.1%SDS at 65°C. All washes were for a minimum duration of 30 minutes. Filters were exposed to phosphorimager screens for 18 hours (2.5.20) and subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16), exposure time 20 hours.

The expression of the 1.9 kb *Rex-1* transcript is shown in A) The expression of the 2.7 and 1.8kb *FGF-5* transcripts are shown in B). The expression of the 1.6kb *Oct-4* transcript is shown in C)

A *Rex-1* EXPRESSION



B *FGF-5* EXPRESSION



C *Oct-4* EXPRESSION

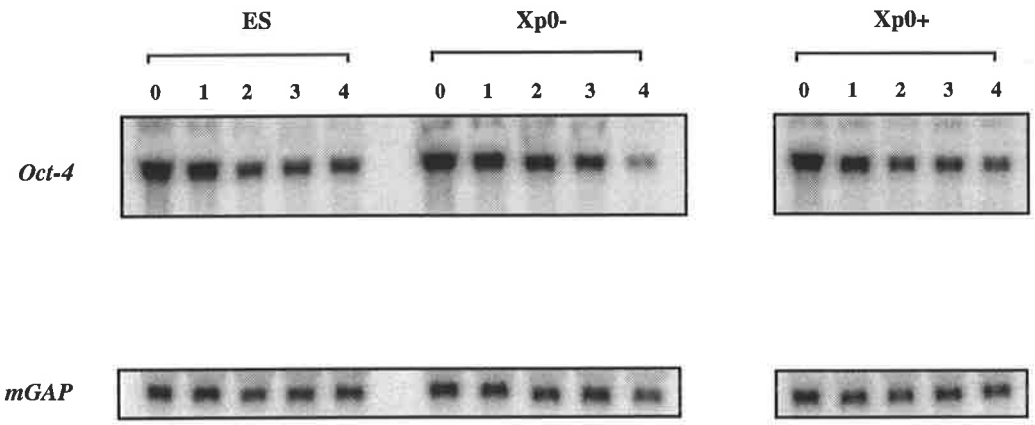
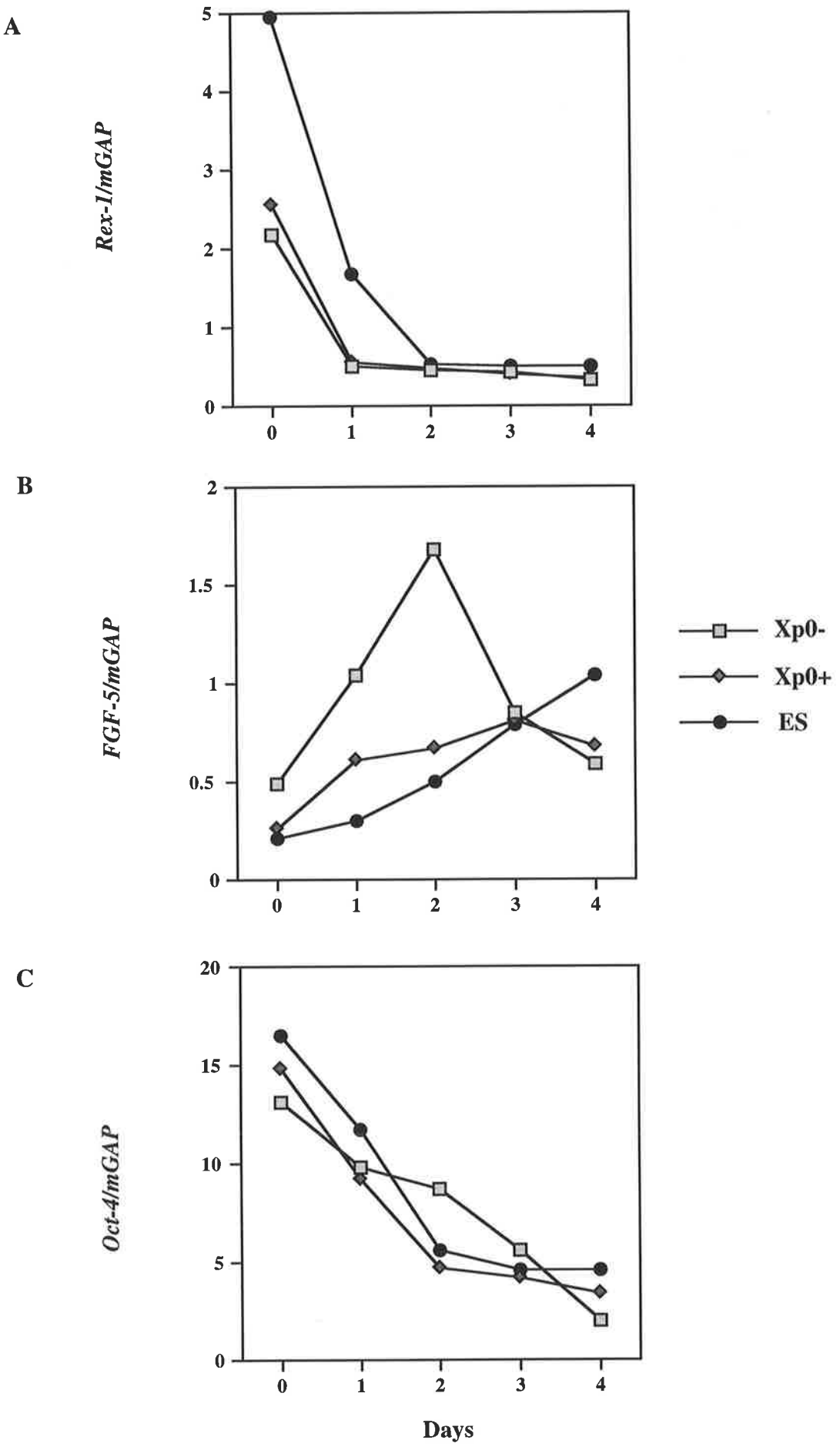


FIGURE 6.14 Comparison of *Rex-1*, *FGF-5* and *Oct-4* expression in embryoid bodies derived from ES, Xp0+ and Xp0- cells.

The level of *Rex-1*, *FGF-5*, *Oct-4* and *mGAP* expression (Fig. 6.13) was quantitated by volume integration (2.5.20). *Rex-1* expression, normalised to *mGAP*, in ES, Xp0+ and Xp0- embryoid bodies from day 0 to day 4 is shown in graph A. *FGF-5* expression normalised to *mGAP* for these embryoid bodies is represented graphically in B. Similarly *Oct-4* expression is shown in graph C.


A
Rex-1/mGAP
5
4
3
2
1
0
0
1
2
3
4
B
FGF-5/mGAP
2
1.5
1
0.5
0
0
1
2
3
4
Xp0-
Xp0+
ES
C
Oct-4/mGAP
20
15
10
5
0
0
1
2
3
4
Days

expression reached a similar level in ES cell cultures. *FGF-5* expression increased as ES, Xp0+ and Xp0- cells differentiated as embryoid bodies. The level of *FGF-5* expression increased dramatically in Xp0- cells and at day 2 reached a maximum level far greater than the levels observed in ES and Xp0+ cultures, before declining. At day 1 and 2 of embryoid body development *FGF-5* expression was higher in Xp0+ cultures than in ES cell cultures. *Oct-4* expression decreased steadily during the differentiation of ES, Xp0+ and Xp0- cells as embryoid bodies.

Consistent with differentiation of the inner cell mass to the primitive ectoderm *in vivo*, *Rex-1* expression decreased and *FGF-5* expression increased during embryoid body formation *in vitro*. Formation of late stage primitive ectoderm occurred earlier in X cell embryoid bodies than ES cell embryoid bodies. This is consistent with the proposed relationship of X cells with the early primitive ectoderm. Late stage primitive ectoderm is the substrate for mesoderm formation during gastrulation *in vivo*. The earlier formation of late stage primitive ectoderm in X cell embryoid bodies may therefore explain the earlier onset of cardiac muscle formation in X cell embryoid bodies when compared to ES cell embryoid bodies.

6.7.2 Formation of Mesoderm in Embryoid Bodies

To determine whether the ability of X cell embryoid bodies to form cardiac muscle was also related to an increased or earlier appearance of mesoderm, the expression of the early mesodermal markers *Brachyury* and *goosecoid* was examined. *Brachyury* expression *in vivo* begins at gastrulation and is restricted to the primitive streak and mesodermal cells emerging from the streak. At later stages of development expression is restricted to the notochord and head process (Herrmann *et al.* 1990). *Goosecoid* expression commences prior to gastrulation in the region of the epiblast where the primitive streak forms. Expression is confined to this region of the primitive streak as it moves anteriorly towards the node. At day 6.9 *goosecoid* expression is no longer detected (Blum *et al.* 1992).

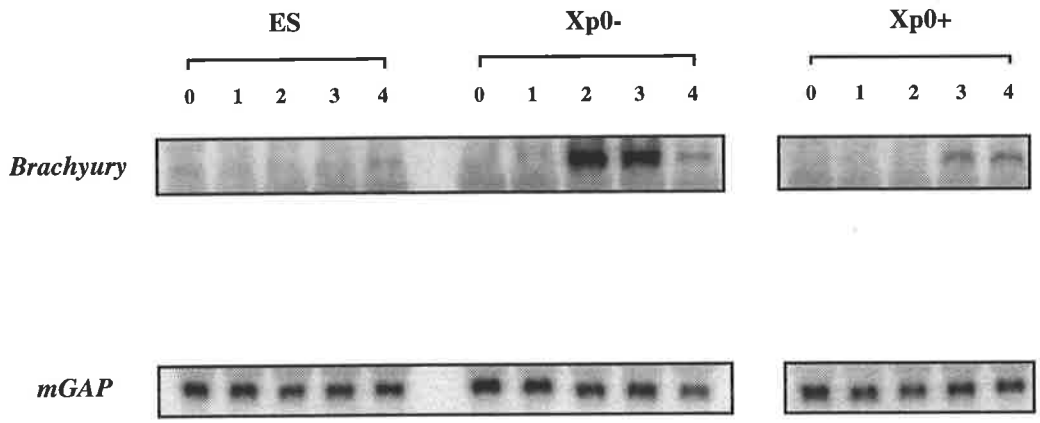
The expression of *Brachyury* and *goosecoid* in ES, Xp0+ and Xp0- embryoid bodies was determined by Northern analysis of total RNA and compared with *mGAP* expression (Figure 6.15). The RNA used in this analysis was derived from ES, Xp0+

FIGURE 6.15 Northern blot analysis of *Brachyury* and *goosecoid* expression in ES, Xp0+ and Xp0- embryoid bodies.

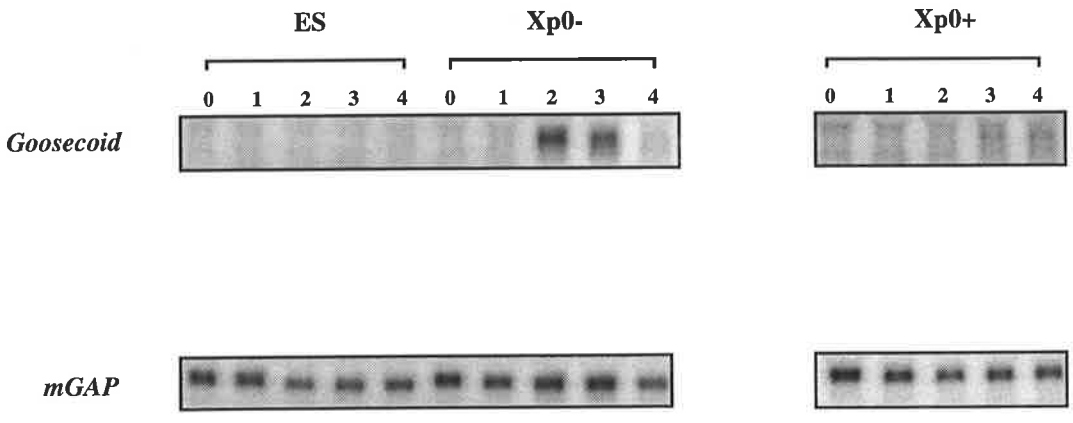
RNA was prepared from ES, Xp0+ and Xp0- embryoid bodies cultures at day 0, 1, 2, 3 and 4 of development (6.7.1). The expression of *Brachyury* and *goosecoid* compared to a *mGAP* loading control was determined by Northern blot analysis of total RNA. Antisense riboprobes were used to detect expression (2.5.15). Filters were washed in 2xSSC/0.1%SDS at 65°C, 0.2xSSC/0.1%SDS at 65°C and finally in 0.2xSSC/0.1%SDS at 75°C. All washes were for a minimum duration of 30 minutes. Filters were exposed to phosphorimager screens for 20 hours (2.5.20). The filters were subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16), exposure time 20 hours.

The expression of the 2.1kb *Brachyury* transcript is shown in A). The expression of the 1.4 kb *goosecoid* transcript is shown in B).

A *Brachyury* EXPRESSION



B *Goosecoid* EXPRESSION



and Xp0- embryoid bodies from days 1 through to 4 (Section 6.7.1). The levels of expression of *Brachyury*, *goosecoid* and *mGAP* were quantitated by volume integration and the ratios of *Brachyury* and *goosecoid* expression normalised against a *mGAP* loading control are presented graphically in Figure 6.16.

As Xp0- cells differentiated as embryoid bodies, a dramatic increase in the expression of *Brachyury* was seen at day 2 of development. By day 3 the level declined slightly and by day 4 *Brachyury* was expressed at low levels. In embryoid bodies derived from Xp0+ cells *Brachyury* expression was first detected at day 3 at lower levels than in Xp0- cells, this level increased slightly at day 4. In ES cell embryoid body cultures *Brachyury* was expressed at a very low level for the first time at day 4.

Goosecoid expression parallelled *Brachyury* expression during embryoid body differentiation. Significant expression could be detected in Xp0- embryoid bodies at day 2 and at a slightly lower level at day 3. *Goosecoid* expression could be detected at low levels in Xp0+ embryoid bodies at day 3 and day 4 but could not be detected at significant levels in ES cells at any stage examined (Figure 6.16 B).

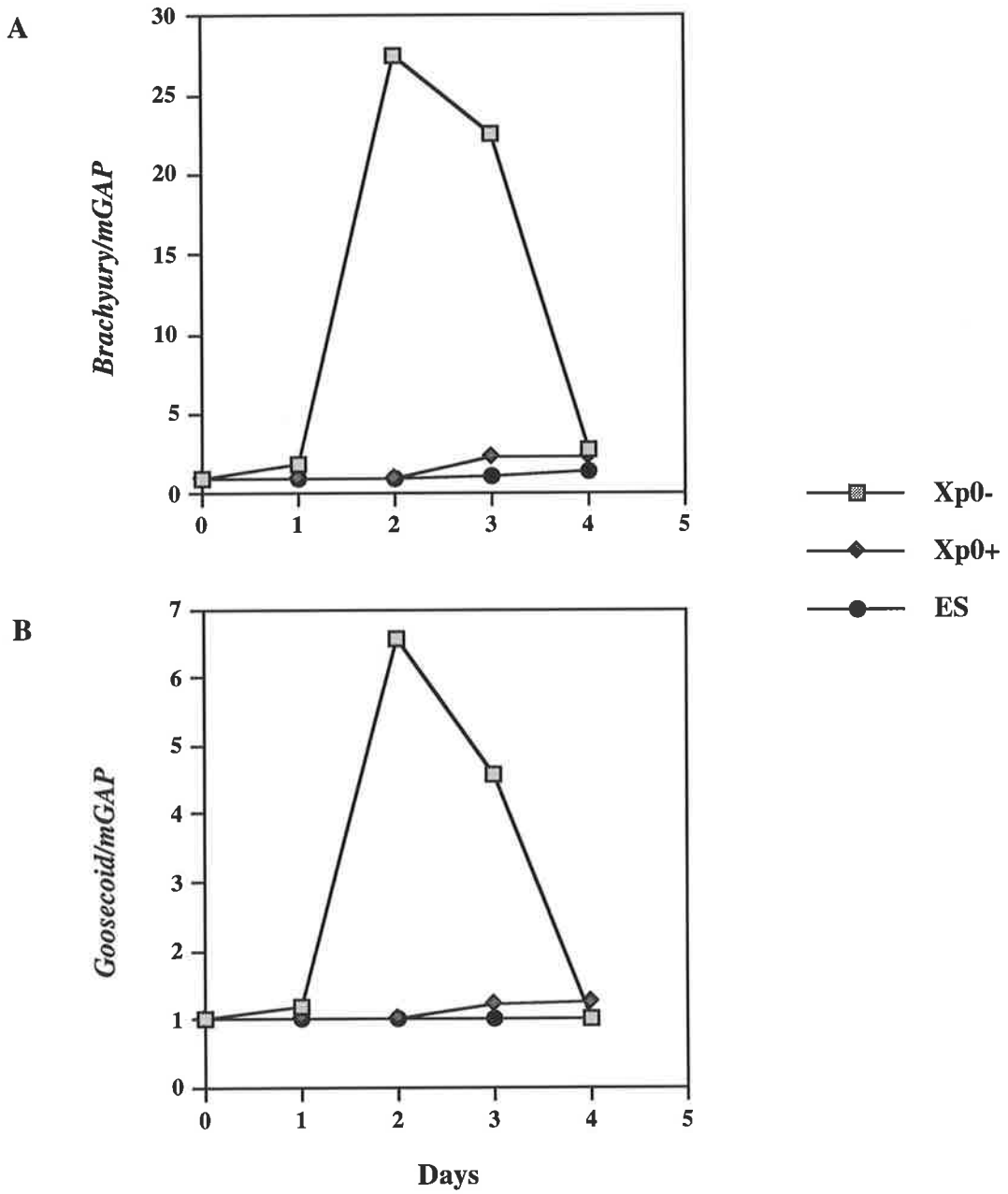
The expression patterns of *goosecoid* and *Brachyury* suggest that X cells form early mesoderm prior to and at a higher extent than ES cell embryoid bodies. This correlates with the earlier and greater capacity for late stage primitive ectoderm formation and cardiac muscle formation compared to ES cell embryoid bodies. The appearance of late stage primitive ectoderm, early mesoderm and cardiac muscle in X cell embryoid bodies is consistent with the proposed relationship between X cells and the early primitive ectoderm.

6.7.3 Formation of Primitive Endodermal Derivatives in Embryoid Bodies

To investigate the presence of primitive endodermal cell types in ES, Xp0+ and Xp0- embryoid bodies, RNA derived from embryoid bodies at days 1, 2, 3 and 4 of development (6.7.1) was analysed for the expression of markers expressed in derivatives of the primitive endoderm. *H19* is expressed in the extraembryonic cells of the murine embryo from day 5.5 (Poirier *et al.* 1991) and is a marker for both visceral and parietal endoderm (Shen and Leder 1992). *SPARC* is expressed in the parietal endoderm of the murine embryo at day 8.0 (Holland *et al.* 1987, Wilkinson *et al.* 1988). *AFP* is

FIGURE 6.16 Comparison of *Brachyury* and *goosecoid* expression in embryoid bodies derived from ES, Xp0+ and Xp0- cells.

The level of *Brachyury*, *goosecoid* and *mGAP* expression (Fig. 6.15) was quantitated by volume integration (2.5.20). *Brachyury* expression, normalised to *mGAP*, in ES, Xp0+ and Xp0- embryoid bodies from day 0 to day 4 is shown in graph A. *Goosecoid* expression normalised to *mGAP* for these embryoid bodies is represented graphically in B.


A
B
Brachyury/mGAP
Goosecoid/mGAP
Days
Xp0-
Xp0+
ES
0
5
10
15
20
25
30
1
2
3
4
6
7

expressed in the visceral endoderm of murine embryos from day 7.0 (Dziadek and Adamson 1978, Dziadek and Andrews, 1983). The expression of *H19*, *SPARC* and *AFP* compared to a *mGAP* loading control was assessed by Northern analysis of total RNA. The results of this analysis are presented in Figure 6.17. The ratio of the expression of each gene normalised against *mGAP* is presented graphically in Figure 6.18.

At day 2 of development *H19* expression was approximately the same in embryoid bodies derived from ES, Xp0+ and Xp0- cells. After this stage the level of *H19* expression increased dramatically in Xp0- cultures. *H19* expression also increased in Xp0+ cultures and to a lesser extent ES cell cultures, however these increases were lower than the increase in Xp0- cultures.

SPARC expression levels also increased during embryoid body development. Xp0- embryoid bodies exhibited higher levels of *SPARC* expression than Xp0+ embryoid bodies which in turn exhibited higher levels than ES cell embryoid bodies at day 3 and 4.

AFP expression was at very low levels in embryoid bodies derived from ES, Xp0+ and Xp0- at day 4 of development and was not significantly different between cultures. Visceral endoderm can be distinguished in the early murine embryo at day 5.5 of development (Kaufman 1992). This suggested that *AFP* expression may be better examined in embryoid bodies at later stages of development. *AFP* expression was analysed by Northern analysis of RNA derived from ES, Xp0+ and Xp0- embryoid bodies at days 4, 6 and 8 of development. The results of this analysis are presented in Figure 6.19. The level of *AFP* expression increased with development, however expression was significantly higher in Xp0+ and Xp0- embryoid bodies than in ES cell embryoid bodies. This has also been shown in RNA derived from seeded ES, Xp0+ and Xp0- embryoid bodies at day 7, 8 and 10 (data not shown).

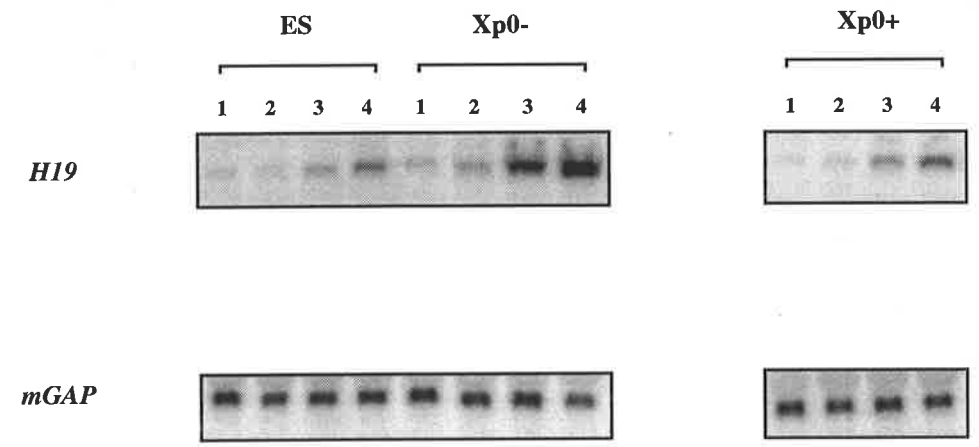
ES cell embryoid bodies can be distinguished from X cell embryoid bodies by the presence of a discernible outer layer of cells reminiscent of primitive endoderm at day 4, and by the presence of visceral endoderm-like cells at day 8. These differences were not reflected by increases in the expression of *H19*, *SPARC* or *AFP* in ES cell embryoid bodies compared to X cell embryoid bodies at relevant stages.

FIGURE 6.17 Northern blot analysis of *H19*, *SPARC* and *AFP* expression in ES, Xp0+ and Xp0- embryoid bodies.

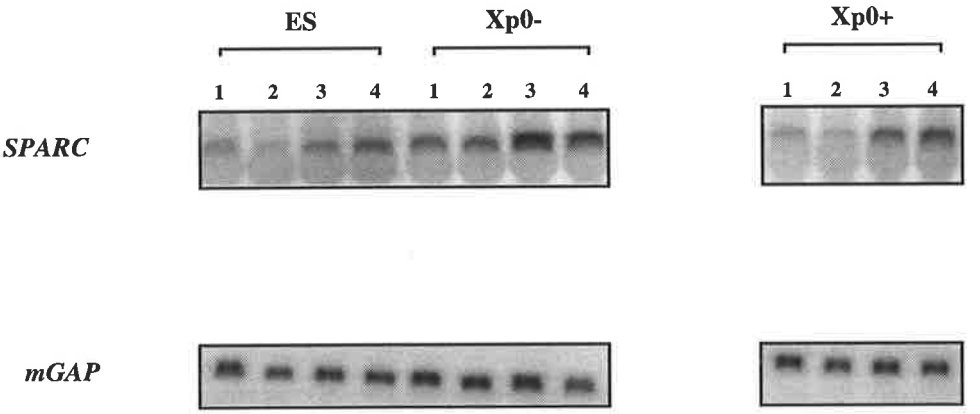
RNA was prepared from ES, Xp0+ and Xp0- embryoid bodies harvested at day 1, 2, 3 and 4 development (6.7.1). The expression of *H19*, *SPARC* and *AFP* compared to a *mGAP* loading control was determined by Northern blot analysis of total RNA (2.5.12). DNA probes were used to detect *H19* and *SPARC* expression (2.5.14). An antisense riboprobe was used to detect *AFP* expression (2.5.15). Filters probed with *H19* and *SPARC* DNA probes were washed in 2xSSC/0.1% SDS at 42°C, 0.2xSSC/0.1% SDS at 50°C and finally in 0.2xSSC/0.1%SDS at 65°C. Filters probed for *AFP* expression were washed in 2xSSC/0.1%SDS at 65°C, 0.2xSSC/0.1%SDS at 65°C and finally in 0.2xSSC/0.1%SDS at 75°C. All washes were for a minimum duration of 30 minutes. Filters were exposed to phosphorimager screens for 20 hours (*SPARC*, *H19*) and 18 hours (*AFP*). The filters were subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16), exposure time 20 hours.

The expression of the 2.6kb *H19*, 2.2kb *SPARC* and 2.2kb *AFP* transcripts are shown respectively in A, B and C.

A *H19* EXPRESSION



B *SPARC* EXPRESSION



C *AFP* EXPRESSION

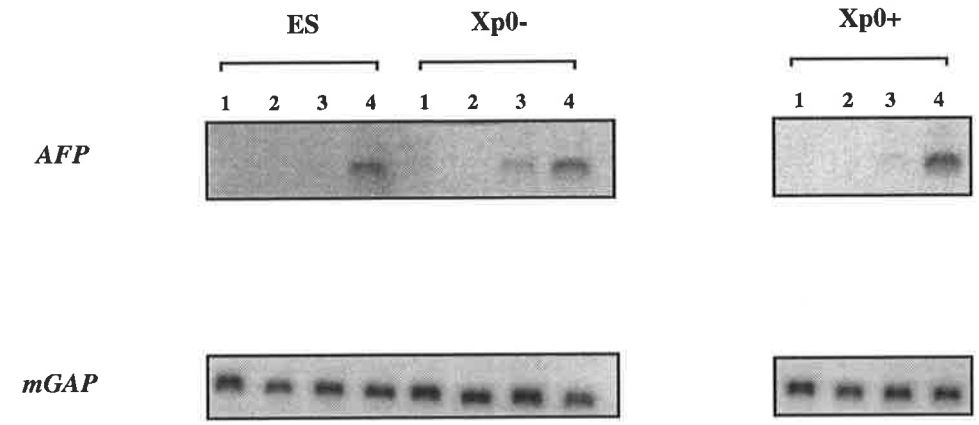


FIGURE 6.18 Comparison of *H19*, *SPARC* and *AFP* expression in embryoid bodies derived from ES, Xp0+ and Xp0- cells.

The level of *H19*, *SPARC*, *AFP* and *mGAP* expression (Fig. 6.17) was quantitated by volume integration (2.5.20). *H19* expression, normalised to *mGAP*, in ES, Xp0+ and Xp0- embryoid bodies from day 1 to day 4 is shown in graph A. *SPARC* expression normalised to *mGAP* for these embryoid bodies is shown in graph B. Similarly *AFP* expression is shown in graph C.

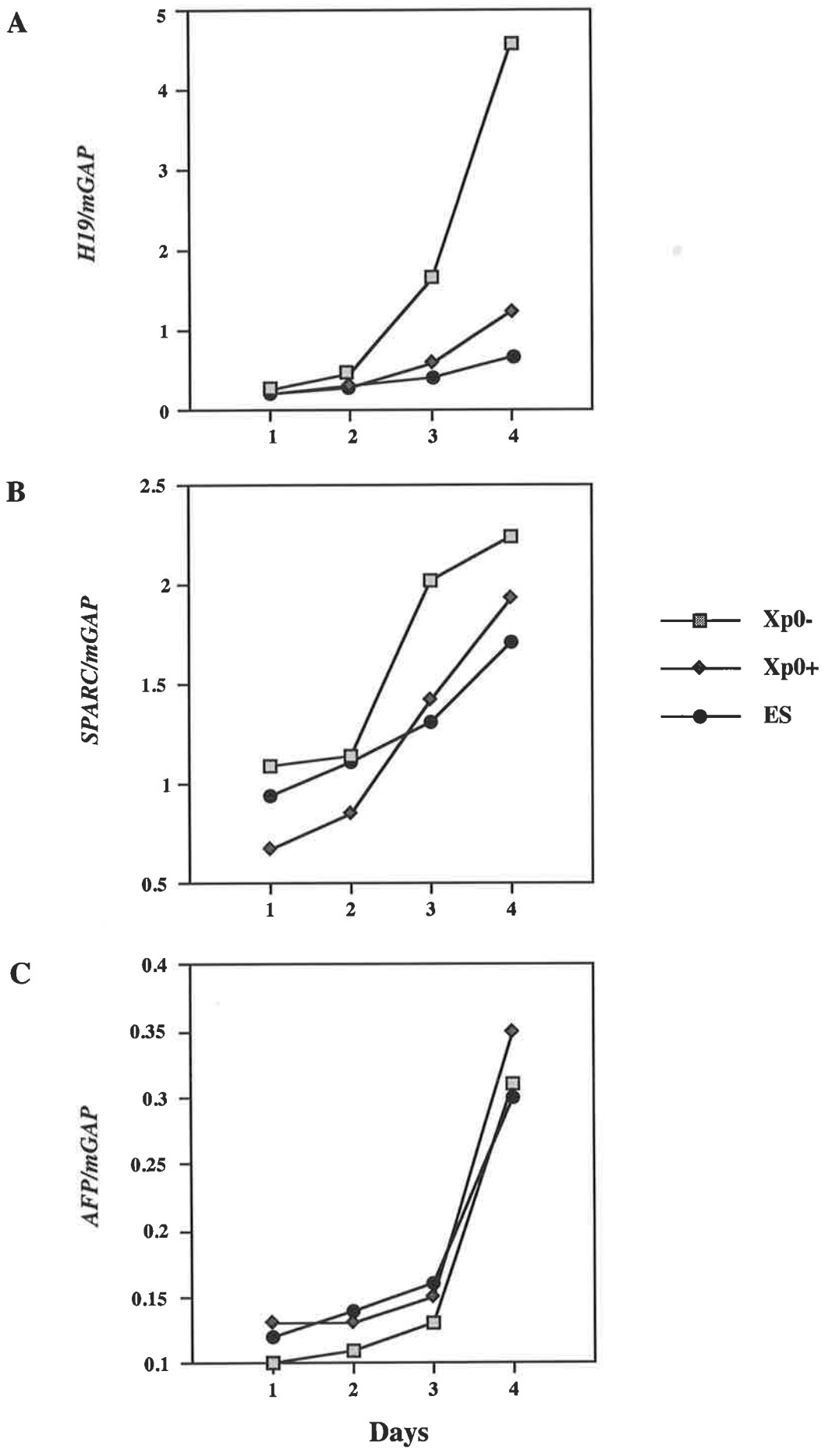
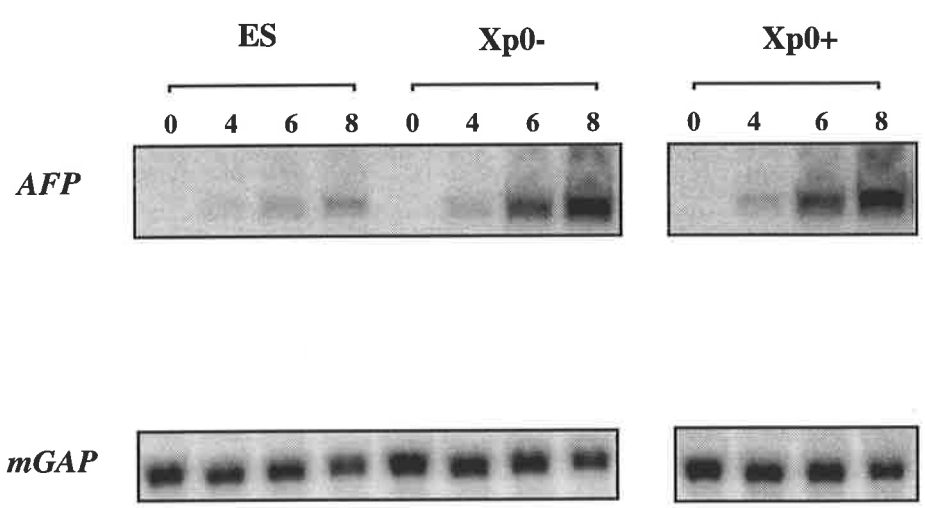

A
H19/mGAP
5
4
3
2
1
0
1
2
3
4
B
SPARC/mGAP
2.5
2
1.5
1
0.5
1
2
3
4
Xp0-
Xp0+
ES
C
AFP/mGAP
0.4
0.35
0.3
0.25
0.2
0.15
0.1
1
2
3
4
Days

FIGURE 6.19 Northern blot analysis of *AFP* expression in ES, Xp0+ and Xp0- embryoid bodies at later stages of development.

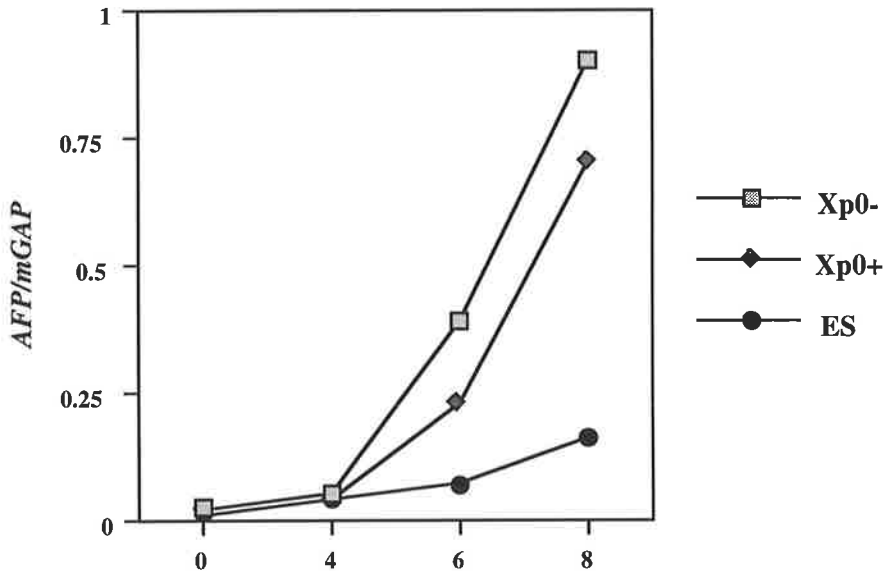
RNA was prepared from ES, Xp0+ and Xp0- cells harvested at day 0 (2.5.10) and at day 4, 6 and 8 of embryoid body development (2.5.11). The expression of *AFP* and *mGAP* was determined by Northern blot analysis of total RNA (2.5.12). Antisense *AFP* riboprobes were used to detect expression (2.5.15). Filters were washed in 2xSSC/0.1%SDS at 50°C, 0.2xSSC/0.1%SDS at 50°C and finally in 0.2xSSC/0.1%SDS at 75°C. All washes were for a minimum duration of 30 minutes. Filters were exposed to phosphorimager screens for 20 hours (2.5.20). The filters were subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16). *AFP* expression in ES, Xp0+ and Xp0- embryoid bodies is shown in A.

The level of *AFP* and *mGAP* expression in ES, Xp0+ and Xp0- embryoid bodies was quantitated by volume integration (2.5.20). The ratio of *AFP* expression to *mGAP* expression is presented graphically in B.

A


ES
Xp0-
Xp0+
0 4 6 8 0 4 6 8 0 4 6 8
AFP
mGAP

B


AFP/mGAP
1
0.75
0.5
0.25
0
0 4 6 8
Xp0-
Xp0+
ES

Embryoid development in ES cells proceeds *via* the initial formation of primitive endoderm and the subsequent formation of primitive ectoderm and mesoderm. The earlier appearance of mesoderm in embryoid bodies derived from X cells complicates interpretation of *H19*, *SPARC* and *AFP* expression in embryoid bodies. Although *H19* can be used as a marker for extraembryonic endodermal cell types, it is also expressed in a variety of mesodermal and endodermal derivatives after gastrulation (Poirier *et al.* 1991). The earlier formation of mesoderm in X cell embryoid bodies as assessed by the expression of *Brachyury* and *goosecoid* suggests that *H19* expression observed in these cultures could be due to the presence of mesodermal and/or endodermal derivatives rather than extraembryonic endodermal cell types. Similarly at later stages of development *SPARC* is expressed in the region of the developing heart (Holland *et al.* 1987). X cell embryoid bodies exhibit a high capacity to form cardiac muscle, and the elevated expression of *SPARC* in X cell embryoid bodies may therefore be related to this. Finally *AFP* while widely used as a marker of visceral endoderm, is also detected within foetal hepatocytes later during development.

Embryoid body formation constitutes a considerably complex differentiation process, the cell types produced are dependent on multiple, sequential inductions between different cell populations. Examination of gene expression at later stages of embryoid body development, particularly after primitive ectoderm and mesoderm formation, may be related to indirect inductions rather than the initial propensity of a cell type to form one tissue over another or express a particular gene.

Accurate assessment of primitive endoderm formation in ES and X cell embryoid bodies requires an earlier and more specific marker. *HNF-4* is a transcription factor expressed in the early primitive endoderm at day 4.5 and subsequently in the visceral endoderm from day 5.5 to 8.5 (Duncan *et al.* 1994). Analysis of expression of this gene in ES, Xp0+ and Xp0- may clarify the differentiation of primitive endoderm within these cultures.

The ability of X cells to form parietal endoderm when aggregated in the presence of RA demonstrates that X cells can form primitive endodermal derivatives. The level of primitive endodermal derivatives in X cell RA-treated aggregates was however lower than that level observed in ES cell RA-treated aggregates. This suggested that X cells have a

lower capacity to form primitive endodermal derivatives than ES cells. This was supported by the layered appearance of ES cell embryoid bodies at day 4, and visceral endoderm cell type at day 8 in ES cell embryoid bodies, compared with X cell embryoid bodies. However the formation of primitive endodermal derivatives in X cell embryoid bodies compared to ES cell embryoid bodies could not be resolved with the available markers.

6.8 Discussion

The aim of the experiments described in this chapter was to define the developmental potential of X cells compared to ES cells. This was achieved by investigation of the differentiation of cells as embryoid bodies and as aggregates in the presence of RA. The ability of X cells to differentiate as embryoid bodies and in the presence of RA, generating multiple, differentiated cell types, demonstrates that X cells constitute a pluripotential cell population. The developmental potential of ES and X cells could be discriminated in a number of ways, related to the formation of primitive endoderm, late stage primitive ectoderm, mesoderm and neural tissue.

6.8.1 The Differentiation Potential of X Cells Compared to ES Cells

Differentiation by aggregation in the presence of RA resulted in the formation of increased levels of cells with the morphology of parietal endoderm and visceral endoderm in ES cell cultures compared to X cell cultures. The presence of parietal endoderm at lower levels in Xp0- cells than ES cell cultures was confirmed by the expression of *SPARC*, a marker of parietal endoderm. During differentiation of ES and X cells as embryoid bodies, ES cells exhibited a layered morphology consistent with the formation of an outer layer of primitive endoderm. A similar layer was not observed in the majority of X cell embryoid bodies. At day 8 of embryoid body development ES cell embryoid bodies could also be distinguished from X cell embryoid bodies by the presence of a visceral endoderm-like cell type. While these observations suggested that X cells exhibited lower capacities for primitive endodermal formation than ES cell embryoid bodies this difference could not be demonstrated *via H19*, *SPARC* or *AFP* expression.

Late stage primitive ectoderm formation in embryoid bodies derived from ES and X cells was examined by the expression of *Rex-1*, *FGF-5* and *Oct-4*. X cell embryoid bodies formed late stage primitive ectoderm earlier than ES cell embryoid bodies.

Several lines of evidence demonstrated a higher capacity of X cells to form mesodermal derivatives when compared to ES cells. Firstly, differentiation of X cells as embryoid bodies was marked by earlier and elevated expression of the early mesodermal markers *Brachyury* and *goosecoid*, and formation of cardiac muscle earlier and to a higher extent than embryoid bodies derived from ES cells. This difference was seen in embryoid bodies cultured in suspension and in embryoid bodies seeded at day 6 of development. Secondly, when X cells were differentiated by aggregation in the presence of RA they exhibited a higher propensity to form fibroblasts, a mesodermal derivative, than ES cells.

Embryoid bodies derived from X cells exhibited a lower capacity for neuron formation than ES cell embryoid bodies. However RA differentiation of aggregates demonstrated that X cells retain the ability to differentiate into neurons. In murine embryogenesis neural induction occurs in a region of primitive ectoderm adjacent to invaginated mesoderm (reviewed in Beddington 1988). Induction of neural specific genes in this region by underlying and anterior mesendoderm has been demonstrated (Ang and Rossant 1993, Ang *et al.* 1994). Inhibition of neural genes by posterior mesendoderm has also been demonstrated (Ang *et al.* 1994). Recently the capacity of primitive streak mesodermal cell lines to induce the expression of *Pax-3* (a neural specific marker) in undifferentiated P19 EC cells has been shown (Pruitt 1994a). Thus mesodermal and endodermal tissues can have specific roles in neural induction and repression.

Visceral endoderm is a ~~second~~ early embryonic cell type that may produce inductive signals regulating primitive ectoderm differentiation. The absorption of nutrients and secretion of serum components necessary for the general growth and maintenance of the embryo has been thought to be the main function of the visceral endoderm in embryogenesis (Hogan *et al.* 1994). The instructive capacity of the visceral endoderm during development is however gradually being recognised. A visceral endodermal cell line, END-2 produces a factor which can induce the differentiation of P19 EC cells into muscle and neurons *in vitro* (Mummery *et al.* 1991, van den Eijen van den Raaij *et al.*

1991). Secondly, the homeobox gene *Hesx-1* is expressed in a small subset of visceral endoderm cells on the anterior side of the early murine embryo at day 7.0 where it appears to play a role in forebrain development during gastrulation (R. Beddington, P. Thomas, personal communication).

These observations highlight the inductive capacity possessed by different tissues within the early murine embryo. Low level neuron formation in X cell embryoid bodies is probably due to the presence or absence of a particular tissue or factor involved in neural induction or repression rather than an intrinsic property of X cells.

Overall the differentiation potential of X cells can be discriminated from ES cells by an increased capacity to form late stage primitive ectoderm and mesodermal derivatives, and a lower capacity to form primitive endodermal derivatives. The ability of X cells to form neurons compared to ES cells was dependent on the differentiation regime employed. The differentiation potential exhibited by X cells is therefore consistent with their gene expression profile and consistent with the proposal that X cells represent the *in vitro* equivalent of early primitive ectoderm.

6.8.2 Gene Expression Correlates with Differentiation Potential

ES cells have a gene expression consistent with their origin within the inner cell mass. Xp0- cells have a gene expression characterised by higher expression of *FGF-5* and lower expression of *Rex-1*, consistent with early primitive ectoderm *in vivo*. Xp0+ cells exhibit a gene expression which is intermediate between Xp0- and ES cells (4.2.1, 5.2, 5.3). In all experiments Xp0+ cells displayed differentiation properties intermediate to those observed with ES and Xp0- cells. The differentiation profile of ES and X cell populations therefore appears to be dependent on the initial gene expression of the population and therefore their relationship with the inner cell mass/primitive ectoderm.

The differences observed between ES and X cells with regard to differentiation potential were not marked by differentiation events specific for ES cells and not X cells, nor the converse. ES and X cells were able to give rise to similar cell types, but were distinguished by the time and extent of their formation. *In vivo* the restriction in differentiation capacity as the ICM forms the primitive ectoderm is thought to be a progressive event rather than an absolute restriction (Smith 1992). Therefore the graded

differentiation observed in ES, Xp0+ and Xp0- cells may reflect the alteration of developmental potential *in vivo* as the inner cell mass forms the primitive ectoderm.

6.8.3 The Formation of Primitive Endoderm *In Vitro* and *In Vivo*

The inner cell mass is distinguished from the primitive ectoderm by the inability of the latter to give rise to the primitive endoderm *in vivo* (Smith 1992). While the exact nature of this restriction remains unclear, the similarities between X cells and early primitive ectoderm suggest that X cells should exhibit a decreased potential for primitive endoderm formation. Several observations supported this prediction. Firstly, when X cells were differentiated in the presence of RA, they exhibited a lower capacity to form primitive endodermal derivatives than ES cells. Secondly, during differentiation as embryoid bodies X cell embryoid bodies lacked the layered morphology consistent with primitive endoderm formation at day 4, and the visceral endoderm cell type at day 8 when compared to ES cell embryoid bodies. Unfortunately, a lower potential for primitive endoderm formation in X cell embryoid bodies compared to ES cell embryoid bodies could not be demonstrated using markers of primitive endoderm derivatives.

The potential for X cells to form primitive endoderm is difficult to predict for several reasons. *In vivo*, cells on the outside of the inner cell mass differentiate into a layer of primitive endoderm at approximately day 4.0 of development (Hogan *et al.* 1994). Cells isolated from the inner cell mass of the embryo after primitive endoderm formation are still capable in certain circumstances of forming primitive endoderm *in vitro* (Gardner 1983). Therefore although the fate of these cells *in vivo* would normally be restricted to primitive ectoderm, this restriction is not seen *in vitro*. Similarly, while X cells in an *in vivo* environment may not give rise to primitive endoderm, they may do so in an *in vitro* environment.

In addition, aggregation appears to be an obligatory step in the formation of extraembryonic endodermal cells types both *in vivo* and *in vitro*. In the early murine blastocyst cells located on the outside of the inner cell mass form primitive endoderm lineages. Similarly cells on the outside of embryoid body aggregates, differentiate into derivatives of the primitive endoderm. Differentiation of ES cells *via* aggregation in the presence of RA resulted in the high level formation of primitive endodermal derivatives.

By contrast differentiation of ES cells with RA in monolayer culture results in the formation of high levels of fibroblasts, and derivatives of primitive endoderm are only observed at a low frequency (J. Rathjen, unpublished observations, Thomas 1994). It therefore appears that the high level appearance of primitive endodermal derivatives in RA-treated aggregates derived from ES cells (6.6) is related to aggregation rather than to RA treatment alone. The mechanism which induces primitive endoderm to form on the outside of embryoid bodies and in RA-treated aggregates may override gene expression differences in ES and X cells.

Furthermore, X cells have the ability to revert to ES cells, which are able to form primitive endoderm. Therefore while X cells themselves may not directly differentiate into primitive endoderm during embryoid body formation, they may be capable of its formation *via* an ES cell intermediate. Sporadic reversion to ES cells and irregular formation of primitive endoderm on the outside surface of X cell embryoid bodies might underlie their disorganised appearance.

6.8.4 The Differentiation Potential of X Cells in Relation to the Two State Model of X Cell Formation and Maintenance

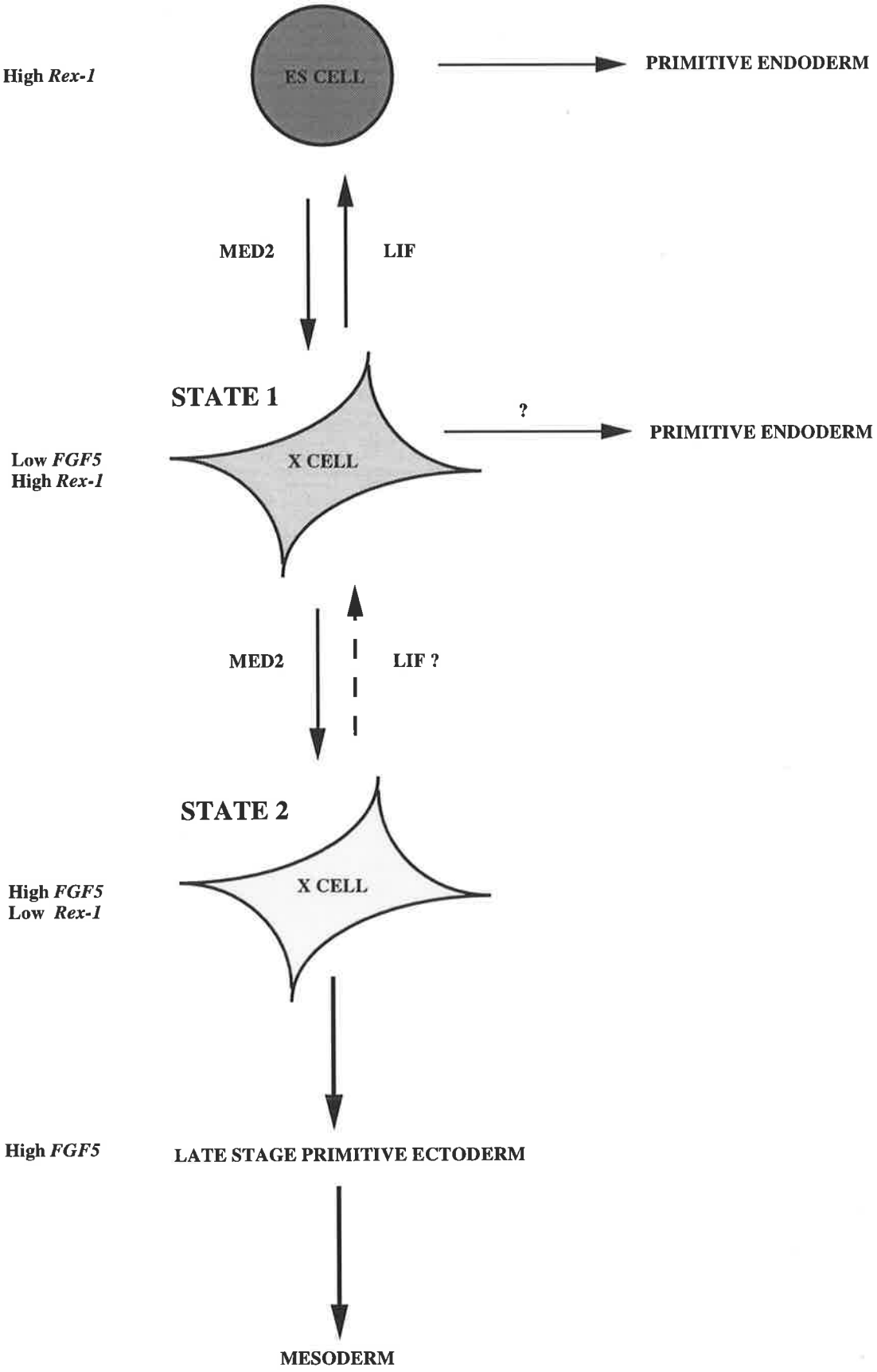
In Chapter 5 the gene expression of passaged X cells as they were cultured at high cell density and their differentiation state at clonal cell density lead to the proposal of a 2 state model for X cell formation and maintenance (Figure 5.10). This model is applied to embryoid body formation and aggregation in RA in Figure 6.20. The differentiation potentials observed in ES, Xp0+ and Xp0- cultures described in this chapter were marked by graded differences. The existence of X cells within two states might further explain the graded nature of ES and X cell differentiation.

ES cells can either differentiate into primitive endoderm or primitive ectoderm, hence mesoderm. X cells in state 1 may revert to ES cells and form primitive endoderm or they may be capable of forming it directly. Alternatively X cells in state 1 may differentiate to state 2. Cells in state 2, characterised by high *FGF-5*, low *Rex-1* may be confined to the formation of late stage primitive ectoderm and/or mesoderm formation.

Xp0+ cultures were proposed to consist of a high proportion of cells in state 1 and a low proportion of cells in state 2, while Xp0- cultures were thought to contain a lower

FIGURE 6.20 The two state model for X cell formation, maintenance and differentiation as applied to differentiation regimes.

The existence of X cells in two states may further explain the behaviour of X cell populations when differentiated as embryoid bodies and by aggregation in the presence of RA. See text for description (6.8.4).


High *Rex-1*
ES CELL
PRIMITIVE ENDODERM
MED2
LIF
STATE 1
?
PRIMITIVE ENDODERM
Low *FGF5*
High *Rex-1*
X CELL
MED2
LIF ?
STATE 2
High *FGF5*
Low *Rex-1*
X CELL
High *FGF5*
LATE STAGE PRIMITIVE ECTODERM
MESODERM

proportion of X cells in state 1 and a higher proportion of X cells in state 2. When Xp0+ and Xp0- cells are differentiated, either by aggregation in the presence of RA or as embryoid bodies, the particular cell types generated, as well as the time and the extent at which they are generated may be related to the proportion of X cells in state 1 and state 2 in the initial X cell culture.

This chapter has highlighted the complexities of studying cell differentiation both *in vitro* and *in vivo*. Notwithstanding these difficulties differences in the developmental potential of X cells compared with ES cells were determined. These differences were generally graded rather than absolute, which is consistent with the developmental lability displayed by the pluripotential cell populations of the murine embryo. Despite the additional complexities which must be taken into account in any *in vitro* system, the differentiation of ES, Xp0+ and Xp0- cells could be correlated with events occurring during early murine development. The results obtained in this chapter support the proposal that X cells are the *in vitro* equivalent of early primitive ectoderm.

CHAPTER SEVEN:
DIFFERENTIATION OF X CELLS
WITH MESODERM INDUCING CYTOKINES

CHAPTER SEVEN: DIFFERENTIATION OF X CELLS WITH MESODERM INDUCING CYTOKINES

7.1 Introduction

Diverse vertebrate species exhibit similar body plans at mid-embryogenesis (Gould 1977). Therefore it is commonly thought that the underlying mechanisms of gastrulation will be conserved between different species. The murine embryo and *Xenopus* embryo have similar fate maps (Lawson *et al.* 1991, Quinlan *et al.* 1995), and many of the genes expressed during gastrulation are conserved both stage and tissue specifically (reviewed in DeRobertis *et al.* 1994).

The identification of biological factors which are able to cause mesoderm induction in *Xenopus* has been the focus of intense research over the last ten years. The first molecules to be implicated in mesoderm induction in *Xenopus* were basic FGF, a member of the fibroblast growth factor (FGF) family and activin A, a member of the transforming growth factor beta (TGFβ) family (for reviews see Slack 1990, Ruiz i Altaba and Melton, 1990, Cooke 1991). An *in vivo* role for these factors during mesoderm induction in *Xenopus* has been suggested by the introduction of dominant negative receptor constructs into early embryos (Amaya *et al.* 1991, Hemmati-Brinvalou and Melton 1992). Introduction of such constructs inhibits mesoderm formation during gastrulation. FGFs, activins and their respective receptors are expressed throughout murine embryogenesis, including gastrulation (Crossley and Martin 1995, Manova *et al.* 1994), however the role that these factors play in such processes is at present unclear.

When ES cells are reintroduced into the blastocyst they have the capacity to contribute to all differentiated cell types constituting the murine embryo, including the germ line (Bradley *et al.* 1984, Robertson 1987). Since ES cells can respond to all of the developmental signals *in vivo*, it should be possible to investigate specific embryonic differentiation events by appropriate manipulation of these cells *in vitro*. While factors which prevent the differentiation of ES cells *in vitro* have been discovered, biological factors which induce the specific differentiation of ES cells have not. A variety of factors shown to be active in a range of developmental processes, including members of the FGF and TGFβ family, have no effect on the differentiation of ES cells *in vitro* (P. Rathjen,

personal communication). MED2 therefore contains a novel biological activity which has the ability to induce the specific differentiation of ES cells.

The gene expression and differentiation analysis described in this thesis suggest that X cells represent the *in vitro* equivalent of primitive ectoderm. *In vivo* the primitive ectoderm is the substrate for mesoderm induction, during gastrulation. If the underlying mechanisms of mesoderm induction in *Xenopus* and mouse are conserved, then basic FGF and activin A might induce the differentiation of X cells.

This chapter describes the differentiation of X cells in response to members of the FGF family and activin A. Consistent with mesoderm induction in *Xenopus*, gene expression analysis suggests that these factors induce the specific differentiation of X cells into alternative mesodermal derivatives. The ability of X cells to differentiate specifically in response to FGF and activin A further distinguishes these cells from ES cells which are unable to respond to these factors. Furthermore, the results of this chapter add substantial evidence to the proposal that X cells represent the *in vitro* equivalent of primitive ectoderm and establish X cells as a model system for the analysis of mesoderm induction *in vitro*.

7.2 Basic FGF Induces Specific Differentiation of X Cells but not ES Cells

Fibroblast growth factors constitute a family of nine structurally and functionally related growth factors. These factors have been implicated in many important biological processes including mitogenesis, angiogenesis, cell migration and differentiation (reviewed in Basilico and Moscattelli 1992, Mason 1994, Baird 1994). FGFs transduce their biological signal through a family of four transmembrane receptor tyrosine kinases (reviewed in Johnson and Williams 1993). FGFs are expressed throughout embryogenesis and certain members have expression patterns consistent with a role in gastrulation (Wilkinson *et al.* 1988, 1989, Haub and Goldfarb 1991, Hebert *et al.* 1990, 1991, Niswander and Martin 1992, deLapeyriere *et al.* 1993, Han and Martin 1993, Mason *et al.* 1994, Crossley and Martin 1995, Mahmood *et al.* 1995). Basic FGF (FGF-2) was one of the first members of the FGF family to be discovered (Gospodarowicz *et al.* 1974, Gospodarowicz and Moran 1974). The effect of basic FGF on mesoderm

induction in *Xenopus* embryos is well characterised. In these studies bFGF induces the formation of ventral mesoderm, including blood, mesenchyme and muscle (Slack *et al.* 1987, Kimelman and Kirschner 1987, Ruiz i Altaba and Melton 1989a, 1989b, Green *et al.* 1990).

7.2.1 The Effect of bFGF on ES Cells and X Cells

ES and Xp0+ cells were seeded at clonal cell density into medium containing LIF and 50% MED2 + LIF, respectively. In mesoderm induction assays bFGF is generally used at a concentration of 20-50ng/ml (Green *et al.* 1990, LaBonne and Whitman 1994). In cell proliferation assays a concentration of 1-20ng/ml is recommended. Cells were therefore cultured in the presence of increasing concentrations of recombinant bovine bFGF (0.1ng/ml to 100ng/ml) for a period of five days and subsequently stained with alkaline phosphatase. Consistent with previous observations (P. Rathjen, personal communication), addition of bFGF to ES cells in the presence of LIF had no effect on their differentiation at any concentration examined. By contrast, 10ng/ml bFGF induced the differentiation of X cells to a characteristic fibroblastic cell type which did not stain for alkaline phosphatase. The morphology of this cell type is shown in Figure 7.1 and has been designated cell type A.

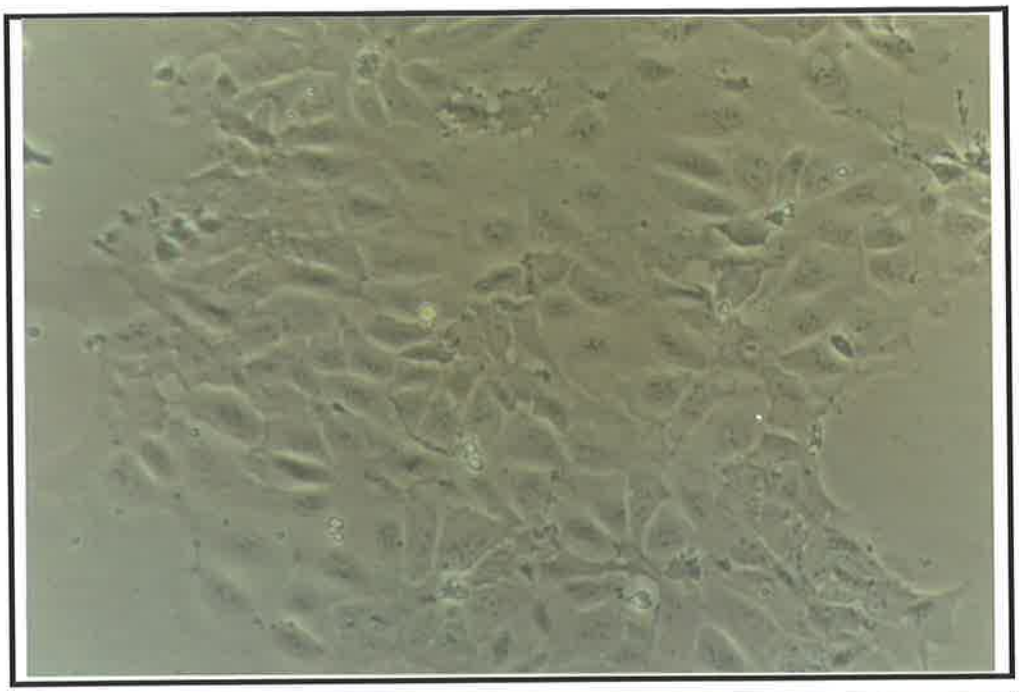
The proportion of ES cell, undifferentiated X cell, semi-differentiated and differentiated colonies present were assessed as previously described in Chapter 3 (Figure 3.4). Colony analyses of ES and X cells cultured with and without 10ng/ml of bFGF are presented in Figure 7.2. The proportion of undifferentiated ES cell colonies observed after 5 days was similar regardless of the presence of bFGF. The addition of bFGF to X cell cultures caused a decrease in the level of undifferentiated X cell colonies from 58% to 21%, with corresponding increases in the levels of differentiated colonies. The level of semi-differentiated and differentiated X cell colonies in the presence of bFGF rose from 31% to 45% and 10% to 34%, respectively. The plating efficiencies of X cells under the two conditions were similar. Therefore it appeared that colonies which comprised undifferentiated and semi-differentiated colonies in control cultures became semi-differentiated and fully differentiated colonies, respectively, in the presence of bFGF. This suggested that bFGF effects X cell differentiation. Cell type A was also observed in

FIGURE 7.1 Basic FGF induces the specific differentiation of X cells:- morphology at day 5.

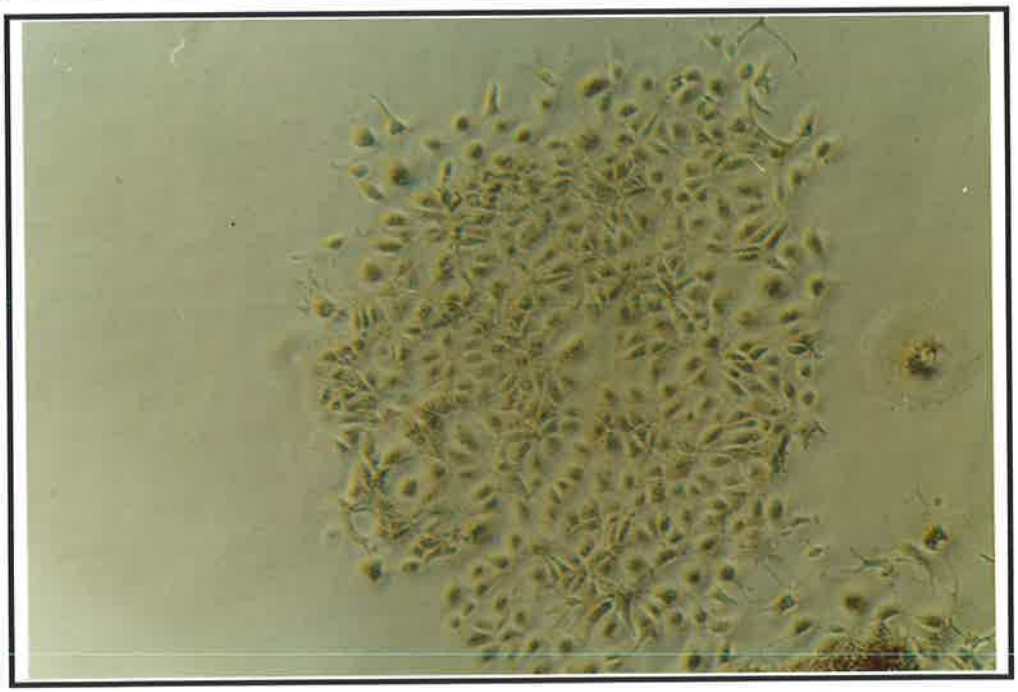
Xp0+ cells were cultured at clonal cell density in MED2 and LIF in the presence of bFGF (10ng/ml) (2.3.21). After five days colonies have the morphology and contain the cell type observed in A. This cell type can be more clearly seen after cultures are stained with alkaline phosphatase and counterstained with haematoxylin (a general cell stain) (2.3.24) in B and C. This cell type does not stain for alkaline phosphatase and has been designated cell type A.

Photographs A and C were taken at a magnification of 50x, B 25x, using phase contrast optics.

A



B



C

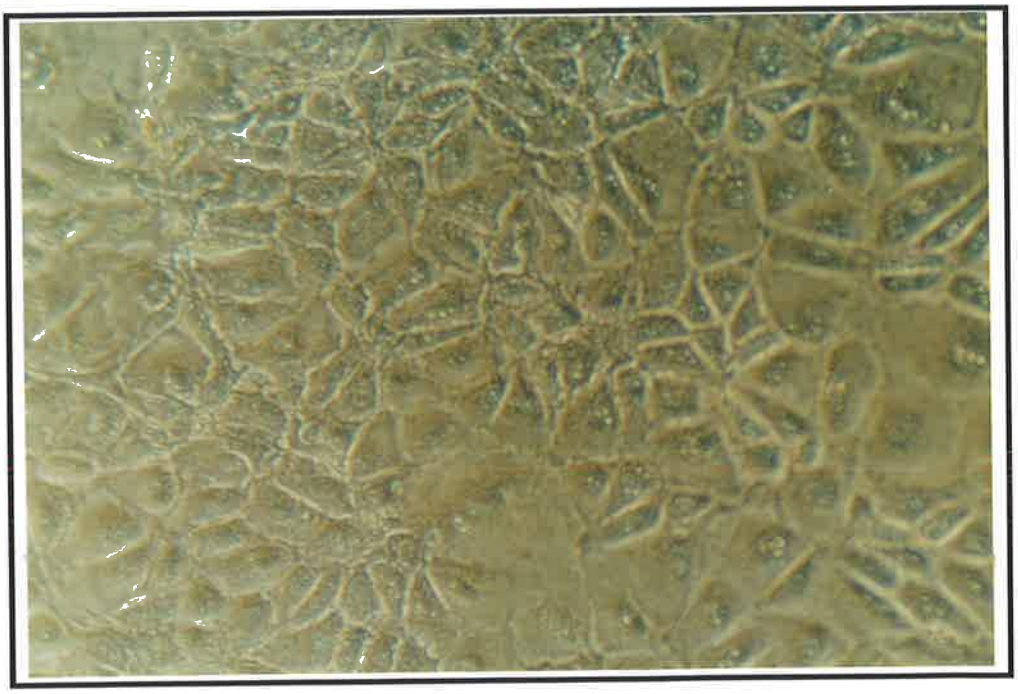


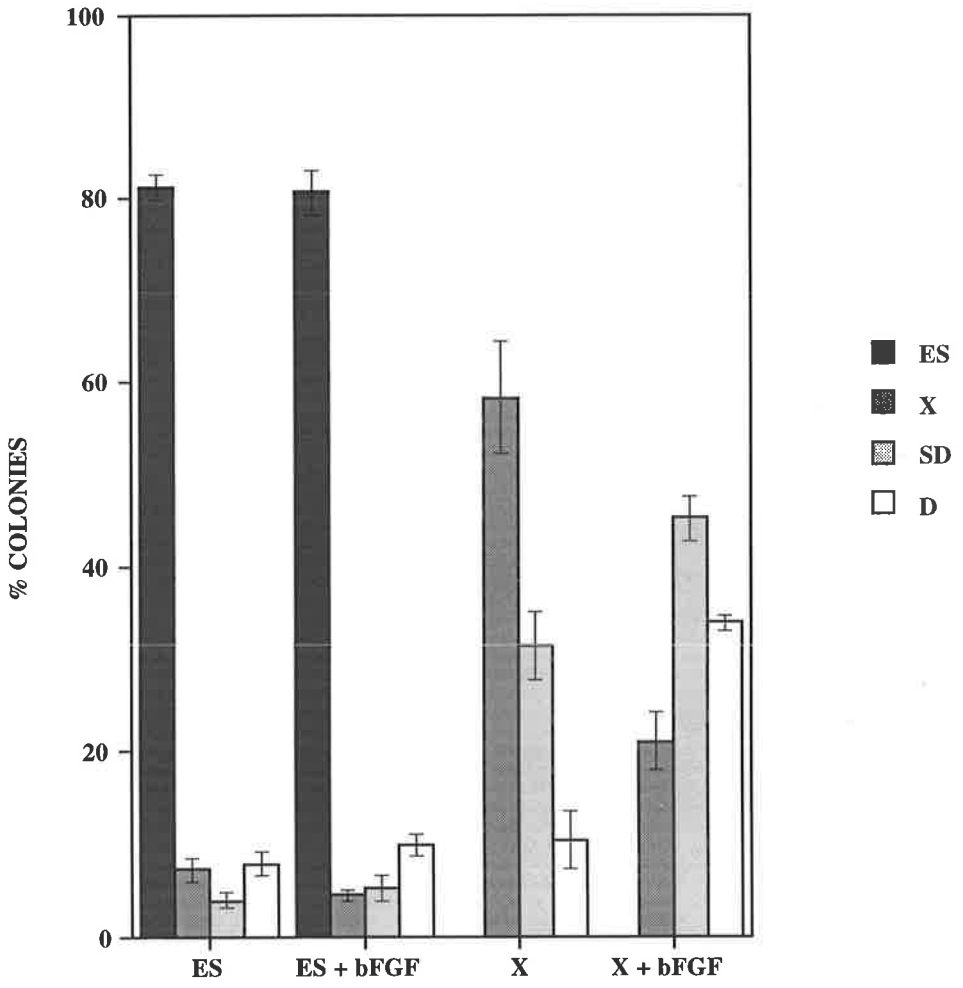
FIGURE 7.2 Basic FGF induces the differentiation of X cells but not ES cells:- colony analysis at day 5.

ES and Xp0+ cells were seeded at clonal cell density into LIF, MED2 and LIF, respectively, with and without bFGF (10ng/ml) (2.3.21). After 5 days cultures were stained with alkaline phosphatase (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig. 3.4). Four duplicate wells were included in each analysis. Results of this analysis are tabulated in A and represented graphically in B.

A

CONDITIONS	*% COLONIES*				*PLATING EFFICIENCY (%)*
	ES	*X*	*SD*	*D*	
ES	*81.1+/-1.4*	*7.2+/-1.2*	*3.9+/-0.8*	*7.8+/-1.3*	*35.2+/-4.1*
ES + bFGF	*80.6+/-2.5*	*4.4+/-0.6*	*5.2+/-1.4*	*9.8+/-1.2*	*36.2+/-2.4*
X	*0*	*58.3+/-6.1*	*31.3+/-3.7*	*10.4+/-3.1*	*42.5+/-3.2*
X + bFGF	*0*	*21.0+/-3.1*	*45.2+/-2.4*	*33.9+/-0.8*	*42.0+/-1.6*

B



X cells cultured without bFGF. However in these cultures it was present at a much lower levels among several morphologically distinct differentiated cell types.

In a separate experiment, cell counts were performed on triplicate wells containing X cells cultured in 50% MED2 and LIF, at clonal cell density, with and without 10ng/ml bFGF. After 5 days cells were trypsinised and total number of cells in each well determined. The results of this analysis are presented in Figure 7.3. The total number of cells present at day 5 in 10ng/ml bFGF was 40% higher than the total number of cells determined for control wells. This implied one or a combination of three possibilities: 1) bFGF has a proliferative effect on X cells, which then differentiate forming cell type A, 2) bFGF has a proliferative effect on cell type A itself or 3) bFGF induces the differentiation of X cells to cell type A which proliferates at a high rate.

7.2.2 Basic FGF Induces X Cell Differentiation

Basic FGF was first identified by its ability to induce the proliferation of BALB-C 3T3 fibroblasts (Gospodarowisz 1974, Godspodarowisz and Moran 1974). Subsequently its mitogenic properties on all cells of mesodermal origin and many cells of neuroectodermal, ectodermal and endodermal origin have been demonstrated (reviewed in Gospodarowicz *et al.* 1986a, 1986b, 1987). This raised the possibility that bFGF may be inducing the proliferation of spontaneous differentiated cells present within the X cell population rather than effecting X cell differentiation.

However, the distribution of colonies in bFGF-treated cultures compared to untreated cultures (Figure 7.2), suggested that this was not the case. Other lines of evidence also support that bFGF effects X cell differentiation. Firstly, examination of X cells cultured in the presence of bFGF at earlier time points suggests that bFGF has an effect on the differentiation of X cells. The proportion of differentiated cells observed in X cells at clonal cell density increases substantially from day 3 to day 5 (3.3). At day 3 the majority of X cell colonies are in the undifferentiated form and differentiated cells are at relatively low levels. A difference in the colony morphology between bFGF-treated and untreated cultures can be seen as early as two days and was particularly evident in the majority of colonies at day 3 (Figure 7.4). X cell colonies in control cultures appeared more compact than those observed in bFGF-treated cultures. bFGF appeared to be

FIGURE 7.3 The effect of bFGF on X cells:- the number of cells at day 5.

Xp0+ cells were seeded at clonal cell density into MED2 and LIF with and without bFGF (10ng/ml). After 5 days cells from triplicate wells were harvested and the mean cell number associated with each condition was determined (2.3.21). Results of the analysis are tabulated in A and represented graphically in B.

A

CONDITIONS	*CELLS*
X	*3.07x10⁴ +/- 0.3*
X + bFGF	*4.37x10⁴ +/- 0.6*

B

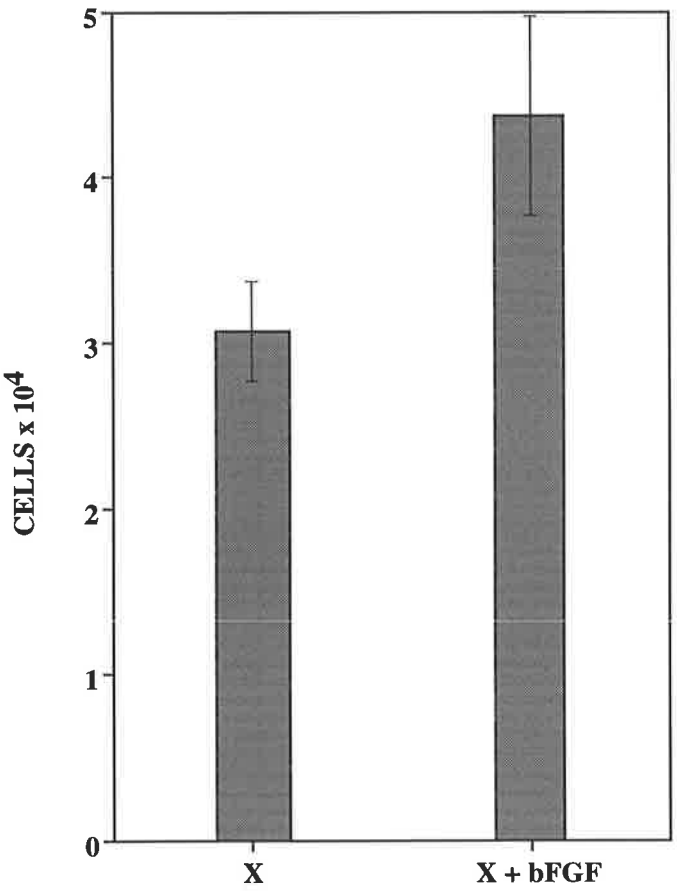
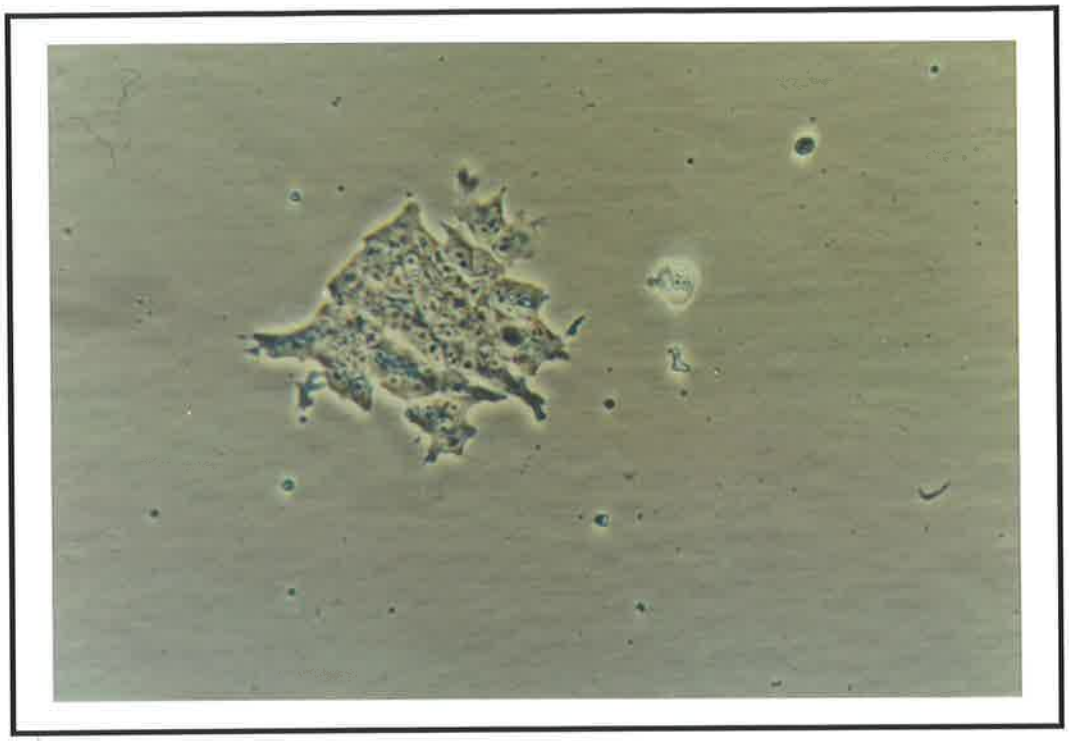


FIGURE 7.4 Basic FGF induces the differentiation of X cells:- morphology at day 3.

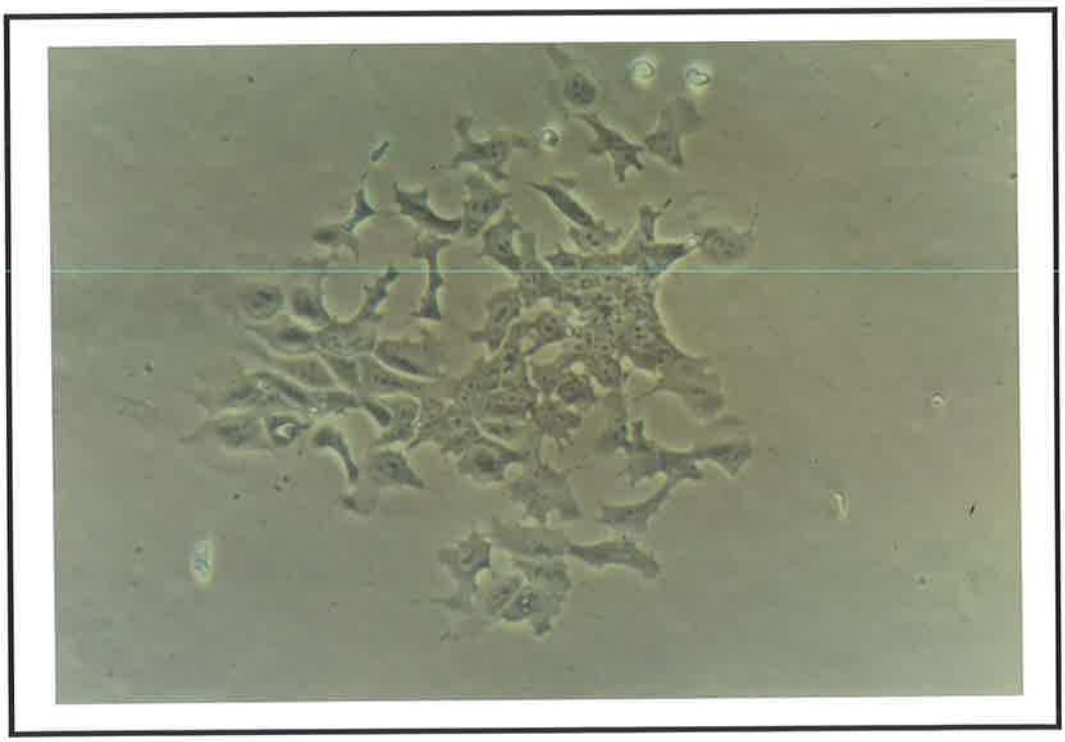
Xp0+ cells were cultured at clonal cell density in the presence of MED2 and LIF with and without bFGF(10ng/ml) (2.3.21). The morphology of X cell colonies within control cultures at day 3 is shown in A. The morphology of X cell colonies within bFGF-treated cultures at day 3 is shown in B.

Photographs were taken under phase contrast at a magnification of 50x.

A



B



causing the migration and differentiation of X cells within colonies at times when differentiated cells were not present at significant levels. X cell colonies in bFGF also appeared larger than X cell colonies in control conditions. This may be an initial morphology change associated with differentiation or may result from a proliferative effect on X cells themselves.

Secondly, the effect of bFGF could not be reproduced by exposure of X cells to other growth factors which induce cell proliferation. Platelet Derived Growth Factor (PDGF) A, like bFGF, is a potent mitogen for a variety of cell types, including fibroblasts (Ross *et al.* 1986). The PDGF receptor is expressed in mesodermal derivatives in murine embryos at 7.5 days of development (Mercola *et al.* 1990, Orr-Urtreger and Lonai 1992) and in the mesodermal derivatives of EC/ES cells (Mummery *et al.* 1986, Mummery and van den Eijnden-van Raaij 1990). Cell type A is likely to be a mesodermal derivative (refer to 7.6.1). X cells cultured at clonal cell density in 50% MED2 and LIF in the presence of PDGF A (1-100ng/ml) appeared identical to control cultures (data not shown). While both PDGFA and bFGF were fully functional in fibroblast proliferation assays (data not shown) only bFGF was able to effect X cell cultures. PDGFA is also inactive in *Xenopus* mesoderm induction assays (Cooke 1991).

Thirdly, the presence of cell type A at relatively homogenous levels in bFGF-treated cultures compared with the heterogeneity of differentiated cell types in untreated cultures also suggests that bFGF directs the differentiation of X cells.

Finally, bFGF reduces the frequency with which X cells revert to ES cells. X cell reversion appears to be an immediate response to LIF in the absence of MED2 (3.7, 5.2). If bFGF has its effect by inducing the proliferation of differentiated cells within X cell colonies then it should not affect X cell reversion. Xp0+ cells were seeded at clonal cell density into medium containing LIF with and without bFGF(10ng/ml). The assay was stained with alkaline phosphatase at day 5 and colonies were analysed as previously described (Figure 7.5).

In the absence of MED2 and in the presence of LIF, X cells reverted to ES cells. When bFGF was added to these cultures, reversion decreased from 60% to 38%. The level of fully differentiated colonies increased from 10% to 18%. The level of semi-

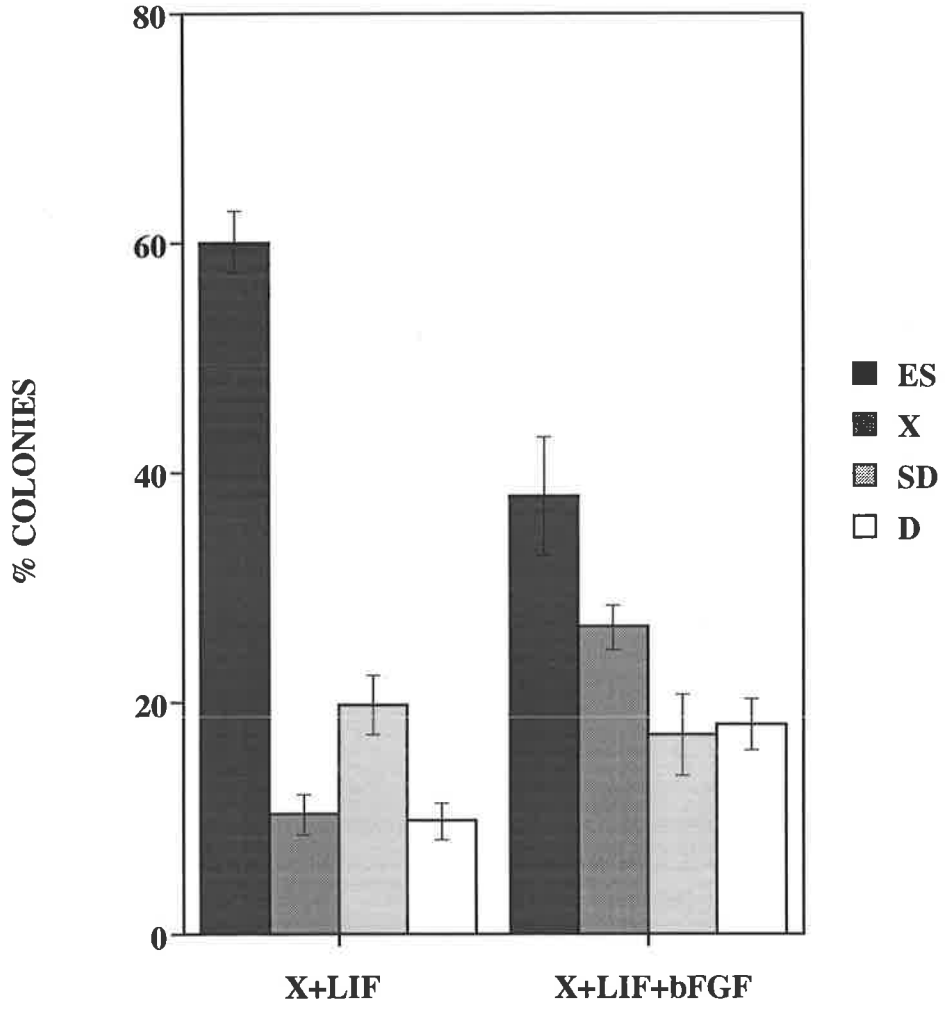
FIGURE 7.5 Basic FGF effects X cell reversion.

Xp0+ cells were seeded at clonal cell density in LIF in the absence of MED2 with and without bFGF (10ng/ml) (2.3.21). Cultures were stained for alkaline phosphatase after 5 days (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig. 3.4). Results were derived from the analysis of four duplicate wells and are tabulated in A and represented graphically in B.

A

CONDITIONS	% COLONIES				PLATING EFFICIENCY (%)
	ES	X	SD	D	
X + LIF	60.1+/-2.7	10.3+/-1.8	19.8+/-2.54	9.8+/-1.6	33.8+/-2.2
X+ LIF + bFGF	37.9+/-5.2	26.6+/-1.9	17.3+/-3.5	18.2+/-2.22	39.0+/-3.2

B



differentiated colonies appeared to be at similar levels while the proportion of undifferentiated X cell colonies had increased from 10% to 27%.

10ng/ml bFGF lowered the frequency with which X cells reverted to ES cells. This implies that bFGF has an effect on X cells and indirectly supports a role for bFGF in X cell differentiation. Basic FGF did not inhibit the reversion of all X cells. This is consistent with its inability to cause the differentiation of all X cells (7.2.1).

These observations suggest that bFGF can induce X cell differentiation. This effect may or may not be direct. bFGF may also have a proliferative effect, whether this effect is on X cells or cell type A or both has not been ascertained at this stage.

7.3. The Effect of Heparin on bFGF X Cell Differentiation

Basic FGF has a strong affinity for the glycosaminoglycan heparin, and this property has facilitated its purification and characterisation (Godspodarowicz *et al.* 1984, Shing *et al.* 1984). The affinity of FGFs for their receptors, and therefore their effect on cell processes, are often dependent on the presence of cell surface heparin sulphate proteoglycans (HSPGs). These effects are therefore potentiated in the presence of heparin (reviewed in Klagsbrun and Baird 1991, Yayon *et al.* 1991, Mason 1994). Inhibitory effects for heparin on FGF function are not as well characterised as stimulatory effects. However, heparin has been reported to inhibit the ability of bFGF to stimulate the proliferation of vascular endothelial cells *in vitro* and to be a potent growth inhibitor of smooth muscle cells (reviewed in Gospodarowisz *et al.* 1986, Gospodarowisz *et al.* 1987a, 1987b).

In section 7.2.1 the effect of bFGF on X cell differentiation was described. While dramatic effects on X cells differentiation were seen, undifferentiated X cell colonies were present at low levels in treated cultures. The ability of heparin to potentiate the effect of bFGF on X cell differentiation was examined.

Xp0+ cells were seeded at clonal cell density into 50% MED2 containing LIF with and without bFGF, in the presence and absence of heparin (10µg/ml). The assay was cultured for 5 days and stained for the presence of alkaline phosphatase activity. The colony types were determined as previously described and are presented in Figure 7.6. Rather than potentiating the effect of bFGF on X cell differentiation, heparin appeared to

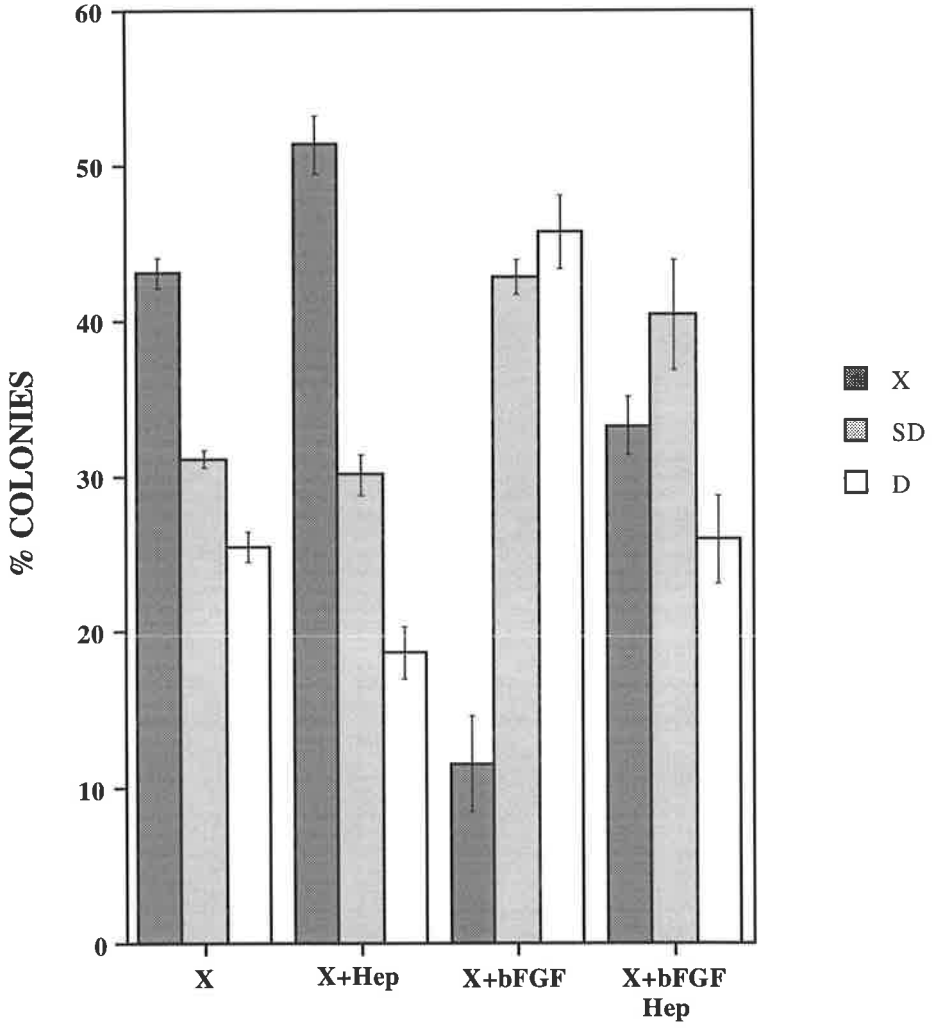
FIGURE 7.6 The effect of heparin addition on the differentiation of X cells with bFGF.

Xp0+ cells were seeded at clonal cell density into MED2 and LIF, with and without bFGF(10ng/ml), in the presence and absence of heparin (10μg/ml) (2.3.21). Cultures were stained for alkaline phosphatase activity at day 5 (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig. 3.4). Results were derived from the analysis of four duplicate wells and results are tabulated in A and represented graphically in B.

A

CONDITIONS	% COLONIES			PLATING EFFICIENCY(%)
	X	SD	D	
X	43.0+/-0.9	32.0+/-0.5	25.0+/-0.9	46.3+/-2.1
X+Hep	51.0+/-1.9	30.0+/-1.4	19.0+/-1.6	43.8+/-2.1
X+bFGF	12.0+/-3.0	43.0+/-1.1	46.0+/-2.4	47.5+/-1.9
X+bFGF+ Hep	32.0+/-1.9	41.0+/-3.5	26.0+/-2.8	41.8+/-2.0

B



impede its effect. When X cells were cultured in the presence of bFGF with heparin a higher proportion of undifferentiated X cell colonies (32%) could be distinguished compared to X cells cultured in bFGF without heparin (12%). Interestingly, heparin resulted in a small yet significant decrease in the level of differentiation observed in untreated X cell cultures. Thus it appears that heparin can also inhibit the action of other factors which induce background differentiation in X cells cultures.

Heparin has also been shown to have an inhibitory effect on mesoderm induction in *Xenopus* (Mitani *et al.* 1989, Johnson *et al.* 1992, Cardellini *et al. 1994*) and has been specifically shown to inhibit mesoderm induction by bFGF (Slack *et al.* 1987). However the mechanism by which heparin exerts an inhibitory effect on this process remains unclear.

7.4 The Effect of Other Members of the FGF Family on the Differentiation of X Cells

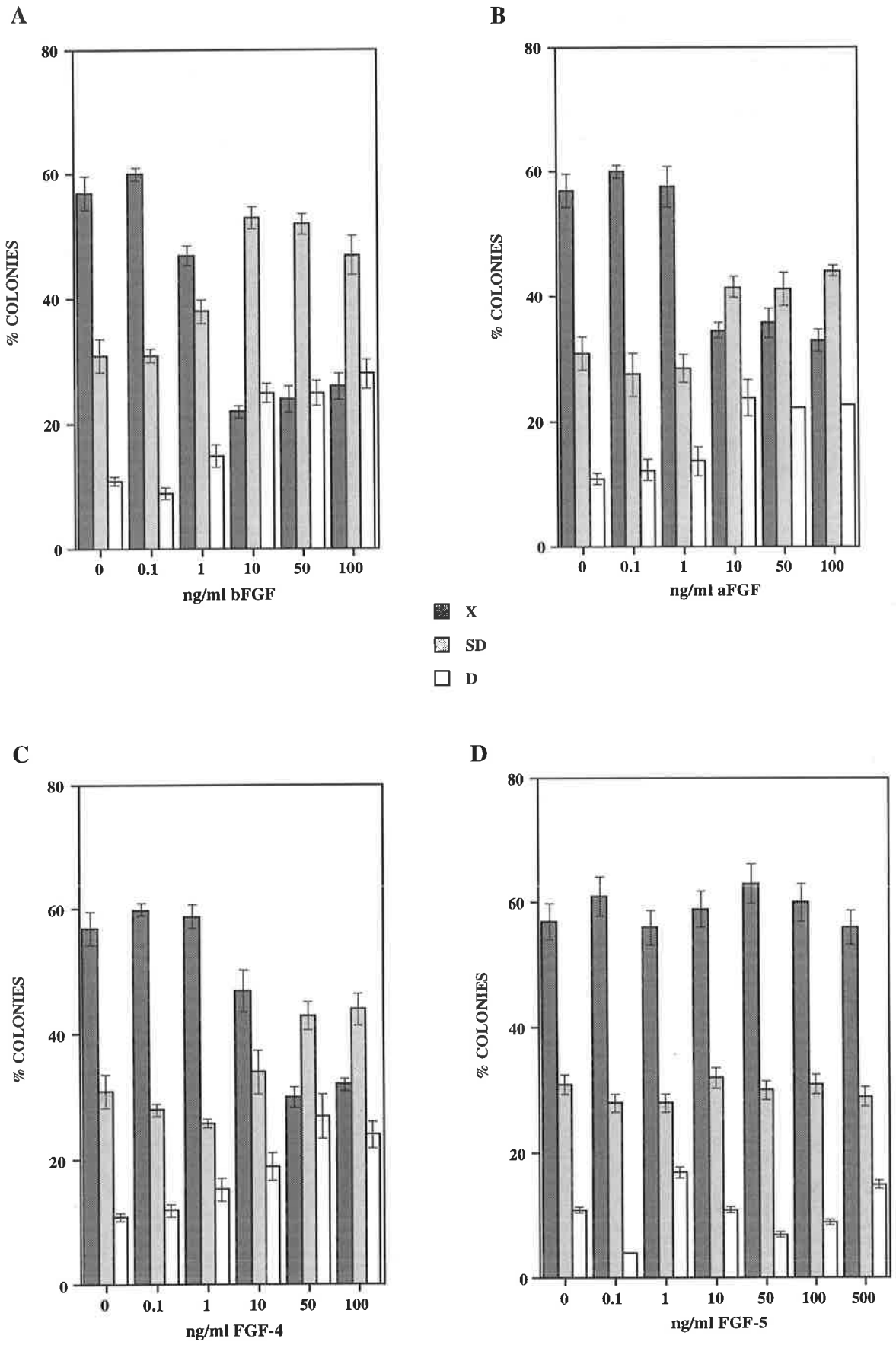
The ability of other members of the FGF family to induce X cell differentiation in a manner analogous to bFGF was investigated. Of particular interest was the effect of FGF-5 on X cell differentiation, since X cells express *FGF-5* themselves (4.2.2). Furthermore, there appears to be a correlation between the level of *FGF-5* expression and the level of differentiation observed in passaged X cells (4.2.1, 5.2, 5.3). The effect of human recombinant FGF-5, acidic FGF (aFGF, FGF-1) and FGF-4 on X cell differentiation was investigated.

Xp0+ cells were seeded at clonal cell density in 50% MED2 and LIF with increasing concentrations of FGF-5 (0.1 to 500ng/ml), aFGF, bFGF and FGF-4 (0.1 to 100ng/ml). Assays were stained at five days for alkaline phosphatase activity and colony types were determined as previously described. Results are presented in Figure 7.7.

Maximal differentiation of X cells was achieved with concentrations of bFGF equal to or greater than 10ng/ml and was characterised by the generation of high levels of cell type A (Figure 7.1). Acidic FGF and FGF-4, although less potent than bFGF in this assay, were also able to induce the differentiation of X cells to cell type A. FGF-5 had no effect on the differentiation of X cells at any concentration examined. Swiss 3T3 fibroblast proliferation assays (2.3.23) indicated that maximal proliferation of fibroblasts

FIGURE 7.7 The effect of aFGF, FGF-4 and FGF-5 on the differentiation of X cells:- a comparison with bFGF.

Xp0+ cells were seeded at clonal cell density in MED2 and LIF in the presence of increasing concentrations of aFGF, bFGF, FGF-4 and FGF-5 (2.3.21). After 5 days assays were stained for alkaline phosphatase activity (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig. 3.4). Results were derived from the analysis of four duplicate wells and results obtained for bFGF, aFGF, FGF-4 and FGF-5 are represented graphically in A, B, C and D respectively.


A
B
C
D
% COLONIES
ng/ml bFGF
ng/ml aFGF
ng/ml FGF-4
ng/ml FGF-5
0
0.1
1
10
50
100
500
20
40
60
80
X
SD
D

was obtained using a concentration of FGF-5 at 500ng/ml in the presence of 10μg/ml Heparin (data not shown). Addition of heparin at any concentration including 500ng/ml had no effect on the differentiation of X cells in response to FGF-5 (data not shown).

Results of this experiment demonstrate that aFGF, bFGF and FGF-4, but not FGF-5, are able to induce X cell differentiation to cell type A. The effects of the different FGFs on X cell differentiation are likely to be mediated at the level of the receptor.

The FGF receptor family constitutes a complex system and four high affinity FGF receptors have been identified. Alternative isoforms of receptors are produced by alternative exon usage. Different FGFs have been shown to have different affinities for the four receptors as well as their different isoforms (Mansukani *et al.* 1992, Wang *et al.* 1994, Clements *et al.* 1992). The presence or absence of HSPGs further complicates receptor binding by different members of the FGF family (reviewed in Klagsbrun and Baird 1991, Mason 1994). While X cells have been shown to express FGFR-1 (Bettess 1993), the exact isoforms of FGFR-1 have not been determined. In addition the expression of the other FGF receptors has not been fully assessed. Therefore the receptor or receptors responsible for the different activities of the FGF members can not yet be determined.

7.5 Activin A Induces the Differentiation of X Cells

Activins are members of the TGFβ family and function as dimers of two β-subunits of which there are two forms βA and βB. Three types of activin have been isolated, activin A (βAβA), activin B (βBβB) and activin AB (βAβB) (Ling *et al.* 1988, Vale *et al.* 1990). Activins interact with two classes of serine/threonine kinase membrane receptors, Type I and Type II, which are distinguishable by their binding properties (Attisano *et al.* 1993, ten Dijke *et al.* 1994). Activin subunits and receptors are expressed throughout murine embryogenesis and are present during gastrulation where they may play a role in mesoderm induction (Albano *et al.* 1993, 1994, Manova *et al.* 1994, Verscheueren *et al.* 1995). *Xenopus* transplantation and animal cap induction studies indicate that activin A induces the formation of dorsal mesoderm, including notochord and neural inducing tissue (for reviews see Slack 1990, Ruiz i Altaba and Melton, 1990, Cooke 1991 and Sive 1993).

7.5.1 Activin A Induces the Differentiation of X Cells but not ES Cells

ES and Xp0+ cells were seeded at clonal cell density into medium containing LIF and 50% MED2 + LIF, respectively. Activin A activity in *Xenopus* mesoderm induction studies has been reported from concentrations as low as 0.2 ng/ml to those as high as 100ng/ml (Green *et al.* 1990, Slack 1990, Asashima 1990). The cells were cultured in the presence of increasing concentrations of recombinant human activin A (1ng/ml to 200ng/ml).

Activin A had no effect on ES cell differentiation at any concentration examined. However addition of activin A to X cells induced their differentiation. Differences between treated and untreated X cell cultures could be seen as early as two days and became obvious at three days (Figure 7.8). After five days the majority of colonies in activin A-treated wells had the colony morphology shown in Figure 7.9. Differentiation with activin A was characterised by the appearance of a fibroblastic cell type which did not stain with alkaline phosphatase. This cell type exhibited a cellular morphology which was different from cell type A observed in bFGF-treated cultures (Figure 7.1) and has therefore been denoted cell type B. Cell type B was not observed in untreated cultures.

Assays were stained for alkaline phosphatase activity and the percentage of ES, X, semi-differentiated and fully differentiated colonies were determined as previously described. Although differentiation of X cell cultures was seen at 50ng/ml activin A, maximal differentiation was seen at 150ng/ml. This contrasts with the low levels of activin A required for mesoderm induction in *Xenopus* (Slack 1990, Green *et al.* 1990). The colony types associated with ES and X cells treated with 150ng/ml activin A are presented in Figure 7.10.

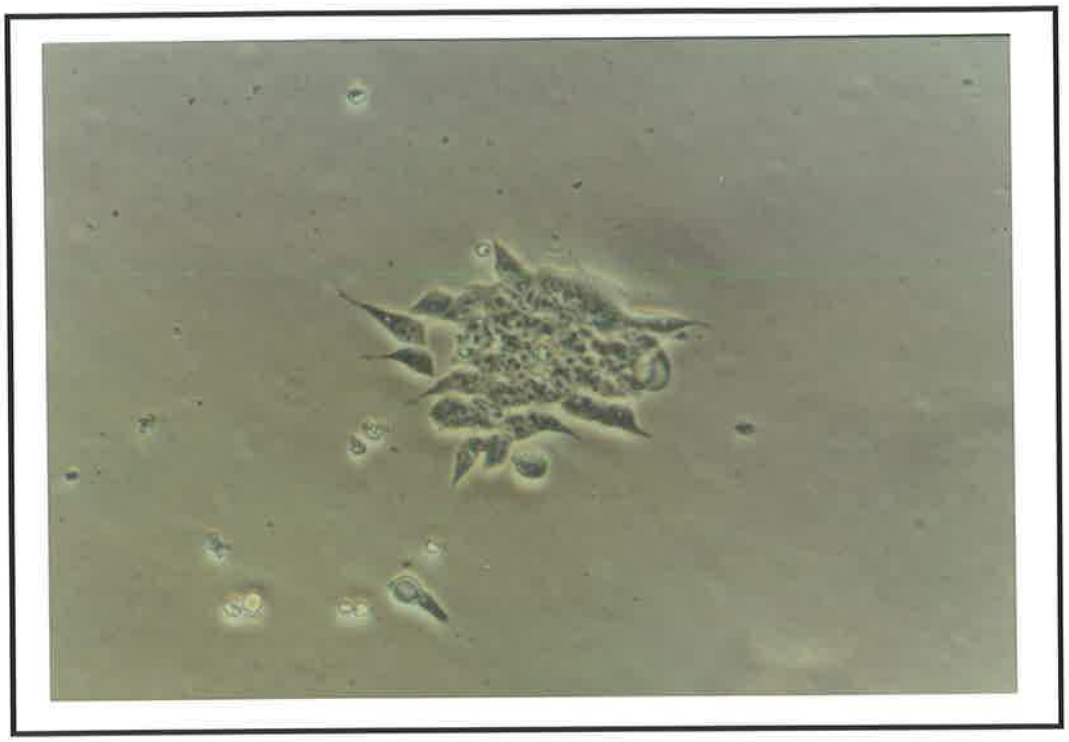
Like bFGF, activin A had no effect on ES cell differentiation. This is consistent with observations made by others (P.Rathjen, personal communication). However, activin A had a dramatic effect on the differentiation of X cells. The percentage of undifferentiated X cell colonies decreased from 52% in untreated cultures to 3% in activin A-treated cultures. The level of semi-differentiated and fully differentiated colonies rose from 39% to 65% and from 9% to 31%, respectively. The dramatic decrease in the level of undifferentiated X cell colonies and the generation of cell type B, which was not

FIGURE 7.8 Activin A induces the differentiation of X cells:- morphology at day 3.

Xp0+ cells were seeded at clonal cell density into MED2 and LIF with and without activin A (150ng/ml). Differentiation of X cell colonies within activin A-treated cultures was evident at day 3. The morphology of X cell colonies within untreated cultures is shown in A. The morphology of X cell colonies in activin A-treated cultures is shown in B.

Photographs were taken under phase contrast at a magnification of 50x.

A



B

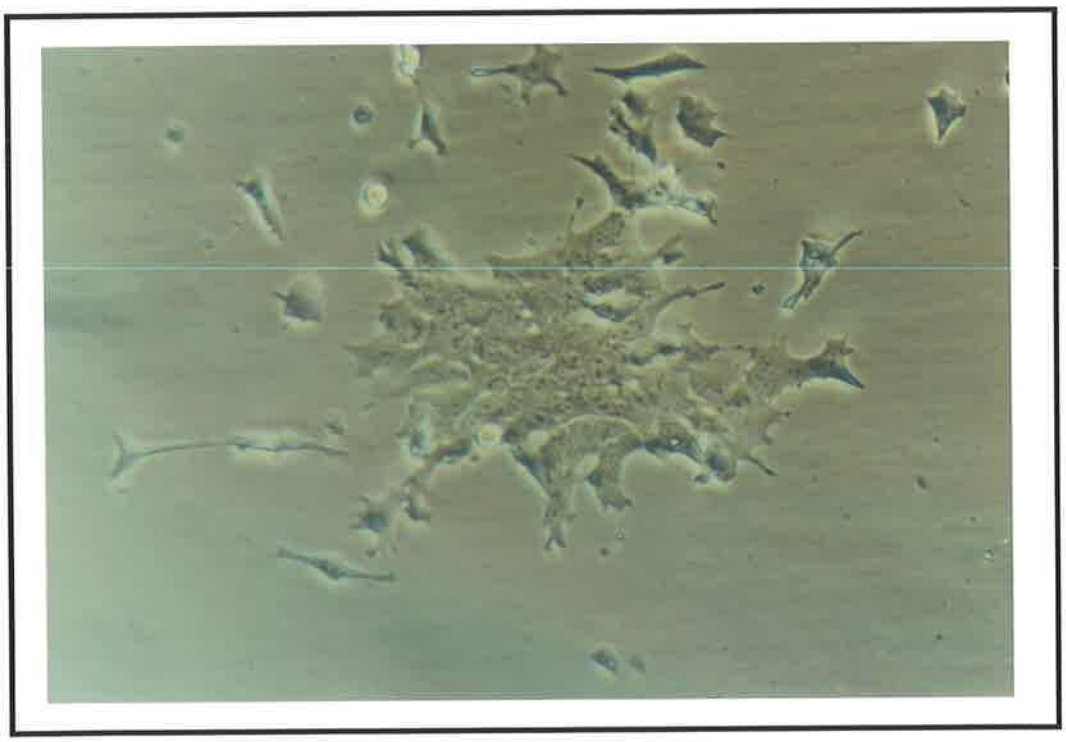
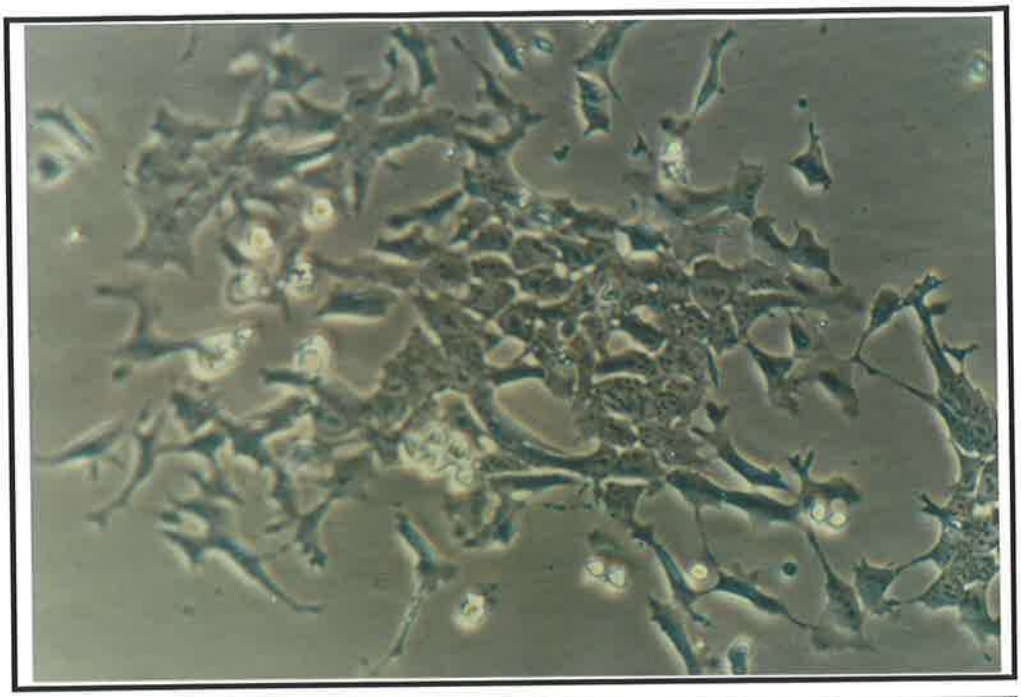


FIGURE 7.9 Activin A induces the differentiation of X cells:- morphology at day 5.

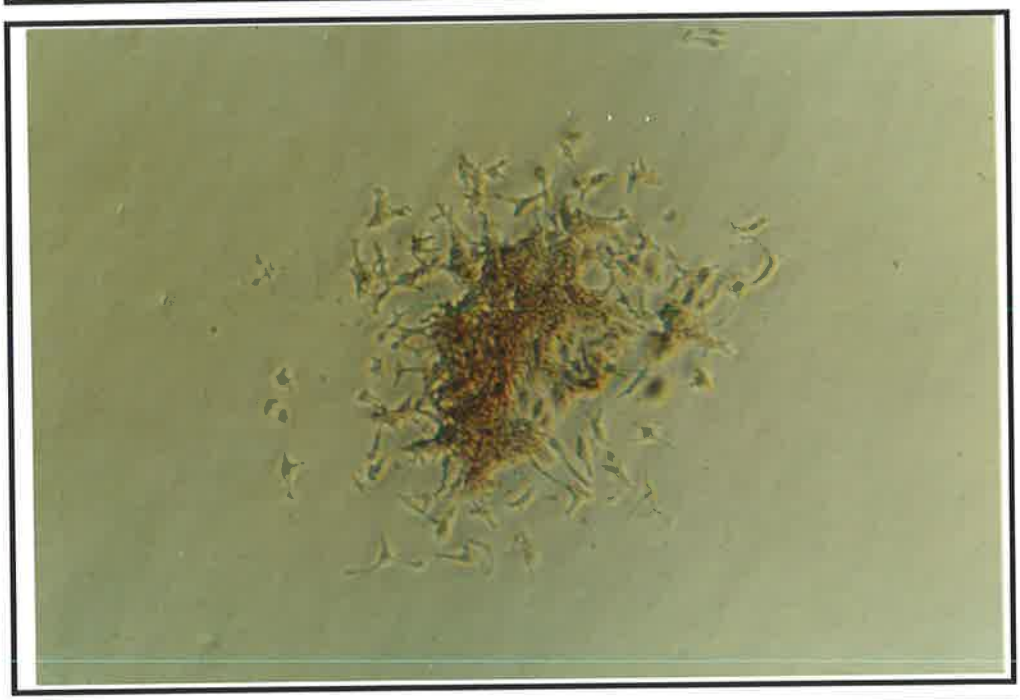
After 5 days X cells cultured in the presence of activin A exhibited a colony morphology shown in A. Cultures were stained for alkaline phosphatase activity and counterstained with haematoxylin (2.3.24). The colony morphology associated with activin A treatment is clearly seen in B. Cellular morphology associated with the differentiated cells within these colonies can clearly be seen in C. This cell type did not stain with alkaline phosphatase and was designated cell type B.

Photographs A and C were taken at a magnification of 50x, B 25x, using phase contrast optics.

A



B



C

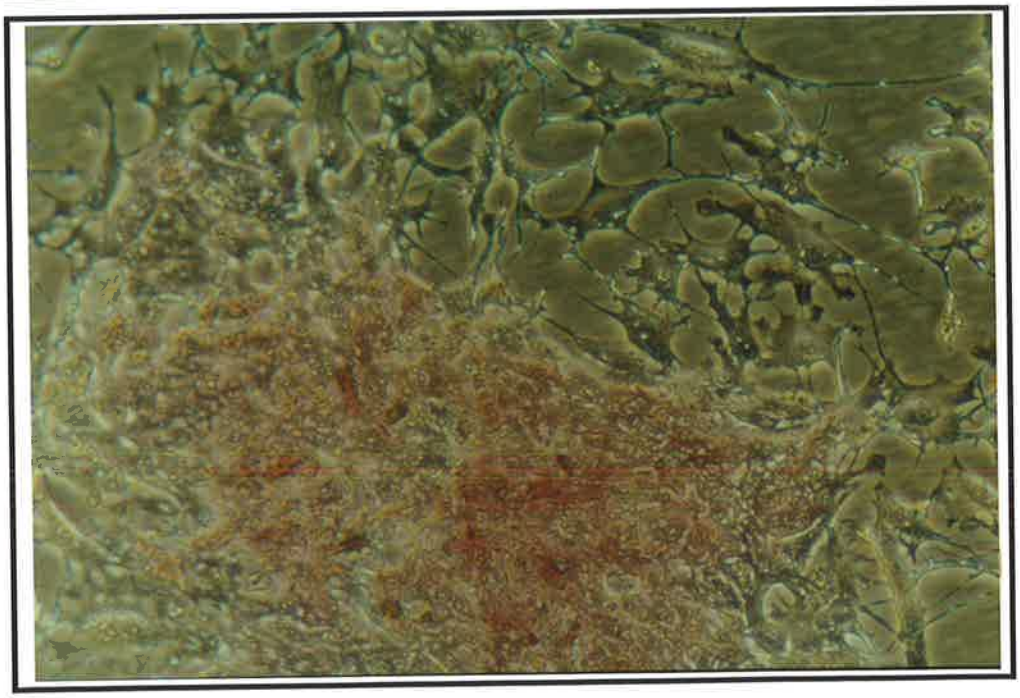


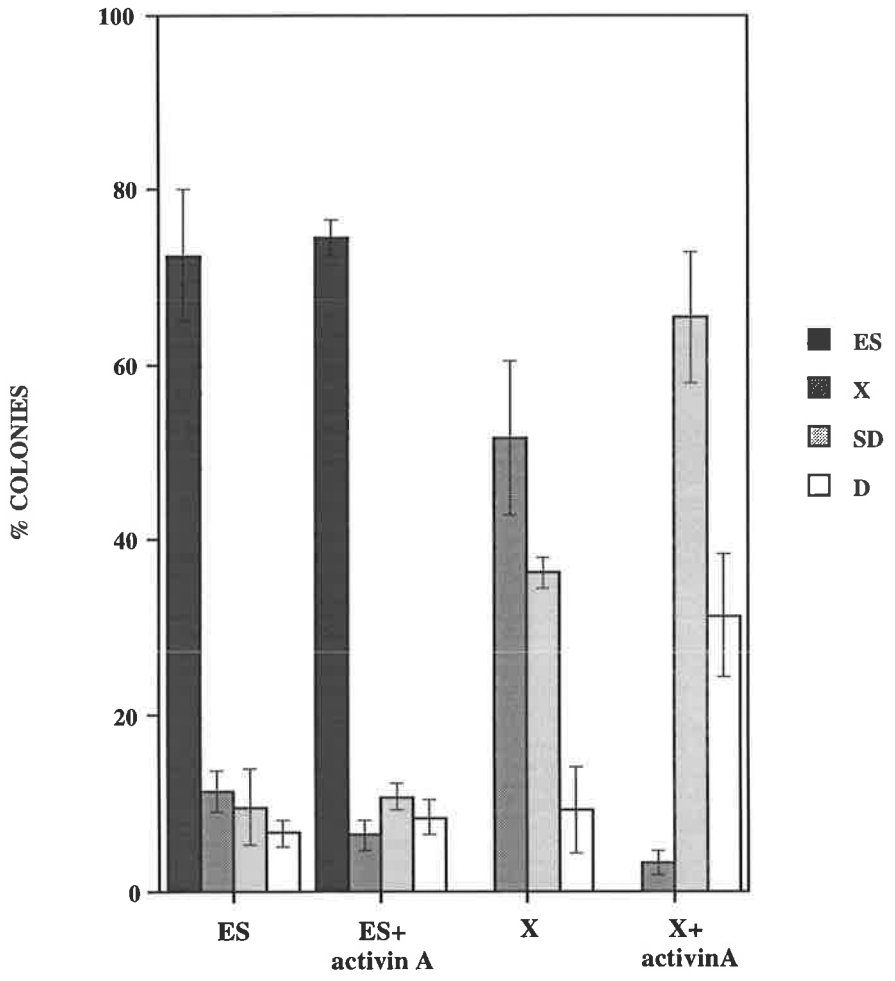
FIGURE 7.10 Activin A induces the differentiation of X cells, but not ES cells:- colony analysis at day 5.

Xp0+ cells were seeded at clonal cell density in MED2 and LIF in the presence and absence of activin A (150ng/ml). Cultures were stained for alkaline phosphatase activity (2.3.24) and the proportion of ES cell colonies (ES), X cell colonies (X), semi-differentiated colonies (SD) and differentiated colonies (D) was determined as previously described (Fig. 3.4). Results were derived from the analysis of duplicate wells, are tabulated in A and represented graphically in B.

A

CONDITIONS	% COLONIES				PLATING EFFICIENCY (%)
	ES	X	SD	D	
ES	72.5+/-7.5	11.3+/-2.3	9.6+/-4.2	6.6+/-1.5	33.6+/-1.6
ES + activin A	74.5+/-2.1	6.4+/-2.1	10.7+/-1.5	8.4+/-2.0	35.1+/-2.3
X	0	51.2+/-8.7	36.3+/-1.7	9.2+/-4.9	39.9+/-5.4
X + activn A	0	3.28+/-1.4	65.4+/-7.5	31.3+/-7.1	41.13+/-3.8

B



present in untreated cultures, upon activin A treatment suggested that activin A was inducing the differentiation of X cells.

7.5.2 Comparison of bFGF-Induced and Activin A-Induced X Cell Differentiation

Both activin A and bFGF induced the differentiation of X cells, however activin A appeared to be more effective at inducing X cell differentiation than bFGF as assessed by the level of undifferentiated X cell colonies (3% and 21%. respectively). At day 3 of differentiation the colony morphology of X cells treated with bFGF and activin A appeared similar (compare Figure 7.4 with 7.8). X cells within colonies treated with these factors appeared migratory, with a spread morphology. After 5 days the majority of colonies observed in bFGF-treated cultures contained cells with a compact fibroblastic morphology, cell type A (Figure 7.1). Activin A-treated cultures had colonies which contained cells which had a highly motile fibroblastic appearance, cell type B (Figure 7.9). Therefore activin A and bFGF directs the alternative differentiation of X cells to morphologically different cell types. This is consistent with alternative cell types formed upon bFGF and activin A treatment in *Xenopus* mesoderm induction studies (7.2, 7.5, Sive 1993).

7.6 The Gene Expression Associated with X Cell Differentiation in Response to Mesoderm Inducing Cytokines

A number of genes expressed stage and tissue specifically during gastrulation have been identified. Of these, the transcription factors *goosecoid* and *Brachyury* have been examined extensively. The homeobox gene *goosecoid* was originally discovered in *Xenopus* (Blumberg *et al.* 1991) and subsequently the murine homologue was isolated (Blum *et al.* 1992). *Goosecoid* expression is conserved stage and tissue specifically between many vertebrate species including the mouse, chick, zebra fish and *Xenopus* (DeRobertis *et al.* 1994). During murine embryogenesis *goosecoid* expression is confined to the posterior region of the egg cylinder where the primitive streak forms. Cells expressing *goosecoid* move anteriorly, ~~becoming localised to~~ marking the node during gastrulation (Blum *et al.* 1992). Thus *goosecoid* is a marker of anterior dorsal mesoderm.

Brachyury was originally identified as the gene mutated in the murine developmental mutation, *T* (Herrmann *et al.* 1990). *Brachyury* is required for mesoderm formation during gastrulation (Wilkinson *et al.* 1990, Herrmann 1991, Beddington *et al.* 1992) and the expression pattern of *Brachyury* is highly conserved among vertebrates (DeRobertis *et al.* 1994). During murine embryogenesis *Brachyury* expression begins at gastrulation and is restricted to the primitive streak and mesodermal cells emerging form the streak. At later stages of development expression is restricted to the notochord and head process (Herrmann *et al.* 1990, Wilkinson *et al.* 1990). *Brachyury* is a marker of posterior and ventral mesoderm.

While both bFGF and activin A induce the formation of ventral mesoderm in isolated *Xenopus* animal caps, only activin A induces dorsal mesoderm in isolated animal caps (reviewed in Sive 1993). Consistent with this, both bFGF and activin A induce the expression of *Brachyury* in *Xenopus* animal cap assays (Smith *et al.* 1991), while only activin A induces *goosecoid* expression (Cho *et al.* 1992).

When isolated egg cylinder stage mouse embryos were treated with activin A, *goosecoid* expression was detected throughout the primitive ectoderm (Blum *et al.* 1992). This implies that an activin-like molecule can direct *in vivo* expression of *goosecoid* in responsive cells during murine embryogenesis. Activin A and bFGF have been shown to induce the expression of *Brachyury* in P19 EC cells (Vidricaire *et al.* 1994).

7.6.1 Gene Expression in bFGF-Treated X Cell Cultures

In situ hybridisation was used to examine the expression of *goosecoid* and *Brachyury* in X cells cultured with and without bFGF. Xp0+ cells were seeded at clonal cell density into 50% MED2 and LIF with and without bFGF (10ng/ml). At day 5 plates were fixed and examined for the expression of *goosecoid* and *Brachyury* using DIG labelled antisense RNA probes.

Goosecoid expression could not be detected using this method. *Brachyury* expression was detected in bFGF-treated cultures and the characteristic expression pattern is shown in Figure 7.11. *Brachyury* expression could not be detected in undifferentiated X cells nor in differentiated cells. Expression appeared to be confined to differentiating X cells within semi-differentiated colonies. *In vivo*, *Brachyury* expression is confined to

FIGURE 7.11 a) and b) Gene expression associated with bFGF-induced X cell differentiation:- *Brachyury* expression.

Photographs outlining *Brachyury* expression in bFGF-treated cultures are shown in a) and b).

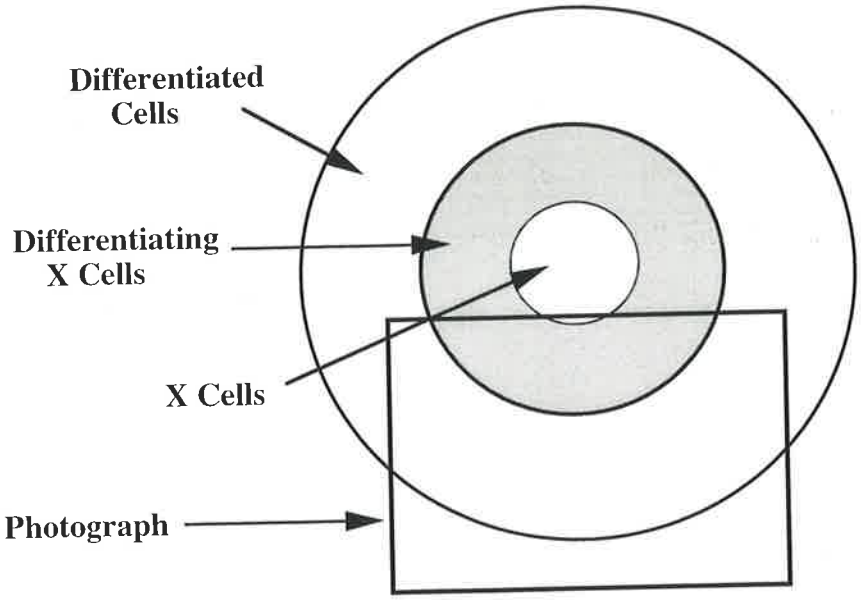
Basic FGF-treated and untreated cultures were fixed at day 5 and *Brachyury* expression analysed by *in situ* hybridisation (2.5.19). *Brachyury* expression was detected in these cultures using a 1.8kb DIG labelled antisense riboprobe (2.5.17). Plates were developed for 9 hours before the colour reaction was stopped. Cells expressing *Brachyury* were distinguished by purple staining.

Undifferentiated X cells are generally found within the centre of semi-differentiated colonies, while differentiated cells are found around the outside. *Brachyury* expression appeared to be confined to the differentiating cells within semi-differentiated colonies. A schematic view of the colony photographed is depicted in A. Photographs in B and C show *Brachyury* expression within this colony. X = undifferentiated X cells.

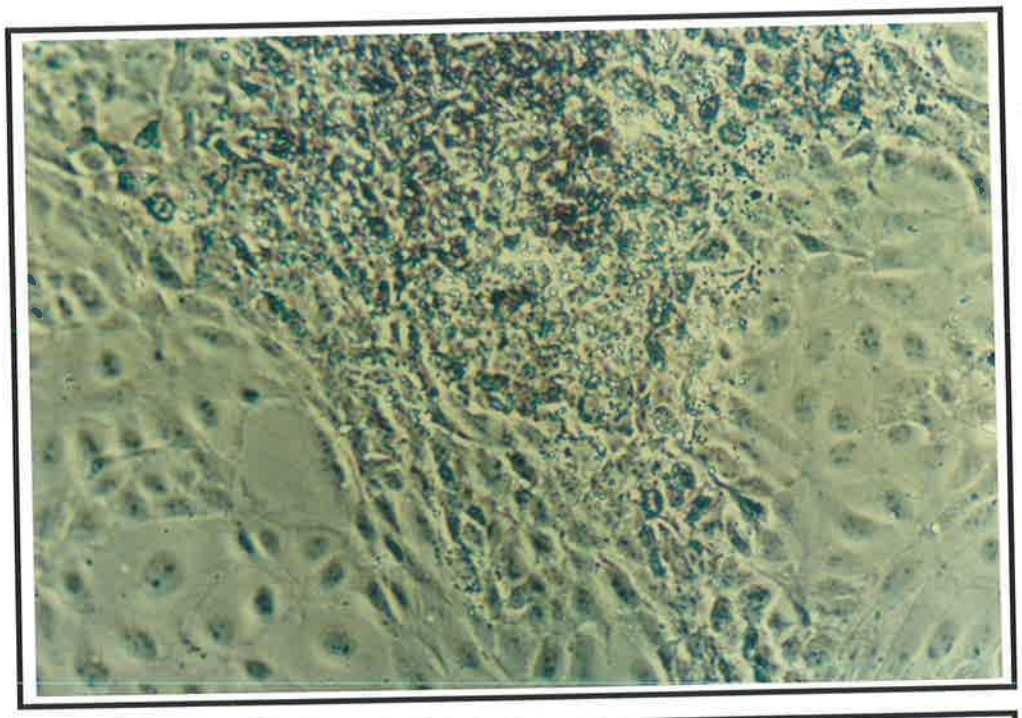
Photographs were taken under phase contrast (B) and bright field optics (C) at a magnification of 50x.

a

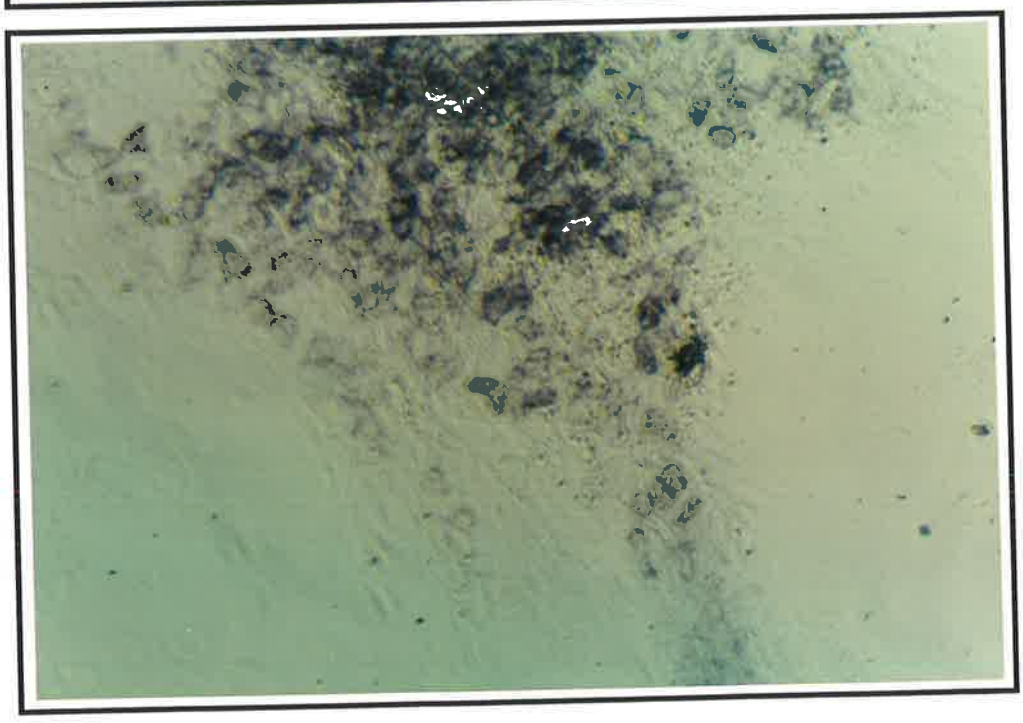
A


Differentiated Cells
Differentiating X Cells
X Cells
Photograph

B

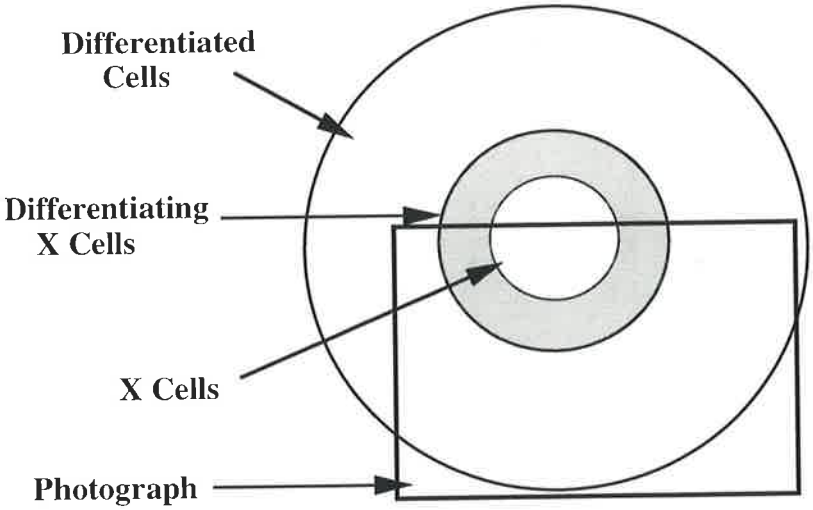


C

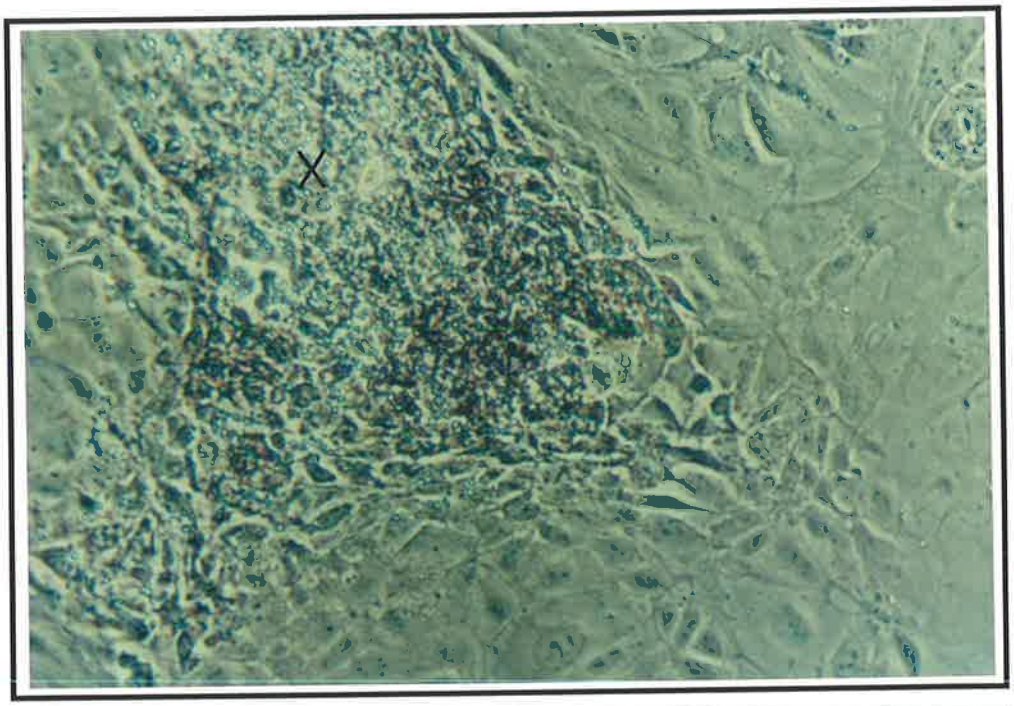


b

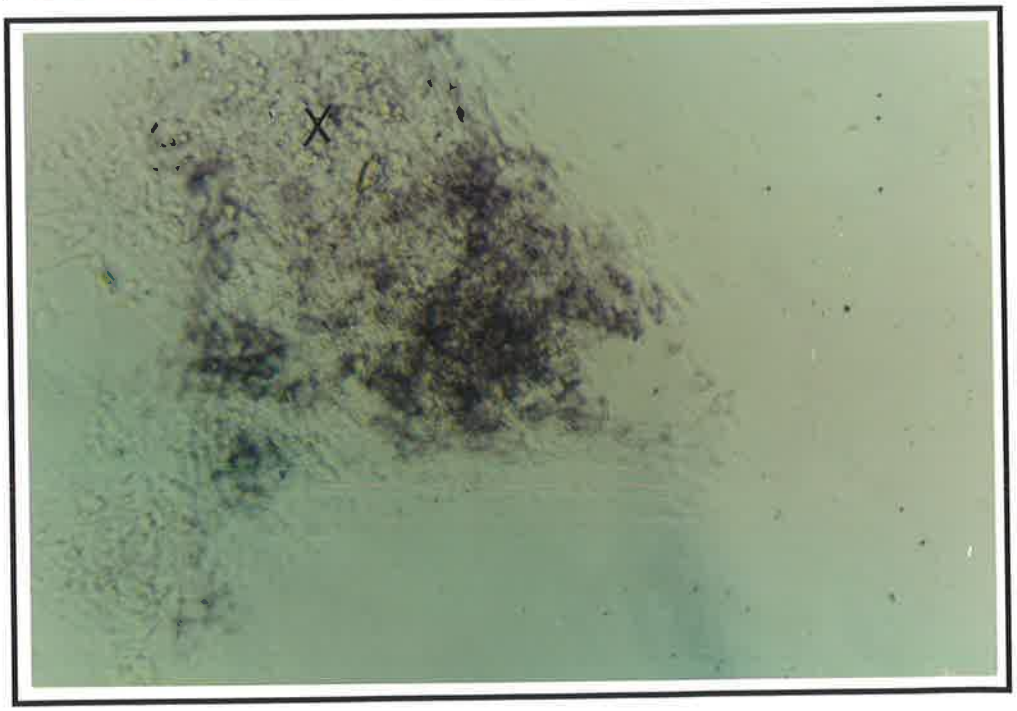
A


Differentiated Cells
Differentiating X Cells
X Cells
Photograph

B


X

C


X

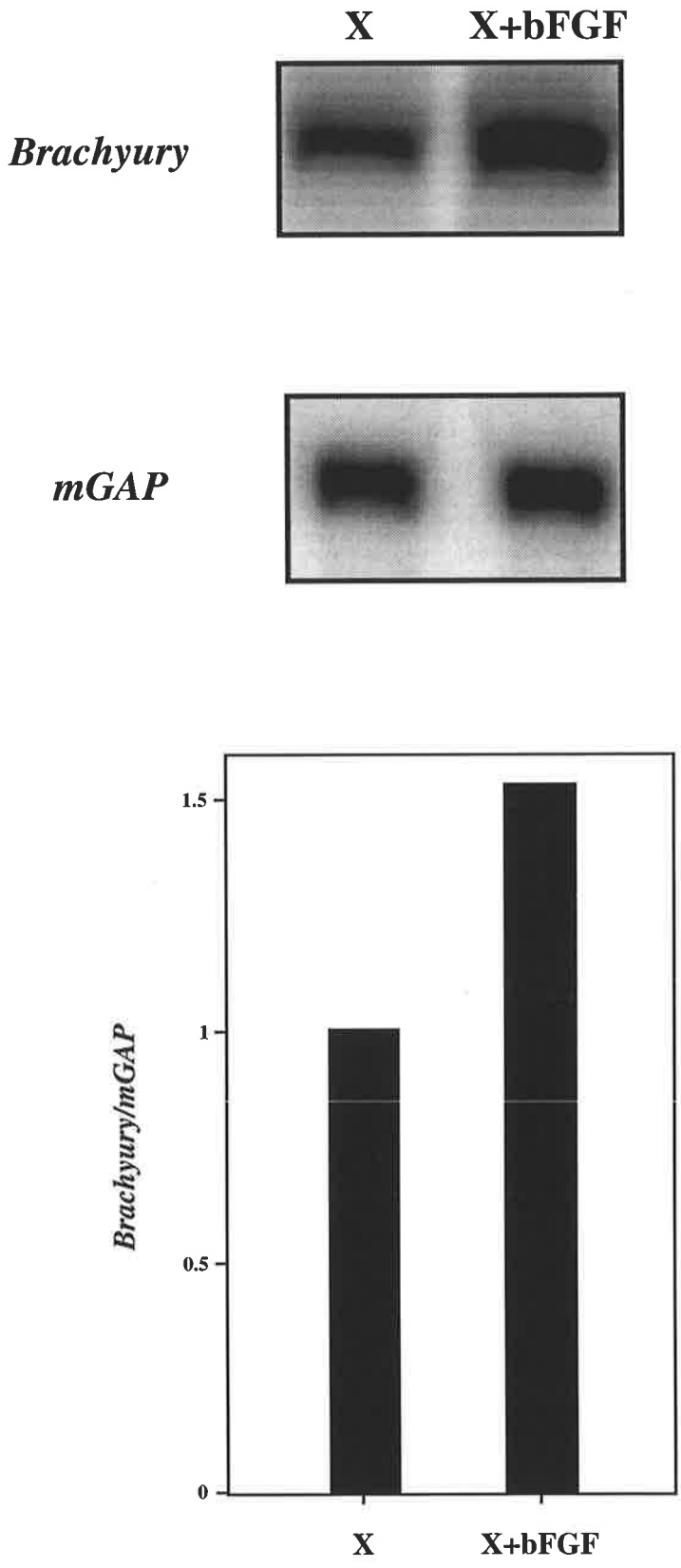
newly formed mesodermal derivatives emanating from the primitive streak and is down regulated in these cells with subsequent differentiation. The *Brachyury* expression pattern observed in bFGF-treated cultures appeared consistent with this, and suggests that bFGF induces the differentiation of X cells to a mesodermal derivative *via* a *Brachyury* expressing intermediate. While bFGF-induced differentiation of X cells was easily observed by the formation of high and homogeneous levels of cell type A, *Brachyury* expression was also detected in differentiating cells within semi-differentiated colonies in untreated X cell cultures. This suggests that spontaneous differentiation of X cells in culture also results in mesodermal derivatives and complicates the interpretation and analysis of *Brachyury* expression specifically related to the presence of bFGF. This complication was resolved by comparing the level of *Brachyury* expression due to background differentiation in control cultures with the level observed in bFGF-treated cultures by Northern analysis.

The level of semi-differentiated colonies in treated cultures is 15% higher than the level observed in untreated cultures (7.2.1). Therefore the level of *Brachyury* expression in bFGF-treated cultures should be higher than the level in untreated cultures. Xp0+ cells were seeded at clonal cell density into replica 150mm tissue culture plates in 50%MED2 with LIF with and without bFGF (10ng/ml). Cells in untreated and treated cultures were harvested for RNA preparation at day 5. The level of *Brachyury* expression, compared with *mGAP*, in untreated and treated X cell cultures was determined by Northern analysis of total RNA. The results of this analysis are presented in Figure 7.12. X cells treated with bFGF exhibited higher levels of *Brachyury* expression than control cultures. The level of *Brachyury* expression in bFGF-treated cultures correlated with the level of semi-differentiated colonies observed in these cultures compared with control cultures. A significant proportion of the colonies within bFGF cultures are fully differentiated (34%) compared to untreated cultures (10%). Therefore while the level of *Brachyury* expression in bFGF-treated cultures is higher than the level in untreated cultures it does not reflect the total level of differentiation which has occurred within the culture. Consistent with *in situ* hybridisation analysis, *goosecoid* expression could not be detected by Northern analysis of RNA derived from these cultures (data not shown).

FIGURE 7.12 Quantitation of the level of *Brachyury* expression within bFGF-treated cultures.

RNA was harvested from X cells cultured at clonal cell density in MED2 and LIF with and without bFGF (10ng/ml) (2.5.10). The expression of *Brachyury* compared to a *mGAP* loading control was analysed by Northern blot of total RNA. An antisense riboprobe was used to detect *Brachyury* expression (2.5.15). The filter was washed in 2xSSC/0.1%SDS at 65°C, 0.2xSSC/0.1%SDS at 65°C and finally in 0.2xSSC/0.1%SDS at 75°C. All washes were for a minimum duration of 30 minutes. The filter was exposed to a phosphorimager screen for 20 hours (2.5.20). The filter was subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16), exposure time 21 hours. The results of this analysis are presented in A.

The level of *Brachyury* and *mGAP* expression was quantitated by volume integration (2.5.20). The level of *Brachyury* expression normalised to *mGAP* observed in treated and untreated cultures is represented graphically in B.


X
X+bFGF
Brachyury
mGAP
1.5
1
0.5
0
Brachyury/mGAP
X
X+bFGF

The nature of the differentiated cell type, cell type A, observed at high frequency in bFGF-treated cultures, was examined by Northern blot and *in situ* analysis. Cell type A did not express *AFP*, *SPARC* or *H19* (data not shown), markers for extraembryonic tissues (Dziadek and Adamson 1978, Dziadek and Andrews, 1983, Holland *et al.* 1987, Wilkinson *et al.* 1988, Poirier *et al.* 1991).

The homeobox gene, *S8*, is expressed predominantly in the mesenchyme of the developing embryo from day 7.5, and expression is confined to fibroblast-like cell lines *in vitro*. *S8* expression is found in mesodermal cells further away from the primitive streak (Opstelten *et al.* 1991, DeJong and Meijlink, 1993). The expression of *S8* in bFGF-treated and untreated X cell cultures, was examined by Northern analysis. The expression of *S8* compared to a *mGAP* loading control is shown in Figure 7.13.

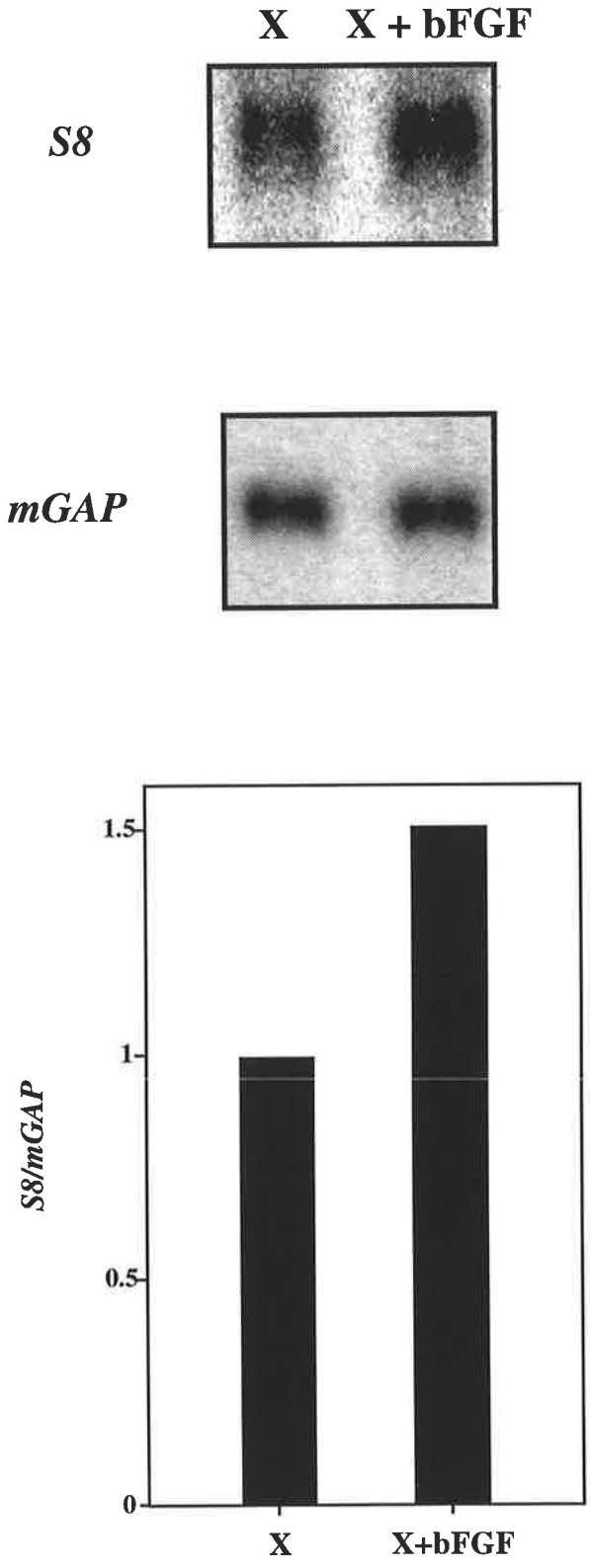
S8 expression could be detected at a higher level in treated cultures than in untreated cultures. *S8* expression was not detected at a significant level in RNA derived from ES and Xp0+ cells (data not shown). Xp0+ cells are formed at high cell density and represent a relatively uniform X cell population (3.6). This indicates that *S8* expression is confined to differentiated cells within treated and untreated cultures. The higher level of *S8* expression observed in treated cultures is consistent with the observation of cell type A at higher levels in these cultures. Attempts to visualise *S8* expression in cell type A by *in situ* hybridisation were unsuccessful. The expression of *S8* at higher levels in bFGF-treated cultures is consistent with bFGF inducing the differentiation of X cells to a mesodermal derivative.

While the interpretation and analysis of gene expression exhibited by bFGF-treated cultures was complicated by the presence of background differentiation in control cultures, several conclusions can be made. Firstly, bFGF does not induce the differentiation of X cells into primitive endodermal derivatives as assessed by *AFP*, *SPARC* and *H19* expression. Secondly, the higher levels of *Brachyury* and *S8* expression in bFGF-treated cultures indicate that bFGF induces the differentiation of X cells to a mesodermal derivative. This mesodermal derivative also arises during spontaneous differentiation in routine X cell culture, perhaps in response to low levels of FGF in serum or MED2, *Brachyury* expressed in intermediate cells and *S8* expressed in terminally differentiated cells. Differentiation to mesoderm *via* intermediate cells that

FIGURE 7.13 Gene expression associated with bFGF-induced X cell differentiation:- *S8* expression.

The expression of *S8* compared to a *mGAP* loading control was analysed by Northern blot of total RNA derived from bFGF-treated and untreated cultures. A DNA probe was used to detect *S8* expression (2.5.14). The filter was washed in 2xSSC/0.1%SDS, 0.2xSSC/0.1%SDS at 42°C and finally in 0.2xSSC/0.1%SDS at 65°C. All washes were for a minimum duration of 30 minutes. The filter was exposed to a phosphorimager screen for 24 hours (2.5.20). The filter was subsequently stripped and reprobed with *mGAP* (2.5.14, 2.5.16), exposure time 22 hours. The results of this analysis are presented in A.

The level of *S8* and *mGAP* expression was quantitated by volume integration (2.5.20). The level of *S8* expression normalised to *mGAP* observed in bFGF-treated and untreated cultures is represented graphically in B.


X
X + bFGF
S8
mGAP
S8/mGAP
1.5
1
0.5
0
X
X+bFGF

express *Brachyury* but not *goosecoid* is consistent with the expression of these genes during mesoderm induction with bFGF in *Xenopus*.

7.6.2 Gene Expression in Activin A-Treated X Cell Cultures

The expression of *goosecoid* and *Brachyury* in X cells cultured with and without Activin A was analysed using *in situ* hybridisation and compared with X cells treated with bFGF. Xp0+ cells were seeded at clonal cell density into 50% MED2 and LIF with and without activin A (150ng/ml) or bFGF (10ng/ml). At day 5 plates were fixed and examined for the expression of *goosecoid* and *Brachyury* using DIG labelled antisense riboprobes.

Brachyury expression could be detected in the differentiating X cells within semi-differentiated colonies in all cultures. The quantity of semi-differentiated colonies in X cells treated with activin A was higher than the level of these colonies in bFGF-treated cultures (compare the % of semi-differentiated colonies shown in Figure 7.2 with that shown in Figure 7.10). Consistent with this 60% of the colonies in activin A-treated cultures exhibited *Brachyury* expression compared with 43% of colonies in bFGF-treated cultures.

Goosecoid expression was detected in activin A-treated cultures but could not be detected in bFGF-treated and untreated cultures. *Goosecoid* expression appeared to be restricted to X cells within the centre of differentiating colonies, whereas *Brachyury* expression was restricted to differentiating cells between X cells and differentiated cells. Photographs showing the expression pattern of *Brachyury* and *goosecoid* within activin A-treated cultures are presented in Figure 7.14 and Figure 7.15 respectively.

Treatment of murine embryos at 6.4 days of development with activin A leads to ectopic expression of *goosecoid* throughout the primitive ectoderm. The expression of *goosecoid* in X cells treated with activin A is therefore consistent with their proposed relationship with primitive ectoderm. The results of these experiments are also consistent with mesoderm induction experiments carried out in *Xenopus* which demonstrate *Brachyury* expression in response to activin A and bFGF, and *goosecoid* expression in response to activin A, but not bFGF. Similarly, *Brachyury* but not *goosecoid* was

FIGURE 7.14 Gene expression associated with activin A-induced differentiation of X cells:- *Brachyury* expression.

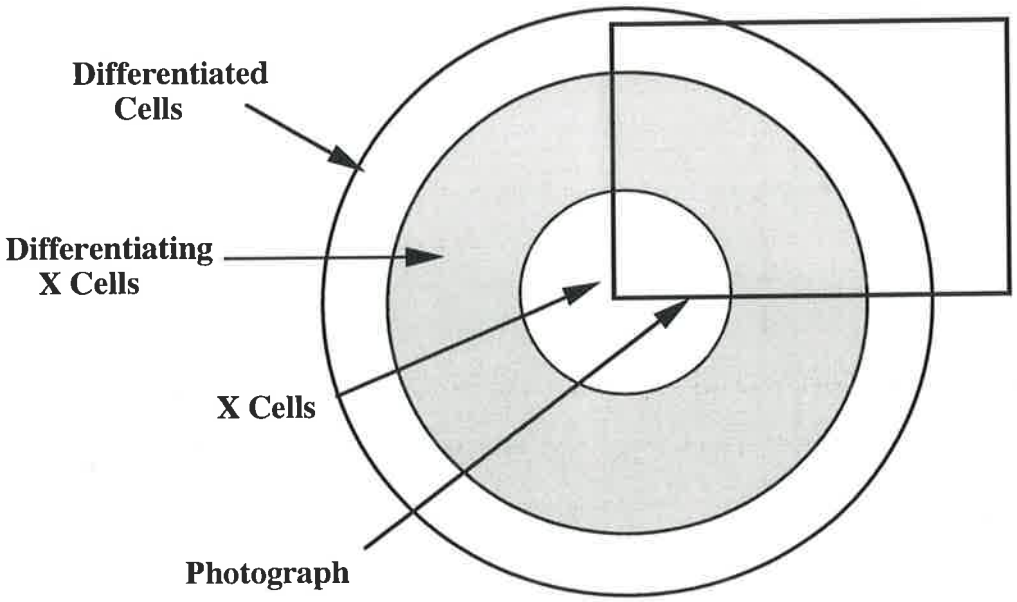
Activin A-treated and untreated cultures were fixed at day 5. *Brachyury* expression within these cultures was analysed by *in situ* hybridisation (2.5.19). *Brachyury* expression was detected in these cultures using a 1.8kb DIG labelled antisense riboprobe (2.5.17). Plates were developed for 9 hours before the colour reaction was stopped. Cells expressing *Brachyury* were distinguished by purple staining.

Undifferentiated X cells are generally found within the centre of semi-differentiated colonies, while differentiated cells are found around the outside. A schematic view of the colony photographed is depicted in A. *Brachyury* expression within this colony is shown in photograph B and appeared to be confined to the differentiating cells within semi-differentiated colonies.

X = undifferentiated X cells

The photograph was taken under phase contrast at a magnification of 50x.

A


Differentiated Cells
Differentiating X Cells
X Cells
Photograph

B

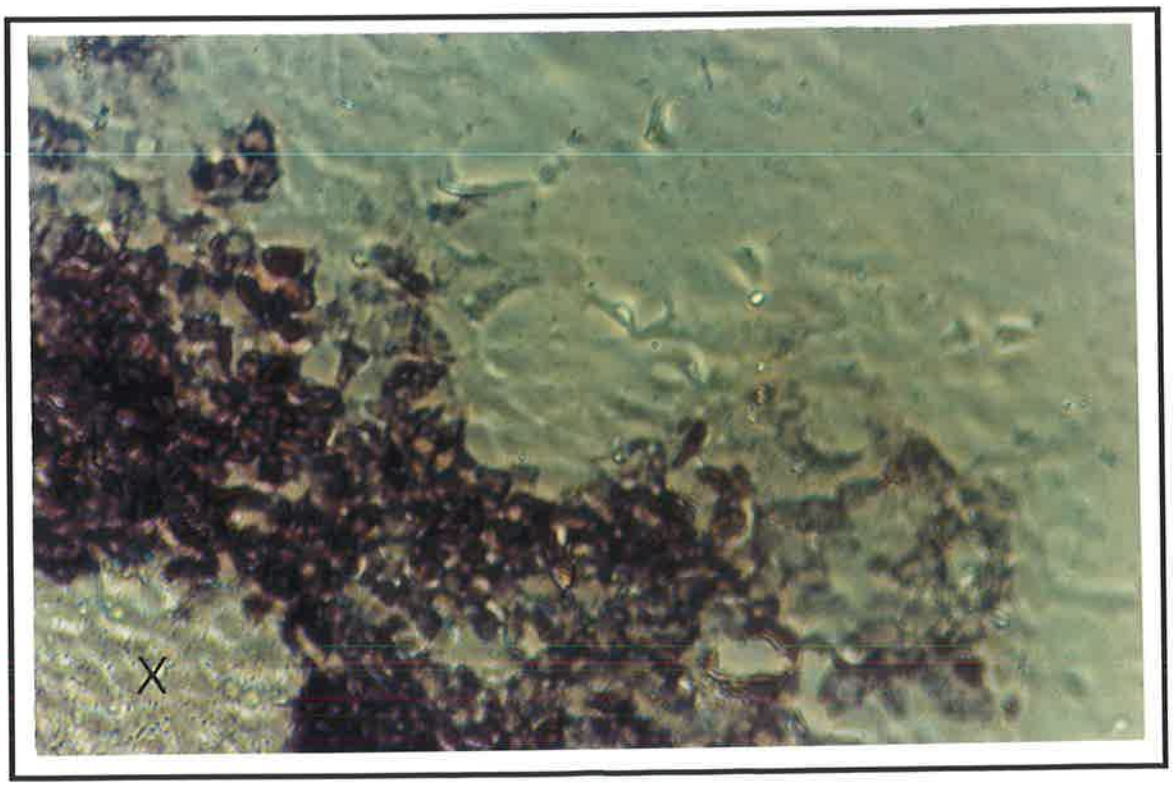

X

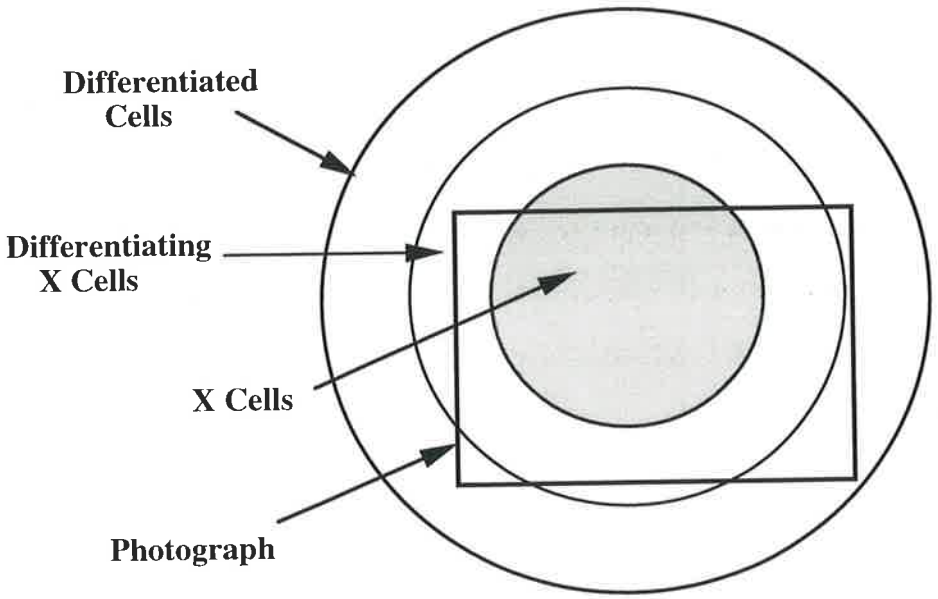
FIGURE 7.15 Gene expression associated with activin A-induced differentiation of X cells:- *goosecoid* expression.

Activin A-treated and untreated cultures were fixed at day 5. *Goosecoid* expression within these cultures was analysed by *in situ* hybridisation (2.5.19). *Goosecoid* expression was detected using a 920bp DIG labelled antisense riboprobe. Plates were developed for 14 hours before the colour reaction was stopped. Cells expressing *goosecoid* were denoted by the presence of purple staining.

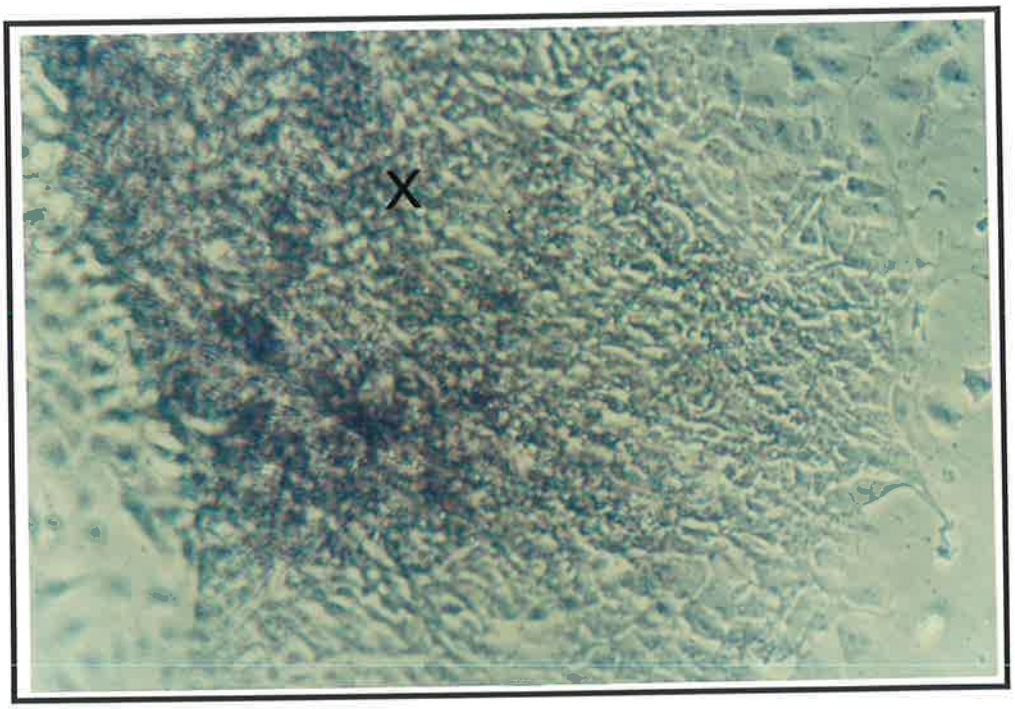
A schematic view of the colony photographed is depicted in A. *Goosecoid* expression within this colony is shown in photographs (B, C) and appeared to be confined to undifferentiated X cells (X) within the centre of colonies.

Photographs were taken under phase contrast (A) and bright field optics (B) at a magnification of 50x.

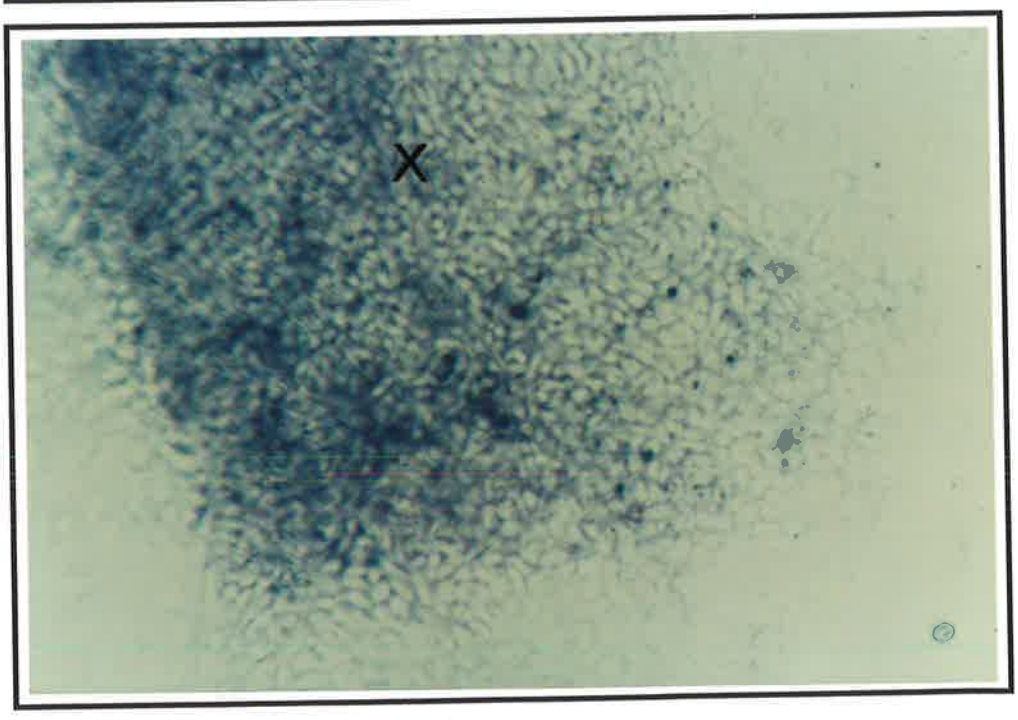
A


Differentiated Cells
Differentiating X Cells
X Cells
Photograph

B


X

C


X

expressed in X cell cultures treated with bFGF, while activin A-treated cultures expressed both *Brachyury* and *goosecoid*.

The pattern of *goosecoid* and *Brachyury* expression observed in activin A-treated cultures also correlates with the expression pattern of these genes during mesoderm induction *in vivo*. *Goosecoid* is expressed in the primitive ectoderm where the primitive streak will form, while *Brachyury* is expressed in the primitive streak and mesodermal cells emanating from the streak. Similarly, *goosecoid* expression appears to be confined to X cells, while *Brachyury* expression is confined to intermediate differentiating cells. The similarities between *in vivo* and *in vitro* expression of these genes is an extremely important result and requires confirmation before definitive conclusions can be made. Complete analysis of the expression pattern of *goosecoid*, *Brachyury* and other marker genes in activin A-treated cultures was prevented by an inability to acquire sufficient quantities of activin A.

7.7 Discussion

Similarities in the fate maps and gene expression associated with murine and *Xenopus* embryogenesis suggest that similar factors will regulate gastrulation. Mesoderm induction studies in *Xenopus* implicate the involvement of FGF-type and activin-type molecules in early developmental processes (Sive 1993). X cells have a gene expression and morphology consistent with cells of the primitive ectoderm which is the substrate for differentiation during gastrulation. This raised the possibility that X cells may differentiate in response to bFGF and activin A.

X cells, but not ES cells, differentiate when treated with either activin A or bFGF. This result defines a further difference between the developmental capacity of ES and X cells. Basic FGF-treated cultures were characterised by a high level of differentiated cells with a compact fibroblast morphology, cell type A. These cells were also observed at lower levels in untreated X cell cultures where they arose through spontaneous differentiation. Activin A-treated cultures were characterised by a highly motile fibroblastic cell type, cell type B, which could not be detected in untreated cultures. Thus bFGF and activin A induced the specific differentiation of X cells into alternative cell types. The appearance of the different cell types in response to activin A and bFGF may

be related to the expression of *goosecoid* and *Brachyury* in these cultures. Differentiating X cells within cultures treated with either activin A or bFGF expressed *Brachyury*. *Goosecoid* expression was only detected in activin A-treated cultures, where expression appeared to be confined to cells within the centre of semi-differentiated colonies. This pattern of *goosecoid* and *Brachyury* expression is consistent both with events occurring during mesoderm induction in *Xenopus* and with their patterns of expression during murine embryogenesis. The results of this chapter therefore provide substantial supporting evidence to the proposal that X cells represent the *in vitro* equivalent of primitive ectoderm. The expression of genes expressed during mesoderm induction *in vivo*, in response to mesoderm inducing cytokines *in vitro*, suggests that the ES/X cell system may constitute a useful model for the analysis of mesoderm induction *in vitro*.

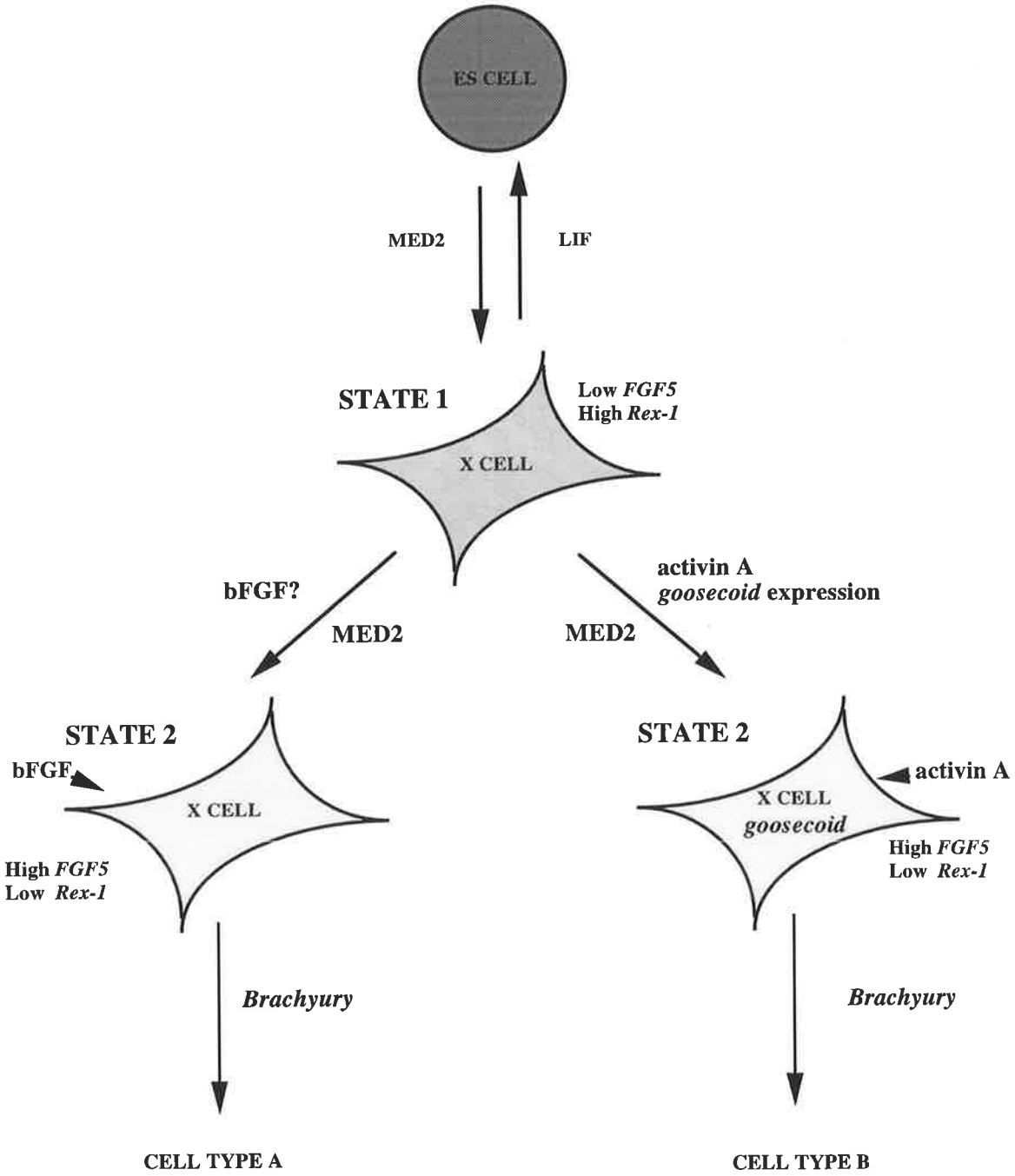
In previous chapters a two state model for X cell formation and maintenance was proposed. The two state model is applied to bFGF-induced and activin A-induced X cell differentiation in Figure 7.16. The gene expression alterations occurring as X cells are cultured at clonal cell density have not been ascertained, it is therefore difficult predict the proportion of cells in state 1 and 2 at any given stage. The persistence of undifferentiated X cells within bFGF-treated cultures suggests that bFGF may direct the differentiation of cells in state 2 which are competent to respond to inductive signals, to cell type A. The effect of bFGF on X cell reversion may also suggest an effect on X cells in state 1, perhaps inducing their progression to state 2. The expression of *goosecoid* in undifferentiated X cells and the formation of cell type B in activin A-treated cultures implies an affect on X cells in state 1 and state 2. While activin A induces the expression of *goosecoid* in cells in state 1 they may require progression to state 2 before differentiating.

Late stage primitive ectoderm is the substrate for mesoderm induction *in vitro*. X cells have a gene expression consistent with early primitive ectoderm. The ability of bFGF and activin A to direct the differentiation of X cells into mesodermal derivatives *in vitro* may suggest that factors directing mesoderm induction *in vivo* are not functionally active and/or present until a later stage of embryogenesis.

While bFGF, aFGF and FGF-4 were able to induce X cell differentiation, FGF-5 had no effect on X cell differentiation. Given that X cells express *FGF-5*, the failure of

FIGURE 7.16 The two state model applied to bFGF-induced and activin A-induced X cell differentiation.

Basic FGF induces the differentiation of X cells to cell type A *via* a *Brachyury* expressing intermediate. Activin A induces the expression of *goosecoid* in X cells and induces their differentiation to cell type B *via* a *Brachyury* expressing intermediate. Activin A and bFGF may have an effect on X cells in state 1 and state 2. See text for description (7.7).


ES CELL
MED2
LIF
STATE 1
Low FGF5
High Rex-1
X CELL
bFGF?
MED2
activin A
goosecoid expression
MED2
STATE 2
bFGF
X CELL
High FGF5
Low Rex-1
Brachyury
CELL TYPE A
STATE 2
activin A
X CELL
goosecoid
High FGF5
Low Rex-1
Brachyury
CELL TYPE B

FGF-5 to induce X cell differentiation is consistent with it having an alternative role such as the establishment of competence (5.4) rather than a direct effect on cell differentiation. FGF-5 is expressed in cells of the primitive ectoderm at day 5.25, prior to the onset of gastrulation and overt differentiation (Haub and Goldfarb 1991, Hebert *et al.* 1991). Thus it is unlikely to have a direct role in induction of cell differentiation. FGF-4 is expressed in the primitive streak in the gastrulating embryo where it may have a role in cell specification (Niswander and Martin 1993). Acidic FGF and basic FGF are expressed during murine embryogenesis (Hebert *et al.* 1990). However, their precise expression patterns prior to and during gastrulation have not been determined.

FGF-3 and FGF-8 are also expressed in the newly migrating mesoderm emanating from the primitive streak during gastrulation (Wilkinson *et al.* 1988, Crossley and Martin 1994) and may play an important role in gastrulation. The effects of these factors on X cell differentiation have not been established. FGFR-1 and 2 are expressed in the primitive ectoderm of gastrulation stage embryos and are therefore potential mediators of FGF signalling (Orr-Urtreger *et al.* 1991). The role that these factors play during gastrulation has been examined by gene targeting. The majority of investigations have proved uninformative as functional inactivation of the relevant genes results in defects either before (FGF-4) or well after gastrulation (FGF-3 and FGF-5) (Mansour *et al.* 1993, Hebert *et al.* 1994, Feldman *et al.* 1995). Targeted disruption of FGFR-1 indicates that this gene is essential for embryonic growth and mesodermal organisation during gastrulation but is not necessary for mesodermal differentiation (Deng *et al.* 1994, Yamaguchi *et al.* 1994).

The expression patterns of activin subunits and receptors in the murine embryo are consistent with a role in mesoderm formation. Activin transcripts are expressed in the uterine tissue surrounding embryos prior to and during gastrulation (Albano *et al.* 1994) and activin receptor type II and type IIB are expressed in the primitive ectoderm during these stages (Manova *et al.* 1995). However functional inactivation of activin subunits and activin receptor type II by gene targeting, have no effect on mesoderm formation *in vivo* (Smith 1995, Matzuk *et al.* 1995a, Matzuk *et al.* 1995b).

Many of the results obtained with gene targeting can be explained by genetic redundancy. Multiple factors and receptors are expressed stage and tissue specifically and

these factors, or other unidentified factors, may compensate for the loss of a given activity. This together with the inaccessibility of the murine embryo at these early developmental stages, makes the analysis of roles that particular factors play during embryogenesis extremely difficult to dissect. The ES/X cell system may ultimately constitute an *in vitro* system more accessible for defining the function of particular factors and/or genes during primitive ectoderm formation and downstream differentiation events associated with gastrulation. The power of being able to study differentiation in relatively homogeneous cell populations *in vitro* underlies much of the progress in our understanding of haemopoiesis. The sporadic differentiation of X cells under current growth protocols complicates the analysis and interpretation of differentiation induced in response to added factors. Current growth protocols employ 50% conditioned medium and addition of LIF for maximal maintenance of X cells. This is not considered optimal for several reasons, LIF has functions in cell differentiation apart from ES and X cell maintenance, and has been reported to suppress mesodermal differentiation *in vitro* and *in vivo* (Pruitt and Natoli 1992, Conquet *et al.* 1992, Vidricare *et al.* 1994), and regulate neuronal differentiation (Murphy *et al.* 1994). Therefore the presence of LIF may influence the outcome of differentiation induced by added factors. In addition, both MED2 and serum are likely to contain factors which influence X cell differentiation.

The following observations suggest that greater X cell stability will be achieved. Firstly, semi-purification of active factors contained within MED2 indicate that X cells are more stable in the absence of contaminating factors (J. Washingon, unpublished observations). Secondly, greater X cell stability has been observed when X cells are cultured on collagen and laminin substrates (J. Rathjen, unpublished observations). Finally, a chemically defined serum free medium has been developed to enable assessment of the effect of cytokines on mesoderm induction in embryoid bodies (Johansson and Wiles, 1995). Growth of X cells in similar medium may constitute a better method for the analysis of X cell differentiation. The development of more stable X cell culture conditions such that X cells can be maintained as relatively homogeneous cell populations is of considerable importance for the use of this system to analyse mammalian mesoderm induction.

CHAPTER EIGHT:
REINTRODUCTION OF X CELLS INTO THE EARLY MURINE EMBRYO

CHAPTER EIGHT: REINTRODUCTION OF X CELLS INTO THE EARLY MURINE EMBRYO

8.1 Introduction

The expression of biochemical markers, differentiation potential and morphology exhibited by X cells are consistent with X cells being an *in vitro* analogue of the pluripotential cells of the primitive ectoderm. If MED2 is removed and LIF is present X cells revert to an ES cell phenotype. The gene expression and *in vitro* differentiation properties of reverted X cells are similar to those possessed by ES cells (5.2, 6.5).

Primordial germ cells (PGC) can be maintained *in vitro* by culture in the presence of Steel factor and LIF. When bFGF is added to PGC culture conditions they revert to ES-like cells termed EG cells (Matsui *et al.* 1992, Resnik *et al.* 1992). This experiment led to the proposal that all pluripotential stem cells of the murine embryo will have a similar potential for interconversion (Rossant 1993). This is consistent with the properties of X cells.

An extremely important property of ES cells and EG cells is their ability to participate in normal development when reintroduced into early murine blastocysts (Bradley *et al.* 1984, Stewart *et al.* 1994). ES cells are capable of contributing progeny to all three germ layers of the embryo, including the germ line. Since reverted X cells have similar properties to ES cells it is possible that they might be capable of similar tissue contribution. Indeed as pluripotential cells, X cells themselves might possess a capacity for tissue contribution. Analysis of the ability of X cells and reverted X cells to contribute to murine tissues *via* blastocyst injection would confirm their pluripotentiality and further define their biological nature.

8.2 Contribution of X Cells and Reverted X Cells to Murine Embryos

The ES cell line E14 was chosen for analysis in blastocyst injection experiments. This cell line contributes to all tissues of the mouse at high frequency when reinjected into murine blastocysts. Like D3 and MBL5, E14 ES cells convert to X cells when cultured in the presence of MED2 (J. Rathjen, unpublished results). Similarly, X cells derived from E14 ES cells reverted to ES cells when cultured in the presence of LIF, in the absence of

MED2 (J.Rathjen, unpublished observations). The profile of neuron and beating muscle formation in embryoid bodies derived from E14 ES, E14 Xp0+ and E14 Xp0- was consistent with that previously described for D3 ES and X cells (data not shown). Furthermore, X cells derived from E14 ES cells differentiated similarly to D3 Xp0+ cells in the presence of bFGF (data not shown).

It has been reasoned that if X cell cultures contain ES cells it must be at very low levels since colonies with the morphology of ES cells are not observed (3.3). However to minimise the possibility of residual ES cell contamination, X cells formed and passaged in the absence of added LIF (Xp1-) and reverted derivatives of these cells (Xp1-R) were chosen for analysis by blastocyst injection.

E14 ES cells were seeded at high cell density and uniformly converted to X cells by culture in MED2 in the absence of added LIF (Xp0-) (2.3.9). These cells were passaged in MED2 for a further 2 days (Xp1-) and prepared for blastocyst injection (2.3.27). Xp1- cells were also reverted to ES cells by culture in the presence of LIF in the absence of MED2 (2.3.10), cultured/passaged in the presence of LIF for a period of six days and prepared for blastocyst injection (2.3.27). All cells prepared for injection were optimal as assessed by morphology (Figure 8.1). Xp1- cultures appeared highly uniform, ES cell colonies were not observed and levels of differentiation were low. Xp1-R cultures contained uniform ES cell colonies.

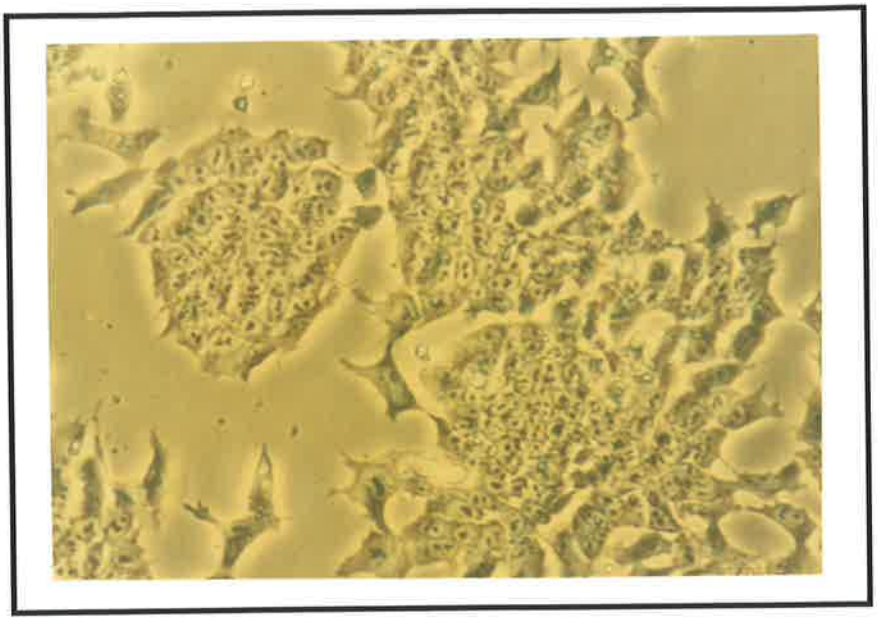
Blastocyst injections were carried out in collaboration with Anita Peura and Steven MacIlfatrick (Bresatec, Adelaide) (2.3.27). Xp1- and Xp1-R cells were injected into CBA/C57F2 blastocysts which were then transferred to pseudo-pregnant foster mothers. Ten mice were derived from blastocysts injected with Xp1- cells and ten mice were derived from blastocysts injected with Xp1-R cells. E14 ES cells are derived from 129/Sv mice. While ES cell contribution can be definitively identified by the presence of chinchilla, contribution can not be assessed based on the presence of agouti unless the host blastocyst injected is black (Silvers 1979, Bultman *et al.* 1992). While low level contribution based on coat colour chimerism could be seen in 7/10 mice derived from blastocysts injected with Xp1-R cells, 0/10 mice derived from blastocysts injected with Xp1- cells exhibited coat colour chimerism (Figure 8.3).

FIGURE 8.1 The morphology of X cells and reverted X cells injected into murine blastocysts.

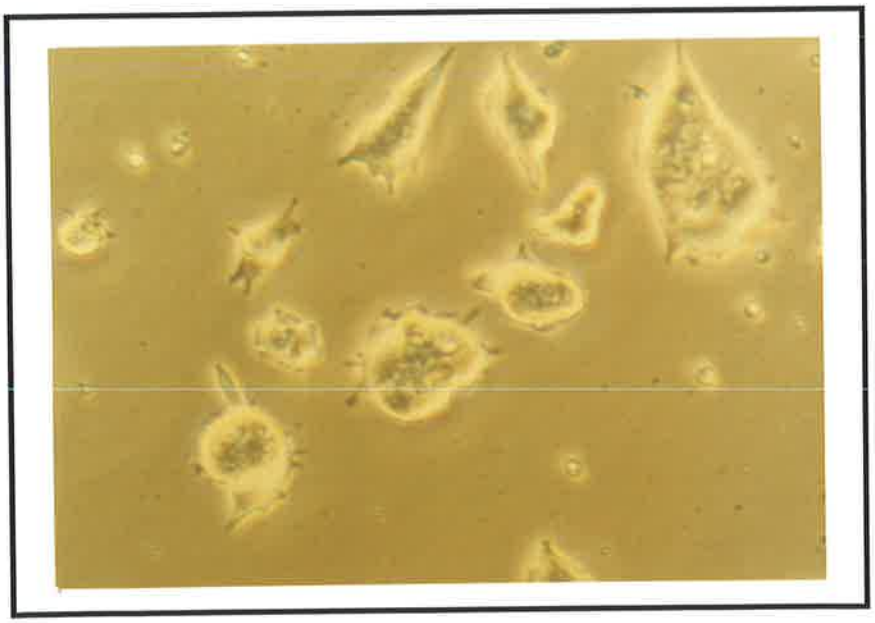
Xp1- cells and reverted X cells (Xp1-R), prepared for blastocyst injection are shown in A and B, respectively.

Photographs were taken at a magnification of 50x using phase contrast microscopy.

A



B



The ubiquitously expressed enzyme glucose phosphate isomerase is a biochemical marker which is commonly used to assess chimerism. GPI exists in three isozymal forms I^a, I^b and a relatively rare form I^c (Bradley, 1988). E14 ES cells are GPI I^aI^a while CBA/C57F2 host embryos are GPI I^bI^b. The contribution of pluripotential cells to chimeric mice can therefore be observed by the presence of GPI I^a in a GPI I^b background.

Tail blood taken from progeny mice was analysed for GPI content (Figure 8.2). Of the mice derived from injection of blastocysts with reverted X cells, 4/10 exhibited contribution to blood. Contribution to blood could not be detected in any of the mice derived from the injection of blastocysts with X cells.

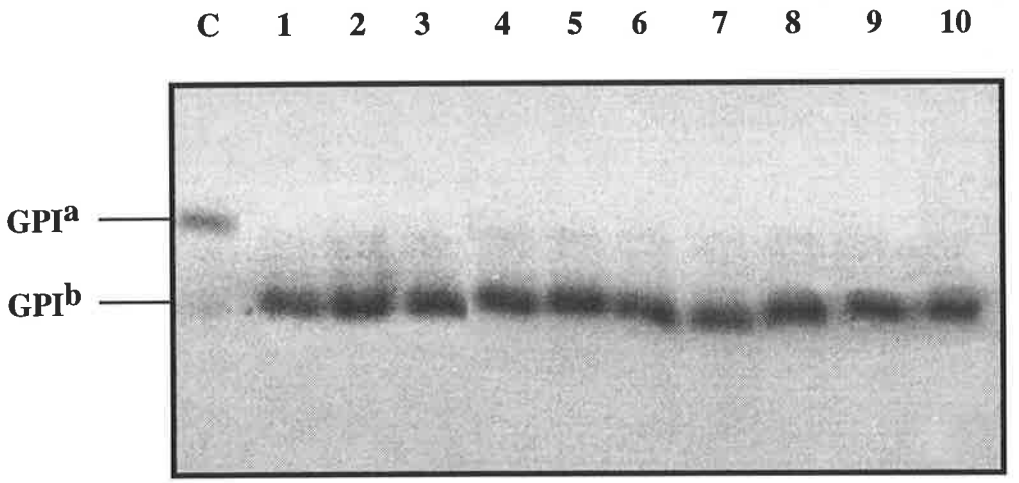
The results of chimeric analysis are summarised in Figure 8.3. 10 mice were derived from blastocysts injected with X cells cultured in the presence of MED2 and the absence of LIF for 4 days. Contribution from X cells to differentiated tissues was unable to be detected in at least two different tissue compartments, the skin as observed by coat colour and the blood as detected by GPI analysis. Of 10 mice derived from blastocysts injected with X cells which had been reverted to ES cells, in the presence of LIF, 7/10 showed contribution to blood and/or skin tissue compartments.

The results of this experiment indicate that X cells can contribute to murine tissues, including blood and skin, provided they have been reverted to ES cells prior to blastocyst injection. These results confirm the difference between X cells and ES cells/reverted X cells deduced from *in vitro* analysis of phenotype, gene expression and differentiation potential. Secondly, they suggest that X cell populations formed by conversion of ES cells *in vitro* do not contain high levels of residual ES cells, since contribution to differentiated tissues from X cells was not seen. Finally, they support the proposal that X cells represent an alternative pluripotential cell population which is capable of tissue contribution.

Complete and accurate interpretation of this experiment is hampered by several limitations. In this experiment only low levels of contribution (5-15%) were observed in chimeric mice. Typically levels greater than 50% are observed and are considered optimal for germ line transmission. Mice were derived from only a limited number of injected blastocysts, which suggests that technical limitations associated with injection may

FIGURE 8.2 Blood GPI analysis of mice derived from blastocyst injections.

Tail blood derived from progeny mice was analysed for GPI isoenzyme content (2.3.27). Lanes 1-10 correspond to mice derived from blastocysts injected with Xp1-cells and lanes 11-20 correspond to mice derived from blastocysts injected with Xp1-R cells.


C 1 2 3 4 5 6 7 8 9 10
GPIa
GPIb

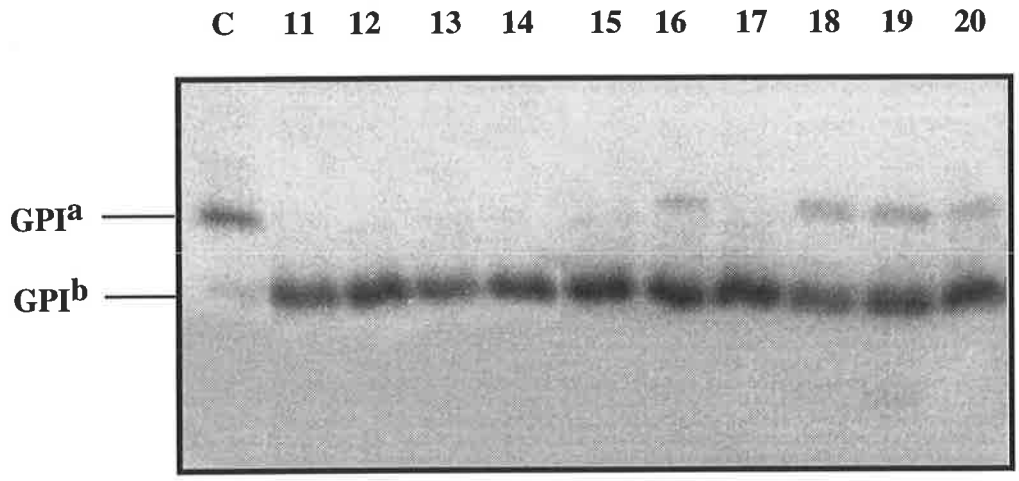

C 11 12 13 14 15 16 17 18 19 20
GPIa
GPIb

FIGURE 8.3 Levels of chimerism observed as assessed by coat colour and blood GPI content in mice derived from blastocysts injected with Xp1- and Xp1-R cells.

Mice numbered 1-10 and 11-20 correspond to those derived from blastocysts injected with Xp1- cells and Xp1-R cells respectively. The percentage of agouti, chinchilla and black coat colouring as well as blood GPI^a content is shown for each mouse.

CELLS INJECTED	MOUSE	SEX	COAT COLOUR			% BLOOD GPI[a]
			% agouti	%chinchilla	%black	
Xp1-	1	F	100	0	0	0
	2	F	100	0	0	0
	3	F	100	0	0	0
	4	F	100	0	0	0
	5	F	0	0	100	0
	6	M	100	0	0	0
	7	M	0	0	0	0
	8	M	0	0	100	0
	9	M	0	0	100	0
	10	M	0	0	100	0
Xp1-R	11	F	95	5	0	0
	12	F	100	0	0	0
	13	M	<100	trace	0	0
	14	M	100	0	0	0
	15	M	100	0	0	0
	16	F	95	5	0	10
	17	F	95	5	0	0
	18	M	95	5	0	15
	19	M	<100	trace	0	15
	20	M	15	0	85	10

explain the low levels of chimerism. A positive ES cell control was not included in these experiments. Therefore it is not possible to determine whether the absence and low level of chimerism seen with Xp1- cells and reverted X cells, respectively, were products of these technical limitations or cellular phenotype.

8.3 Contribution of Reverted XB17 Cells to Murine Embryos

XB17 cells are a clonal X cell line originally isolated from Day 6 D3 embryoid bodies in the presence of MED2 and LIF (4.4.1, J. Rathjen and J.Washington, unpublished results). ES cells were not isolated from the same embryoid bodies in the presence of LIF. Therefore XB17 cells originate from an X cell and not an ES cell. Use of this cell line in blastocyst injection experiments allows the possibility of contaminating ES cells to be excluded. XB17 cells have a morphology and gene expression profile similar to X cells derived from ES cells, and exhibit the capacity to revert to ES cells when cultured in the presence of LIF, in the absence of MED2 (5.2). Furthermore, XB17 cells are able to form embryoid bodies and are able to differentiate in response to bFGF and activin A in a similar manner to X cells (data not shown).

XB17 cells were passaged in the presence of MED2 and LIF (2.3.11) and at passage 20 cells were prepared for blastocyst injection (2.3.27). Passage 20 cells were also reverted to ES cells by culture in the presence of LIF in the absence of MED2 (2.3.11), cultured/passaged in the presence of LIF for four passages and then prepared for blastocyst injection. All cells prepared for injection were optimal as assessed by morphology (Figure 8.4).

XB17p20 and XB17Rp4 cells were injected into CBA/C57F2 blastocysts which were then transferred to pseudo-pregnant foster mothers (2.3.27). Mice were not derived from blastocysts injected with XB17p20 cells. This is likely to be the result of sub-optimal injection and husbandry conditions rather than a direct effect of the cells (8.2). Four mice were derived from blastocysts injected with reverted XB17 cells. Low level contribution could be assessed by the presence of 5% agouti coat colour in one mouse (Figure 8.5).

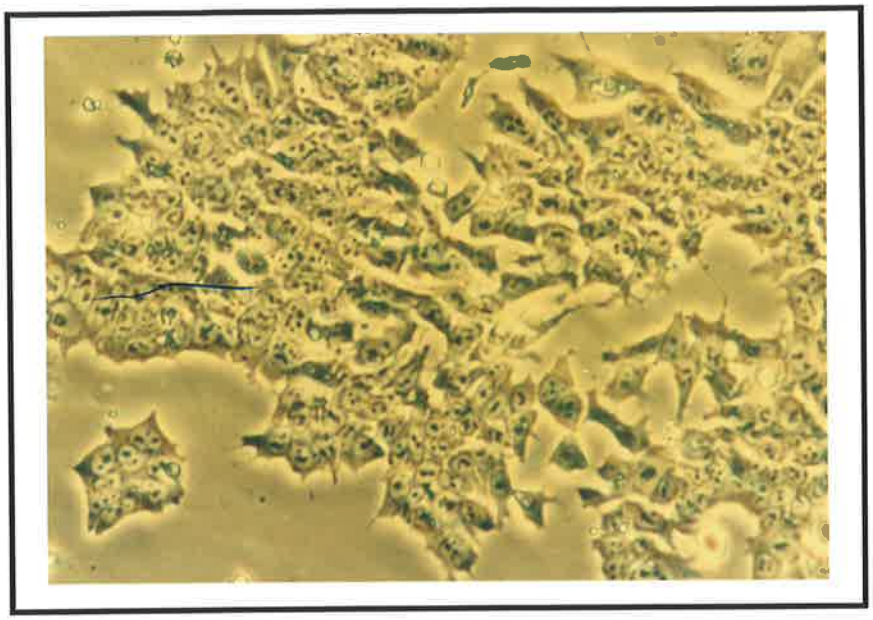
While the ability of XB17 cells to contribute to murine tissues could not be assessed in this experiment, it is considered unlikely in view of the inability of Xp1- cells

FIGURE 8.4 The morphology of XB17 cells and reverted XB17 cells injected into murine blastocysts.

XB17 and reverted XB17 cells prepared for blastocyst injection are shown in A and B respectively.

Photographs were taken at a magnification of 50x using phase contrast microscopy.

A



B

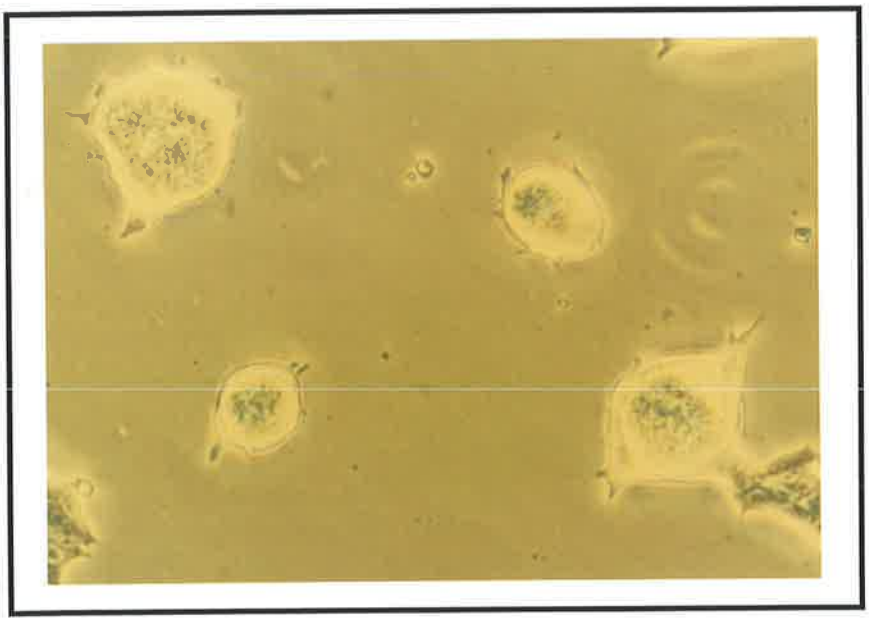


FIGURE 8.5 Levels of chimerism observed as assessed by coat colour in mice derived from blastocysts injected with reverted XB17 cells.

Four mice were derived from blastocysts injected with reverted XB17 cells. The percentage of agouti, chinchilla and black coat colouring observed, is shown for each mouse.

CELLS INJECTED	MOUSE	SEX	COAT COLOUR		
			% agouti	%chinchilla	%black
XB17 Rp4	1	F	100	0	0
	2	F	100	0	0
	3	M	100	0	0
	4	M	5	0	95

to contribute (8.2). The contribution of reverted XB17 cells to chimeric mice is extremely significant and a rather impressive result given the origin, manipulation, extended passage and cultures conditions associated with these cells. XB cells were derived clonally from embryoid bodies in the absence of ES cells, therefore any contribution is attributable to reverted cells derived directly from XB17 cells rather than contaminating ES cells. This result directly demonstrates the pluripotential nature of X cells and further defines their use as an alternative pluripotential cell population capable of tissue contribution.

8.4 Discussion

The results of experiments presented in this chapter indicate that X cells can contribute to murine tissues, including blood and skin, even after extended passage *in vitro*. Contribution appears to require reversion of X cells to ES cells by culture in LIF prior to blastocyst injection. These results demonstrate that X cells and XB cells are pluripotent and provides further evidence discriminating ES cells from X cells. The inability of X cells to contribute directly to murine tissues may be due to their altered cell adhesion properties and consequent inability to combine with the cells of the ICM.

Experiments are currently being repeated using E14EGT2a ES cells, which have been shown to contribute to tissues of the mouse including the germ line at high levels in our laboratory (L. Whyatt, unpublished observations). The ability of X cells derived from these ES cells cultured in both the presence and the absence of added LIF are being assessed. In addition, a more extensive analysis of tissue contribution by XB cells is underway. The results derived from these experiments will allow a more precise interpretation of the ability of X cells versus ES and reverted X cells to contribute to murine tissues.

The ability of X cells to contribute to chimeras has profound implications for mammalian transgenesis which is currently limited by an inability to isolate embryonic stem cells capable of germ line contribution from species other than the mouse and Chinese hamster (Smith 1992). The conditioned medium MED2 converts ES cells to X cells and allows isolation of X cells from embryoid bodies. Experimental evidence presented in this thesis suggests that X cells are the *in vitro* equivalent of the primitive ectoderm of the murine embryo. Preliminary evidence suggests that MED2 can maintain

embryonic primitive ectoderm *in vitro* (9.7) (J. Rathjen, unpublished observations). Distinct from the inner cell mass and primordial germ cells, the primitive ectoderm represents an alternative pluripotential stem cell population within the murine embryo, and may represent an alternative target tissue for the isolation of pluripotential stem cells from mammalian embryos. Pluripotential stem cell lines isolated in this way must be capable of germ line contribution. Further experiments will demonstrate the feasibility of using X cells as an alternative source of pluripotential cells capable of germ line contribution. Furthermore, they will give an insight into the methodology that will ultimately be required for the successful integration of cells of the primitive ectoderm into the inner cell mass of murine embryos. The establishment of any alternative method for pluripotential stem cell isolation in mice should have direct application in the isolation of pluripotential stem cells from other mammalian species.

CHAPTER NINE:
FINAL DISCUSSION

CHAPTER NINE: FINAL DISCUSSION

9.1 The Embryological Equivalent of X cells

Mammalian development requires the coordinated differentiation of pluripotential embryonic stem cells. Access to homogeneous populations of embryonic stem cells *in vitro* potentially gives rise to an invaluable system for the analysis of factors which control stem cell proliferation, maintenance and differentiation during embryogenesis. While biological factors capable of ES cell maintenance have been identified, biological factors involved in the specific differentiation of ES cells have remained elusive. Identification of a conditioned medium (MED2) which induced the specific and uniform differentiation of ES cells therefore represented an important discovery. Culture of ES cells in MED2 in either the presence or absence of exogenous LIF caused their uniform differentiation to a morphologically distinct cell type, termed X cells (1.8). The overall aim of the work presented in this thesis was to characterise X cells and to define their embryonic equivalent. The approaches employed involved extensive analysis of X cell gene expression and differentiation potential.

9.2 X Cell Gene Expression is Consistent with the Early Primitive Ectoderm

X cells were not equivalent to an early mesodermal cell type or primitive endodermal cell type based on the lack of expression of marker genes specific for these cell populations *in vivo*. Expression of *Oct-4*, alkaline phosphatase and SSEA-1 confined the embryonic equivalent of X cells to a pluripotential cell population in the murine embryo. The expression of *uvomorulin* in X cells restricted the pluripotential cell equivalent to either the inner cell mass, primitive ectoderm or an unidentified pluripotential cell population within the embryo. Marker genes which discriminate between these alternatives included *FGF-5*, *Rex-1* and *Gbx-2*. *Rex-1* and *Gbx-2* expression are expressed by cells of the inner cell mass and absent in primitive ectoderm at day 6.0 of embryonic development (Rogers *et al.* 1991, G. Chapman, J. Rathjen, unpublished observations). *FGF-5* is not detected in the inner cell mass at day 3.25 but is detected in the primitive ectoderm at day 5.25 (Haub and Goldfarb, 1991). X cells exhibited higher

levels of *FGF-5* expression and lower levels of *Gbx-2* and *Rex-1* expression than ES cells. This implied that X cells correspond to the early primitive ectoderm, consistent with cytokeratin expression, cell morphology and cell cycle time (1.8.3).

Precise gene expression alterations occurring during the inner cell mass to primitive ectoderm transition *in vivo* have not been fully characterised. The exact onset of *FGF-5* expression and down regulation of *Gbx-2* and *Rex-1* expression within these tissues has not been determined. Thorough analysis of the expression of these genes will more fully define this transition *in vivo* and the exact stage of pluripotential cell development to which X cells correspond.

9.3 X Cell Differentiation Capacity is Consistent with the Early Primitive Ectoderm.

The developmental fates of the ICM and primitive ectoderm within the embryo are different. A relationship between X cells and the primitive ectoderm implies an alteration in the developmental capacity of X cells compared to ES cells. In particular it might be expected that X cells would exhibit a lower capacity to form primitive endoderm, a higher capacity to generate late stage primitive ectoderm and derivatives, and respond to mesoderm inducing cytokines.

9.3.1 Differentiation of X Cells in Response to Retinoic Acid

X cells differentiated by aggregation in the presence of RA exhibited a higher capacity to form fibroblasts, a mesodermally-derived cell type, and a lower capacity to for primitive endodermal derivatives than ES cells differentiated under identical conditions. This correlates well with the proposed relationship of ES cells to the inner cell mass and X cells to the early primitive ectoderm.

9.3.2 Differentiation of X Cells as Embryoid Bodies

The pluripotential nature of X cells was confirmed by their ability to differentiate as embryoid bodies. X cells could be distinguished from ES cells during embryoid body differentiation in a several ways. Analysis of marker gene expression indicated that X cells formed late stage primitive ectoderm and mesodermal derivatives earlier and at higher

levels than ES cells. This observation was consistent with the gene expression of X cells and supports the proposed relationship between X cells and the early primitive ectoderm.

ES cell embryoid bodies were distinguished by a higher potential to form neurons than X cell embryoid bodies. High levels of neurons were however observed in X cell aggregates differentiated in the presence of RA. This suggested that the lack of neuron formation in X cell embryoid bodies was due to the absence or presence of an earlier cell type or regulatory factor rather than an inherent property of X cells.

The presence of an outer cellular layer reminiscent of primitive endoderm in ES cell embryoid bodies but not X cell embryoid bodies at day 4 of development, coupled with the observation of a visceral endoderm-like cell type in ES cell and not X cell seeded embryoid bodies, suggested that X cells might be deficient in primitive endoderm formation during embryoid body differentiation. This could not be demonstrated using available gene expression markers. While the embryonic ICM and primitive ectoderm are distinguished by the inability of the latter to give rise to primitive endoderm *in vivo*, this restriction in developmental capacity appears to be progressive (Smith 1992). Therefore the ability of X cells to form primitive endoderm *in vitro* may reflect the true developmental capacity of early primitive ectoderm *in vivo*. Alternatively, the ability to form primitive endoderm may be related to the reversion capability of X cells.

9.3.3 Differentiation of X Cells in Response to Mesoderm Inducing Cytokines

The ability of X cells to differentiate in response to mesoderm inducing cytokines including bFGF and activin A distinguished these cells from ES cells which failed to respond to these cytokines *in vitro*. The morphology of the cell types produced and the gene expression alterations were consistent with the formation of mesoderm. Activin A and bFGF induced specific but alternative differentiation of X cells. bFGF-induced X cell differentiation was characterised by expression of *Brachyury*, while activin A-induced X cell differentiation was characterised by the expression of both *Brachyury* and *goosecoid*. While *Brachyury* expression was confined to a differentiating X cell intermediate, *goosecoid* expression was confined to undifferentiated X cells within the centre of colonies. The alternative expression of *Brachyury* and *goosecoid* in response to

activin A and bFGF is consistent with their expression in response to these factors during mesoderm induction in *Xenopus*. Furthermore, the pattern of *Brachyury* and *goosecoid* expression observed in differentiating X cell cultures was consistent with the expression patterns of these genes during gastrulation *in vivo*. *Goosecoid* is expressed in the primitive ectoderm where the primitive streak will form while *Brachyury* expression is confined to early mesodermal cells emanating from the streak during gastrulation. The ability of X cells to differentiate in response to bFGF and activin A into early mesoderm is consistent with their proposed relationship to the primitive ectoderm, and suggests that X cells may comprise an *in vitro* assay system for the identification and functional analysis of inductive factors during gastrulation.

9.4 X Cells and *In Vivo* Differentiation Events

In vivo the term primitive ectoderm is applied to cells of the inner cell mass after the formation of primitive endoderm (Rossant 1988). It is not until the formation of the proamniotic cavity that a morphological difference in the organisation of these pluripotential cell populations becomes apparent. The gene expression associated with X cells and related differentiation capacity suggest that the differentiation of the inner cell mass to primitive ectoderm is a progressive differentiation event marked by subtle alterations in gene expression and differentiation potential. The restrictions occurring in pluripotential stem cell populations during early embryogenesis are thought to involve the progressive and heterogeneous loss of developmental capacity (Smith 1992). The formation of X cells from ES cells *in vitro* may therefore be informative about the nature of the inner cell mass to primitive ectoderm transition *in vivo*.

9.5 X Cell Reversion

X cells exhibit the capacity to revert phenotypically to ES cells when cultured in LIF in the absence of MED2. Gene expression analysis demonstrated that reverted X cells exhibit similar gene expression to ES cells. Furthermore, analysis of the differentiation potential of reverted X cells compared with their X cell counterparts revealed that these cells behave as ES cells when differentiated as embryoid bodies, and in contribution to murine tissues following blastocyst injection. Reverted X cells were also

able to reconvert to X cells when cultured in MED2. Pluripotential stem cell interconversion has also been demonstrated for primordial germ cells (PGC) (Matsui *et al.* 1992, Resnick *et al.* 1992). Culture of PGCs in the presence of LIF, Steel factor and bFGF induces their reversion to ES cells. It has been predicted that cells of the primitive ectoderm should also convert to a common ES cell intermediate (Rossant 1993). The correlation between X cells and the cells of the primitive ectoderm and their ability to revert to ES cells supports this proposal. Stem cell interconversion may underlie the developmental lability associated with murine embryogenesis. The ability of pluripotential stem cells to reprogram their fate during development may constitute a method for ensuring developmental progression in adverse circumstances.

9.6 The Relationship Between X Cell Gene Expression and Differentiation Capacity

Under current growth conditions LIF addition appears to be required for stable maintenance of X cells in the undifferentiated state. This is not surprising given the reversion capability of X cells and the requirement of LIF for maintenance of PGC and ES cells *in vitro* (Matsui *et al.* 1991, Pesce *et al.* 1993, Cheng *et al.* 1994, Smith *et al.* 1988). Interestingly, correlations can be drawn between the presence of added LIF, gene expression and differentiation capability. X cells formed at high cell density in the presence of MED2 and LIF exhibit lower *FGF-5* expression and higher *Rex-1* expression than X cells formed in the presence of MED2 in the absence of added LIF. Consistent with their gene expression and therefore a relationship to the primitive ectoderm, Xp0+ cells exhibit differentiation capabilities intermediate to those exhibited by Xp0- and ES cells.

As X cells are formed and passaged in MED2, gene expression alterations characteristic of an inner cell mass to primitive ectoderm transition occur. The presence of LIF appears to impede this transition. This correlates with the observation that LIF inhibits primitive ectoderm formation during embryoid body differentiation *in vitro* (Shen and Leder 1992). X cells cultured in the absence of added LIF exhibit dramatic increases in *FGF-5* from passage 0 to 2. This appears to correlate with the increased level of differentiation observed in these cultures. Furthermore, passaged X cells exhibit

reversion and differentiation capacities which appear to be dependent on their initial gene expression, such that X cells with higher levels of *FGF-5* and lower levels of *Rex-1* and *Gbx-2* exhibit higher levels of differentiation and lower levels of reversion. *FGF-5* has been proposed to confer differentiation competence in uncommitted embryonic cell populations (Hebert *et al.* 1992, Conlon 1994). Consistent with this, FGF-5 itself does not appear to induce X cell differentiation. *FGF-5* expression at high levels in X cell populations may therefore alter their competence to respond to other factors which induce differentiation.

The gene expression of X cells after passage 2 appeared to undergo cyclic alterations. While *FGF-5* increased and *Rex-1* decreased from p0 to p2, *FGF-5* decreased and *Rex-1* increased after this stage, only to increase and decrease again. Alterations in gene expression in X cells passaged in the presence of LIF appeared to undergo cyclic alterations at a slower rate. To explain the reversion capacity, differentiation capacity and cyclic alterations in gene expression a two state model for X cell formation, maintenance and differentiation was proposed. This model proposed that X cells could exist in two states related to gene expression. X cells in state 1 exhibited a high capacity to revert and a lower capacity to differentiate, and a gene expression profile characterised by lower *FGF-5*, higher *Rex-1* and *Gbx-2*. X cells in state 2 exhibited a low capacity to revert and a high capacity to differentiate, and exhibited a gene expression profile characterised by higher *FGF-5*, lower *Rex-1* and *Gbx-2*.

While it is appreciated that current *in vitro* growth protocols may play a large part in X cell stability and behaviour, a two state model may explain a progressive restriction of developmental potential as the ICM forms the primitive ectoderm *in vivo*. Subtle alterations in gene expression and therefore differentiation potential during this period may explain much of the developmental lability associated with murine embryos *in vivo*.

9.7 The Primitive Ectoderm as an Alternative Target for Pluripotential Cell Isolation

The isolation of clonal XB cell lines from embryoid bodies at stages of development where primitive ectoderm is present (J. Rathjen, J. Washington, unpublished observations), supports the postulated relationship between X cells and the

primitive ectoderm. The demonstration that reverted XB cells can contribute to mouse embryos upon blastocyst injection suggested that the primitive ectoderm within the murine embryo itself would possess similar properties for isolation, reversion and embryonic contribution. Preliminary experiments employing MED2 to aid the isolation of primitive ectoderm cells from day 5.5 murine embryos have supported this suggestion (J. Rathjen unpublished observations). MED2 allows the proliferation of embryonic primitive ectoderm cells *in vitro*. These cells revert to an ES cell phenotype when cultured in LIF and have been maintained in this form for up to three weeks before subject to expression analysis. The primitive ectoderm thus promises to constitute an alternative target tissue for pluripotential stem cell isolation. Experimental evidence described in this thesis, suggests that these cells will be capable of tissue contribution when recombined with murine blastocysts.

The ability to investigate embryogenesis and generate transgenic animals in other mammalian species has been hampered by the inability to isolate species specific stem cells. The isolation of pluripotential cells from the primitive ectoderm may constitute an alternative approach to the isolation of stem cells from species which do not give rise to ES cells *via* traditional methods. Isolation of pluripotential stem cells from other mammalian species, particularly from livestock and human, is of enormous intellectual, medical and commercial importance.

9.8 X Cells and the Primitive Ectoderm

The work described in this thesis provides strong evidence that X cells represent the *in vitro* equivalent of primitive ectoderm. The expression of over twenty different marker genes for a variety of embryonic cell types is consistent with this identification. X cells are pluripotent and distinct from ES cells, yet represent a natural ES cell differentiation intermediate (4.3.1, Thomas 1994). Furthermore, X cells have a high capacity to form late stage primitive ectoderm and mesodermal derivatives and are able to differentiate in response to mesoderm inducing cytokines, expressing genes consistent with early mesoderm formation *in vivo*. Finally, they exhibit differentiation and reversion properties consistent with those predicted for the cells of the primitive ectoderm.

Work carried out concomitant with this research show that X cells can be isolated from embryoid bodies at stages of development where primitive ectoderm is present (J. Rathjen, J. Washington, unpublished observations). ES cells were not isolated from the same embryoid bodies in the presence of LIF. XB cells exhibit a gene expression and differentiation capability similar to X cells derived from ES cells. Furthermore, primitive ectoderm cells of the murine embryo can be maintained in MED2 and subsequently reverted to an ES cell phenotype (J. Rathjen, unpublished observations). Taken together, these observations provide substantial evidence in favour of X cells representing the *in vitro* equivalent of primitive ectoderm.

9.9 Future Work

A requirement for future work is the development of more stable X cell culture conditions. Conditioned medium is inherently depleted of nutrients and will contain additional factors not involved in X cell formation or maintenance that may effect the stability of X cells. The factors responsible for X cell formation and maintenance are currently being purified. Identification and purification of these factors is therefore a pre-requisite for the establishment of more stable X cell culture conditions. Preliminary evidence also suggests that X cells are more stable when cultured on collagen IV, fibronectin and laminin substrates (J. Rathjen, unpublished observations). Greater X cell stability will allow the precise function of LIF in X cell maintenance to be established, and should aid complete characterisation of bFGF-induced and activin A-induced X cell differentiation. Establishment of stable X cell culture conditions is particularly important for the successful use of X cells as an *in vitro* system for the identification and/or functional analysis of factors and genes involved in murine development.

The ability to recreate the inner cell mass to primitive ectoderm transition *in vitro* will enable molecular characterisation of factors involved in and genes expressed during this stage of murine development *in vivo*. This system has already been used by members of the laboratory for the identification of genes expressed specifically in the inner cell mass and primitive ectoderm *in vivo*. Utilising differential display PCR, genes with specific expression patterns in ES cells but not X cells and the converse have been identified. Preliminarily characterisation of a gene specifically expressed in ES cells and

not X cells *in vitro* has indicated that it is expressed in the inner cell mass but not the primitive ectoderm of the murine embryo (T. Shultz, unpublished observations). This supports the use of ES/X cells as an model system for the identification of factors and genes involved in early murine embryogenesis.

The differentiation of ES cells to X cells is marked by an increase in *FGF-5* expression and a decrease in *Rex-1* and *Gbx-2* expression. While this alteration in gene expression is consistent with the inner cell mass to primitive ectoderm transition, the precise temporal and spatial expression patterns of these genes *in vivo* have not been determined. Analysis of the expression of these genes *via* whole mount *in situ* hybridisation in early murine embryos at successive stages of development will more clearly define the position of X cells during primitive ectoderm formation and will be informative of the inner cell mass to primitive ectoderm transition in general.

The differentiation capacity of X cells as embryoid bodies has been examined at a very simplistic level. Further examination may identify additional differences between the cell lineages generated by ES and X cell embryoid bodies, and may provide insights into the underlying mechanisms of neuron and cardiac muscle formation. Differences in the differentiation capabilities of ES and X cells may ultimately serve as a way to identify genes or factors specifically involved in specific developmental events by function or differential gene expression analysis. Furthermore, treatment of X cell embryoid bodies with known cytokines may lead to the generation of defined cell lineages *in vitro*. For example, the propensity which X cells possess for mesoderm formation when combined with haemopoietic regulators, may allow development of a system for the generation of haemopoietic stem cell populations *in vitro*.

While X cell embryoid body differentiation may constitute a system for the isolation of progenitor stem cells, it is a relatively complex and heterogeneous cell differentiation system. Access to homogeneous X cell cultures which can be differentiated into specific cell types in response to added factors is likely to constitute a far better system for progenitor stem cell formation, characterisation and/or isolation. The ability to generate precursor stem cell populations *in vitro* is fundamental to the successful application of gene therapy.

The specific differentiation of X cells to mesodermal cell populations, in response to mesoderm inducing cytokines, suggest that X cells will constitute an accessible *in vitro* system for the identification and/or functional analysis of factors and genes involved in gastrulation. Assignment of function to genes during embryogenesis has been hampered by genetic redundancy and embryonic complexity. The ability to investigate gene function within the primitive ectoderm *in vitro* would therefore be extremely valuable.

In vitro culture systems have proved invaluable for the identification and isolation of biological factors directing haemopoietic stem cell differentiation. By analogy, the culture of murine embryonic stem cells *in vitro*, long promised to provide a powerful system for the dissection of biological events occurring during mammalian development. The ability to form primitive ectoderm from ES cells *in vitro* represents a significant advance in this direction. The primitive ectoderm responds to signals which direct embryonic differentiation. Access to this cell type *in vitro* should not only allow the identification of further developmental restrictions occurring during mammalian development, it may allow defined cell lineages to be generated *in vitro*. With the identification of X cells as the *in vitro* equivalent of primitive ectoderm, the full potential of ES cells as an *in vitro* assay system for analysis of mammalian development may at last be realised.

REFERENCES

REFERENCES

Adamson, E.D. (1988) Cell-lineage-specific gene expression in development. In:- *Experimental approaches to mammalian embryonic development.* Cambridge University Press, Cambridge pp 321-364.

Adamson, E.D. and Ayers, S.E. (1979) The localisation and synthesis of some collagen types in developing mouse embryos. *Cell* **16** 953-965.

Albano, R.M., Groome, N. and Smith, J.C. (1993) Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. *Development* **117** 711-723.

Albano, R.M., Arkell, R., Beddington, R.S.P. and Smith, J.C. (1994) Expression of inhibin subunits and follistatin during postimplantation development: decidual expression of activin and expression of follistatin in the primitive streak, somites and hindbrain. *Development* **120** 803-813.

Amaya, E., Musci, T.J. and Kirschner, M.W. (1991) Expression of a dominant negative mutant FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66** 257-270.

Amaya, E., Stein, P.A., Musci, T.J. and Kirschner, M.W. (1993) FGF signalling in the early specification of mesoderm in *Xenopus*. *Development* **118** 477-487.

Andrews, J.E. (1994) Investigation of the role of the homeobox gene *Nkx2.5* in heart development. *Honours Thesis , The Dept. of Medical Biology, The Walter and Eliza Hall Institute of Medical Research.*

Ang, S-L. and Rossant, J. (1993) Anterior mesendoderm induces mouse *Engrailed* genes in explant cultures. *Development* **118** 139-149.

Ang, S-L and Rossant J. (1994) *HNF-3β* is essential for node and notochord formation in mouse development. *Cell* **78** 561-574.

Ang, S-L., Conlon, R.A., Jin, O. and Rossant, J. (1994) Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* **120** 2979-2989.

Asashima, M., Nakano, H., Simada, K., Kinoshita, K., Ishii, K., Shibai, H. and Ueno, N. (1990) Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* **198** 330-335.

Attisano, L., Carcamo, J., Ventura, F., Weis, F.M.B., Massague, J. and Wrana, J.L. (1993) Identification of human activin and TGFβ type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75** 671-680.

Baird, A. (1994) Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors. *Curr. Opin. Neurobiol.* **4** 78-86.

Basilico, C. and Moscatelli, D. (1992) The FGF family of growth factors and oncogenes. *Adv. Cancer Res.* **59** 115-165.

Bastian, H. and Gruss, P. (1990). A murine *even-skipped* homologue, *evx-1*, is expressed during embryogenesis and neurogenesis in a biphasic manner. *EMBO. J.* **9** 6: 1839-1852.

Beddington, R.S.P. (1988) Analysis of tissue fate and prospective potency in the egg cylinder. In:- *Experimental Approaches to Mammalian Development* . pp 121-147 Cambridge; Cambridge University Press.

Beddington, R.S.P and Robertson, E.J. (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105** 733-737.

Beddington, R.S.P, Rashbass, P. and Wilson, V. (1992) *Brachyury* - a gene affecting mouse gastrulation and early organogenesis. *Development (Suppl)* 157-165.

Beddington, R.S.P. and Smith J.C. (1993) The control of vertebrate gastrulation: inducing signals and responding genes. *Curr. Opin. Genet. Dev.* **3** 655-661.

Bettess, M.D. (1993) Gene expression in murine pluripotential stem cells. *Honours Thesis , Department of Biochemistry, University of Adelaide.*

Blum, M., Gaunt, S.J., Cho, K.W.Y., Steinbeisser, H., Blumberg, B. Bittner, D. and De Robertis E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene *goosecoid*. *Cell* **69** 1097-1106.

Blumberg, B., Wright, C.V.E., DeRobertis, E.M. and Cho, K.W.Y. (1991) Organiser specific homeobox genes in *Xenopus laevis* embryos. *Science* **253** 194-196.

Bradley, A., Evans, M., Kaufman, M.H. and Robertson, E.J. (1984) Formation of germ line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* **309** 255-256.

Bradley, A. (1987) Production and analysis of chimeric mice. In:- *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach.*. pp 113-151. Edited by E. J Robertson, IRL Press, Oxford.

Brandon, E.P., Idzerda, R.L. and Mcknight, G.S. (1995a) Targeting the mouse genome: a compendium of knockouts (part I). *Curr. Biol.* **5** 625-634.

Brandon, E.P., Idzerda, R.L. and Mcknight, G.S. (1995b) Targeting the mouse genome: a compendium of knockouts (part II). *Curr. Biol.* **5** 758-765.

Brulet, P., Babinet, C., Kemler, R. and Jacob, F. (1980) Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. *Proc.Natl.Acad.Sci. USA* **77** 4113-4117.

Bultman, S.J., Michaud, E.J. and Woychik, R.P. (1992) Molecular characterisation of the mouse agouti locus. *Cell* **71** 1195-1204.

Burdsal, C.A., Damsky, C.H. and Pederson, R.A (1993) The role of E-cadherin and intergrins in mesoderm differentiation and migration at the mammalian primitive streak. *Development* **118** 829-844

Capecchi, M.R. (1989) The new mouse genetics: altering the genome by gene targeting. *TIG* **5** 3:70-76.

Cardellini, P. Polo, C. and Coral, S. (1994) Suramin and heparin: aspecific inhibitors of mesoderm induction in the *Xenopus laevis* embryo. *Mech. of Dev.* **45** 73-87.

Chapman, G. (1994) Isolation and characterisation of the murine homeobox gene *MMoxB. Honours Thesis , Department of Biochemistry, University of Adelaide.*

Cheng, L., Gearing, D.P., White, L.S., Compton, D.L., Schooley, K. and Donovan, P.J. (1994) Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development* **120** 3145-3153.

Cho, K.W.Y., Blumberg, B., Steinbeisser, H. and DeRobertis, E.M. (1991) Molecular nature of spemann's organiser: the role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67** 1111-1120.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal. Biochem.* **162** 156-159.

Clements, D.A., Wang, J-K., Dionne, C.A. and Goldfarb, M. (1993) Activation of Fibroblast growth factor (FGF) receptors by recombinant human FGF-5. *Oncogene* **8** 1311-1316.

Conlon, F.L., Barth, K.S. and Robertson. E.J. (1991) A novel retrovirally induced embryonic lethal mutation in the mouse: assessment of the developmental fate of embryonic stem cells homozygous for the 413.d proviral integration. *Development* **111** 969-981.

Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B. and Robertson, E.J. (1994) A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. *Development* **120** 1919-1938.

Conover, J.C., Ip, N.Y., Poueymirou, W.T., Bates, B., Goldfarb, M., Dechiara, T.M. and Yancopoulos, G.D. (1993) Ciliary neutrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* **119**, 559-565.

Conquet, F., Peyriras, N., Tiret, L. and Brulet, P. (1992) Inhibited gastrulation in mouse embryos overexpressing the leukemia inhibitory factor. *Proc.Natl.Acad.Sci. USA* **89** 8195-8199.

Cooke J. (1991) Inducing factors and the mechanism of body pattern formation in vertebrate embryos. *Curr. Topics Dev. Biol.* **25** 45-73.

Copeland, N.G., Jenkins, N.A. Gilbert, D.J., Eppig, J.T., Maltais, L.J., Miller, J.C., Deitrich, W.F, Weaver, A., Lincoln, S.E., Steen, R.G., Stein, L.D., Nadeau, J.H. and Lander, E.S. (1993) A genetic linkage map of the mouse: current applications and future prospects. *Science* **262** 57-66.

Cornell, R.A., Musci, T.J. and Kimelman, D. (1995) FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. *Development* **121** 2429-2437.

Crossley, P.H. and Martin, G.R. (1995) The mouse *FGF-8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121** 439-451.

Damjanov, I., Damjanov, A. and Damsky, C.H. (1986) Developmentally regulated expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in peri-implantation mouse embryos and extraembryonic membranes. *Dev. Biol.* **116** 194-202.

DeJong, R. and Meijlink, F. (1993) The homeobox gene *S8*: mesoderm-specific expression in presomite embryos and in cells cultured *in vitro* and modulation in differentiating pluripotent cells. *Dev. Biol.* **157** 133-146.

Deng, C-X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M. and Leder, P. (1994) Murine *FGFR-1* is required for early postimplantation growth an axial organisation. *Genes Dev.* **8** 3045-3057.

deLapeyriere, O., Ollendorf, V., Planche, J., Ott, M.O., Pizette, S., Coulier, F. and Birnbaum, D. (1993) Expression of the *FGF-6* gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118** 601-611.

DeRobertis, E.M., Fainsod, A., Gont, L.K. and Steinbeisser, H. (1994) The evolution of vertebrate gastrulation. *Development (Suppl.)* 117-124.

Dietrich, W.F., Miller, J.C., Steen, R.G., Merchant, M., Danron, D., Nahf, R., Gross, A., Joyce, D.C., Wessel, M., Dredge, R.D., Marquis, A., Stein, L.D., Goodman, N., Page, D.C. and Lander, E.S. (1994) A genetic map of the mouse with 4,006 simple sequence length polymorphisms. *Nature Genetics* **7** 220-245.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985) The *in vitro* development of blastocyst derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp.Morph.* **87** 27-45.

Duncan, S.A., Manova, K., Chen, W.S., Hoodless, P., Weinstein, D.C., Bachvarova, R.F. and Darnell, J.E. (1994) Expression of *HNF-4* in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo: *HNF-4* is a marker for primary endoderm in the implanting blastocyst. *Proc. Natl. Acad. Sci. USA* **91** 7598-7602.

Dush, M.K. and Martin, G.R. (1992) Analysis of mouse *evx* genes: *evx-1* displays graded expression in the primitive streak. *Dev. Biol.* **151** 273:287.

Dziadek, M.A. and Adamson, E. (1978) Localisation and synthesis of alpha fetoprotein in post-implantation mouse embryos. *J. Embryol. Expt. Morphol.* **43** 289-313.

Dziadek, M.A. and Andrews G.K. (1983) Tissue specificity of alpha fetoprotein messenger RNA expression during mouse embryogenesis. *EMBO J.* **2** 4: 549-554.

Edwards, D.R., Parfett, C.L.J. and Denhardt, D. (1985) Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of species to B2 repetitive elements. *Mol. Cell. Biol.* **5** 3280-3288.

Evans, M.J. (1972) The isolation and properties of a clonal tissue culture strain of pluripotential mouse teratoma cells. *J. Embryol. Exp. Morphol.* **28** 163-176.

Evans, M.J. and Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292** 154-156.

Feldman, B., Poueymirou, W., Papaioannou, V.E., Dechiara, T.M. and Goldfarb, M. (1995) Requirement of *FGF-4* for postimplantation mouse development. *Science* **267** 246-248.

Franke, W.W., Grund, C., Kuhn, C., Jackson, B.W. and Illmansee, K. (1982) Formation of cytoskeletal elements during mouse embryogenesis III primary mesenchymal cells and the first appearance of vimentin filaments. *Differentiation* **23** 43-59.

Friedrich, G. and Soriano, P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5** 1513-1523.

Gardner R.L. (1983) Origin and differentiation of extra-embryonic tissues in the mouse. *Int. Rev. Exp. Pat.* **24** 63-133.

Gardner, R.L., Lyon, M.F., Evans, E.P. and Burtenshaw, M.D. (1985) Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. *J. Embryol. Exp. Morphol.* **88**: 349-363.

Gassmann, M., Donoho, G. and Berg, P. (1995) Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. *Proc.Natl.Acad.Sci. USA* **92** 1292-1296.

Gearing, P.D., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A. and Metcalf, D. (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukemia inhibitory factor (LIF). *EMBO J.* **6** 3995-4002.

Gearing, D.P. and Bruce, G (1992) Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. *New Biol* **4** 61-65.

Gearing, D.P., Comeau, M.R., Friend, D.J., Gimpel, S.D., Thut, C.J., McGourty, J., Brasher, K.K., King, J., Gillis, S., Mosley, B., Ziegler, S.F. and Cosman, D. (1992) The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* **255** 1434-1437.

Ginsberg, M., Snow, M.H.L. and McLaren, A. (1990) Primordial germ cells in the mouse embryo during gastrulation. *Development* **110** 521-528.

Gospodarowicz, D. (1974) Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* **249** 123-127.

Gospodarowicz, D. and Moran, J. (1974) Effect of fibroblast growth factor, insulin, dexamethasone and serum on the morphology of BALB/c3T3 cells. *Proc.Natl.Acad.Sci. USA.* **71** 4648-4652.

Gospodarowicz, D., Cheng, J., Lui, G-M., Baird, A. and Bohlent, P. (1984) Isolation of brain fibroblast growth factor by heparin-sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc.Natl.Acad.Sci.* **81** 6963-6967.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986a) Review: Fibroblast growth factor. *Mol. Cell. Endocrin.* **46** 187-204.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986b) Review: Molecular and biological characterisation of fibroblast growth factor, an angiogenic factor which controls the differentiation of mesoderm and neuroectoderm derived cells. *Cell. Diff.* **19** 1-17.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1987) Fibroblast growth factor: structural and biological properties. *J. Cell. Phys. (Suppl.)* **5** 15-26.

Gossler, A. Joyner, A.L., Rossant, J. and Skarnes, W.C. (1989) Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* **244** 463-465.

Gould, S.J. (1977) *Ontogeny and Phylogeny, Cambridge , Mass: Harvard University Press.*

Green, J.B.A., Howes, G., Symes, K., Cooke, J. and Smith, J.C. (1990) The biological effects of XTC-MIF: quantitative comparison with Xenopus bFGF. *Development* **108** 173-183.

Han, J-K. and Martin, G.R. (1993) Embryonic expression of *FGF-6* is restricted to the skeletal muscle lineage. *Dev. Biol.* **158** 549-554.

Harrison, S.M., Dunwoodie, S.L., Arkell, R.M., Lehrach, H. and Beddington, R.S.P. (1995) Isolation of novel tissue-specific genes from cDNA libraries representing individual constituents of the gastrulating mouse embryo. *Development* **121** 2479-2489.

Haub, O. and Goldfarb, M. (1991) Expression of the *fibroblast growth factor-5* gene in the mouse embryo. *Development* **112** 397-406.

Hebert, J.M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G.R. (1990) Isolation of cDNAs encoding four mouse FGF family members and characterisation of their expression patterns during embryogenesis. *Dev. Biol* **138** 454-463.

Hebert, J.M., Boyle, M. and **Martin, G.R.** (1991). mRNA localization studies suggest that the murine *FGF-5* plays a role in gastrulation. *Development* **112**, 407-415.

Hebert, J.M., Rosenquist, J., Gotz, J. and Martin, G.R. (1994) *FGF-5* as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* **78** 1017-1025.

Hemmati-Brivanlou, A. and Melton, D.A. (1992) A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359** 609-614.

Herrmann, B.G., Labeit, S., Poutska, A., King, T.R. and Lehrach, H. (1990) Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* **343** 617-622.

Herrmann, B.G. (1991). Expression pattern of the *Brachyury* gene in whole-mount T^{Wis}/T^{Wis} mutant embryos. *Development* **113**, 913-917.

Hilton, D.J., Nicola, N.A., Gough, M.A. and Metcalf, D. (1988) Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation-inducing activity on murine myeloid leukemic lines. *J. Biol. Chem.* **263** 9238-9243.

Hogan, B.L.M., Taylor, A. and Adamson, E.D. (1981) Cell interactions modulates embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291** 235-237.

Hogan, B., Constantini, F. and Lacy, E. (1986) *Manipulating the Mouse Embryo: a Laboratory Manual. First Edition* , Cold Spring Harbour Laboratory Press.

Hogan, B., Beddington, R. Constantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo: a Laboratory Manual. Second Edition* , Cold Spring Harbour Laboratory Press.

Holland, P.W.H., Harper, S.J., McVey, J.H. and Hogan, B.J. (1987) *In vivo* expression of mRNA for the Ca^{++}-binding protein *SPARC* (Osteonectin) revealed by *in situ* hybridization. *J. Cell Biol.* **105** 473-482.

Hooper, M., Hardy, K., Handyside, A., Hunter, S. and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germ line colonization by cultured cells. *Nature* **326** 292-295.

Hosler, B.A., LaRosa, G.J., Grippo, J.F. and Gudas, L. (1989). Expression of *Rex-1*, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol. Cell. Biol.* **9** 12, 5623-5629.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S.J. Yasukawa, K., Kishimoto, T., Anderson, D.J., Stahl, N. and Yancopoulos, G.D. (1992) CNTF and LIF act on neuronal cells *via* shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69,** 1121-1132.

Johansson, B.M. and Wiles, M.V. (1995) Evidence for involvement of activin A and bone morphogenetic protein-4 in mammalian mesoderm and hematopoietic development. *Mol. Cell. Biol.* **15** 1:141-151.

Johnson, D.E. and Williams, L.T. (1993) Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res.* **60** 1-41.

Johnson, K.E., Boucaut, J-C. and DeSimone, D.W. (1992) Role of the extracellular matrix in amphibian gastrulation. *Curr. Topics Dev. Biol.* **27**.

Johnson M.H. and Maro, B. (1988) Time and space in the mouse early embryo: a cell biological approach to cell diversification. In:- *Experimental approaches to mammalian development*, Cambridge University Press, Cambridge, pp 35-65.

Jones-Villeneuve, E.M.V., McBurney, M.W., Rogers, K.A. and Kallins, V.I. (1982) Retinoid Acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell. Biol.* **94** 253-262.

Joyner, A.L. (1991) Gene targeting and gene trap screens using embryonic stem cells: new approaches to mammalian development. *Bioessays* **13** 12:649-656.

Kaufman, M.H. (1992) *The Atlas of Mouse Development.* Academic Press.

Keller. G., Kennedy, M., Papayannopoulou, T. and Wiles, M.V. (1993) Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol.* **13** 1:473-486.

Kidder, G.M. (1992) The genetic program for preimplantation development. *Dev. Genetics.* **13** 319-325.

Kimelman, D. and Kirschner, M. (1987) Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA encoding for FGF in the early *Xenopus* embryo. *Cell* **51** 869-877.

Klagsbrun, M. and Baird, A. (1991) A dual receptor system is required for basic fibroblast growth factor activity. *Cell* **67** 229-231.

Krieg, P.A. and Melton, D.A. (1987) *In vitro* RNA synthesis with SP6 RNA polymerase. *Meth. Enz.* **155**, 397-415.

LaBonne, C. and Whitman, M. (1994) Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development* **120** 463-472.

Latham, K.E., Garrels, J.I., Chang, C.and Solter,D. (1991) Quantitative analysis of protein synthesis in mouse embryos. 1. extensive reprogramming at the one- and two-cell stage. *Development* **112** 921-932.

Lawson, K.A., Meneses, J.J. and Pedersen, R.A. (1991) Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113** 891-911.

Leivo, I., Vaheri, A., Timpl, R. and Wartiovaara, J. (1980) Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* **76** 100-114.

Liang, P. and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. *Science* **257** 967-970.

Lindenbaum, M.H. and Grosveld, F. (1990) An *in vitro* globin gene switching model based on differentiated embryonic stem cells. *Genes and Dev.* **4** 2075-2085.

Ling, N., Ueno, N., Ying, S-Y., Esch, F., Shimasaki, S., Hotta, M., Cuvevas, P. and Guillemin, R. (1988) Inhibins and activins. *Vitamins and Hormones* **44** 1-46.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I., and **Harvey, R.P.** (1993). *Nkx2.5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendents. *Development* **119**, 419-431.

Lobe, C.G. (1992) Transcription factors and mammalian development. *Curr. Topics Dev. Biol.* **27** 351-383.

Lyons, I., Parsons, L.M., Hartley, L., Ruili, L., Andrews, J.E., Robb, L. and Harvey, R.P. (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx 2.5*. *Genes. Dev.* **9** 1654-1666.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C. and Mason, I. (1995) A role for *FGF-8* in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* **5** 7: 797-806.

Manova, K., DeLeon, V., Angeles, M., Kalantry, S., Giarre, M., Attisano, L., Wrana, J. and Bachvarova, R.F. (1995) mRNAs for activin receptors II and IIB are expressed in mouse oocytes and in the epiblast of pregastrula and gastrula stage mouse embryos. *Mech of Dev.* **49** 3-11.

Mansour, S.L., Goddard, J.M. and Cappechi, M.R. (1993) Mice homozygous for a targeted disruption of the proto-oncogene *int-2* have developmental defects in the tail and inner ear. *Development* **117** 13-28.

Mansukhani, A., Dell'Era, P., Moscatelli, D., Kornbluth, S., Hanafusa, H. and Basilico, C. (1992) Characterisation of the murine *BEK* fibroblast growth factor (FGF) receptor: activation by three members of the FGF family and requirement for heparin. *Proc.Natl.Acad.Sci.* **89** 3305-3309.

Maro, B. Gueth-Halonet, C., Aghion, J. and Antony C. (1991) Cell polarity and microtubule organisation during mouse early embryogenesis. *Development (Suppl.)* **1** 17-25.

Martin, G.R. (1981) Isolation of a pluripotent cell line form early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc.Natl.Acad.Sci.* **78** 7634-7636.

Martin, G.R. and Evans, M.J. (1975a) Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc.Natl.Acad.Sci.* **72** 1441-1445.

Martin, G.R. and Evans, M.J. (1975b) Multiple differentiation of clonal teratocarcinoma stem cells following embryoid body formation *in vitro*. *Cell* **6** 467-474.

Mason, I.J., Taylor, A., Williams, J.G., Sage, H. and **Hogan, B.L.M.** (1986). Evidence from molecular cloning that *SPARC*, a major product of mouse parietal endoderm, is related to an endothelial cell 'culture shock ' protein of Mr 43 000. *EMBO J.* **5** 7, 1465-1472.

Mason, I.J. (1994) The ins and outs of fibroblast growth factors. *Cell* **78**, 547-552.

Mason, I.J., Fuller-Pace, F., Smith, R. and Dickson, C. (1994) *FGF-7* (keratinocyte growth factor) expression during murine embryogenesis suggests roles in myogenesis, forebrain regionisation and epithelial-mesenchymal interactions. *Mech. of Dev.* **45** 15-30.

Matsui, Y., Toksoz, D., Nishikawa, S., Williams, D., Zsebo, K. and Hogan, B.L.M. (1991) Effect of Steel factor and leukemia inhibitory factor on murine primordial germ cells in culture. *Nature* **353** 750-752.

Matsui, Y., Zsebo, K. and Hogan, B.L.M. (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70** 841-847.

Matzuk, M.M., Kumar, T.R., Vassalll, A., Bickenbach, J.R., Roop, D.R., Jaenisch, R. and Bradley, A. (1995a) Functional analysis of activins during mammalian development. *Nature* **374** 354-356.

Matzuk, M.M, Kumar, T.R. and Bradley, A. (1995b) Different phenotypes for mice deficient in either activins of activin receptor type II. *Nature* **374** 356-359.

McGinnis, W. and Krumlauf, R. (1992) Homeobox genes and axial patterning. *Cell* **68** 283-302.

McBurney, M.W., Jones-Villeneuve, E.M.V., Edwards, M.K.S. and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299** 165-167.

Melton, D.A. (1991) Pattern formation during animal development. *Science* **252** 234-241.

Mercola, M., Wang, C., Kelly, J., Brownlee, C., Jackson-Grusby, L., Stiles, C. and Bowen-Pope, D. (1990) Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev. Biol.* **138** 114-122.

Metcalf, D. (1989) The molecular control of cell division, differentiation, commitment, and maturation in haemopoietic cells. *Nature* **339** 27-30.

Metcalf. D. (1991) The Florey Lecture, 1991. The colony-stimulating factors: discovery to clinical use. *Phil. Trans. R. Soc. Lond. B.* **333** 147-173.

Mitani, S. (1989) Retarded gastrulation and altered subsequent development of neural tissues in heparin-injected Xenopus embryos. *Development* **107** 423-455.

Monaghan, A.P., Kaestner, K.H., Grau, E. and Schutz, G. (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF3-α, β, γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119** 567-578.

Monk, M. and McLaren, A. (1981) X-chromosome activity in foetal germ cells of the mouse. *J. Embryol. Exp. Morphol.* **63** 75-84.

Mummery, C.L., Feijen, A., Moonlenaar, W.H., van der Brink, C.E. and de Laat, S.W. (1986) Establishment of a differentiated mesodermal line from P19EC cells expressing functional PDGF and EGF receptors. *Exp. Cell. Res.* **165** 229-242.

Mummery, C.L. and van den Eijnden-van Raaij, A.J.M. (1990) Review: Growth factors and their receptors during differentiation and early murine development. *Cell Diff. Dev.* **30** 1-18.

Mummery C.L., van Achterberg, T.A.E., van den Eijnden-van Raaij, A.J.M., van Haaster, L., de Laat, S.W., and Piersma, A.H. (1991) Visceral endoderm-like cell lines induce differentiation of murine P19 embryonal carcinoma cells. *Differentiation* **46** 51-60.

Murphy, M., Reid, K., Ford, M., Furness, J.B. and Bartlett, P. (1994) FGF2 regulates proliferation of neural crest cells, with subsequent neuronal differentiation regulated by LIF or related factors. *Development* **120** 3519-3528.

Murtha, M.T., Leckman, J.F. and Ruddle, F.H. (1991) Detection of homeobox genes in development and evolution. *Proc.Natl.Acad.Sci. USA* **88** 10711-10715.

Niswander, L. and Martin, G.R. (1992) *FGF-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114** 755-768.

Opstelten, D-J. E., Vogels, R., Robert, B., Kalkhoven, E., Zwartkruis, F., De Laaf, L., Destree, O. H., Deschamps, J., Lawson, K.A., and Meijlink, F. (1991). The mouse homeobox gene, *S8*, is expressed during embryogenesis predominantly in mesenchyme. *Mech. of Dev.* **34**, 29-42.

Orr-urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P. (1991) Developmental expression of two murine fibroblast growth factor receptors *flg* and *bek*.. *Development.* **113** 1419-1434.

Orr-urtreger, A. and Lonai, P. (1992) Platelet derived growth factor-A and its receptor are expressed in separate, but adjacent cell layers of the mouse embryo. *Development* **115** 1045-1058.

Papaioannou, V.E., McBurney, M.W., Gardner, R.L. and Evans, M.J. (1975) Fate of teratocarcinoma cells injected into early mouse embryos. *Nature* **258** 70-73.

Pease, S., Braghetta, P., Gearing, D., Grail, D. and Williams, L. (1990) Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev. Biol.* **141** 344-352.

Pedersen, R.A. (1988) Potency, lineage and allocation in preimplantation mouse embryos. In *Experimental approaches to mammalian development.*, Cambridge University Press, Cambridge, pp 3-33.

Pedersen, R.A. (1994) Studies of *in vitro* differentiation with embryonic stem cells. *Reprod. Fertil. Dev.* **6** 543-52.

Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paoni, N.F. and Wood, W.I. (1995) Cardiotrophin-1: biological activities and binding to the leukemia inhibitory factor receptor/gp130 signalling complex. *J. Biol. Chem.* **270** 18 10915-10922.

Pesce, M., Farrace, M.G., Piacentini, M., Dolci, S. and DeFelici, M. (1993) Stem cell factor and leukemia inhibitory factor promote primordial germ cell growth survival by suppressing programmed cell death (apoptosis). *Development* **118** 1089-1094.

Piquet-Pellorce, C., Grey, L., Mereau, A., and Heath, J.K. (1994) Are LIF related cytokines functionally equivalent ? *Exp. Cell Research.* **213**, 340-347.

Poirier, F., Chan, C.T.J., Timmons, P.M., Robertson, E.J., Evans, M.J., and Rigby, P.W.J. (1991) The murine *H19* gene is activated during embryonic stem cell differentiation *in vitro* and at the time of implantation in the developing embryo. *Development* **113**, 1105-1114.

Potocnik, A.J., Nielsen, P.J. and Eichmann, K. (1994) *In vitro* generation of lymphoid precursors from embryonic stem cells. *EMBO J.* **13** 22:5274-5283.

Pruitt, S.C. (1994a) Primitive streak mesoderm-like cell lines expressing *Pax-3* and *Hox* gene autoinducing activities. *Development* **120** 37-47.

Pruitt, S.C. (1994b) Discrete endogenous signals mediate neural competence and induction in P19 embryonal carcinoma stem cells. *Development* **120** 3301-3312.

Pruitt, S.C. and Natoli, T.A. (1992) Inhibition of differentiation by leukemia inhibitory factor distinguishes two induction pathways in P19 embryonal carcinoma stem cells. *Differentiation* **50** 57-65.

Quinlan, G.A., Williams, E.A., Tan, S-S. and Tam, P.P.L. (1995) Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: implication for body plan organisation during early embryogenesis. *Development* **121** 87-98.

Rathjen, P.D., Nichols, J., Toth, S., Edwards, D.R., Heath, J.K. and Smith, A.G. (1990). Developmentally programmed induction of differentiating inhibiting activity and the control of stem cell populations. *Genes and Dev.* **4** 2308-2318.

Resnick, J.L., Bixler, L.S., Cheng, L. and Donovan, P.J. (1992) Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359** 550-552.

Richa, J and Solter, D. (1988) Role of cell surface molecules in early mammalian development. In:- *Experimental approaches to mammalian embryonic development.* Cambridge University Press, Cambridge.

Robertson, E.J., Bradley, A., Kuehn, M. and Evans, M. (1986) Germ line transmission of gene introduced into cultured pluripotential cells by retroviral vector. *Nature* **323** 445-448.

Robertson, E.J. (1987) *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach.*, IRL Press, Oxford.

Robertson E.J. (1991) Using embryonic stem cells to introduce mutations into the mouse germ line. *Biol. Reprod.* **44** 238-245.

Rogers, M.B., Hosler, B.A., and **Gudas, L.** (1991). Specific expression of a retinoic acid-regulated, zinc-finger gene, *Rex-1* , in preimplantation embryos, trophoblast and spermatocytes. *Development* **113**, 815-824.

Rohwedel, J., Maltsev, V., Bober, E., Arnold H-H., Hescheler, J. and Wobus, A.M. (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis *in vivo*: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev. Biol.* **164** 87-101.

Rosenthal, M.D., Wishnow, R.M. and Sato, G.H. (1970) In vito growth and differentiation of clonal populations of multipotential mouse cells derived from a transplantable testicular teratocarinoma. *J. Natl. Cancer. Inst.* **44** 1001-1014.

Rosner, M.H., Vigano. A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W.J. and Staudt, L.M. (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345** 686-692.

Rosner, M.H., Vigano. A., Rigby, P.W.J., Arnheiter, H. and Staudt, L.M. (1991) *Oct-3* and the beginning of mammalian development. *Science* **253** 144-145.

Ross, R., Raines, E.W. and Bowen-Pope, D.F. (1986) The biology of platelet derived growth factor. *Cell* **46** 155-169.

Rossant, J. (1993) Immortal germ cells? *Curr. Biol.* **3** 1:47-49.

Rossant, J. (1988) Development of extra-embryonic cell lineages in the mouse embryo. In:- *Experimental approaches to mammalian embryonic development.* Cambridge University Press, Cambridge. pp 97-120.

Rossant, J and Pederson, R.A. (1988) *Experimental approaches to mammalian embryonic development.* Cambridge University Press, Cambridge.

Rudnicki, M.A. and McBurney, M.W. (1987) Cell culture and induction of differentiation of embryonal carcinoma cell lines. In:- *Teratocarcinomas and embryonic stem cells.* Edited by E.J. Robertson, IRL Press. pp 19-49.

Ruiz i Altaba, A. and Melton D.A. (1989a) Interaction between peptide growth factors and homeobox genes in the establishment of antero-posterior polarity in frog embryos. *Nature* **341** 33-38.

Ruiz i Altaba, A. and Melton D.A. (1989b) Involvement of the Xenopus homeobox gene *Xhox-3* in pattern formation along the anterior-posterior axis. *Cell* **57** 317-326.

Ruiz i Altaba, A. and Melton D.A. (1990) Axial patterning and the establishment of polarity in the frog embryo. *TIG* **6** 2: 57-67

Sasaki, H. and Hogan, B.L.M. (1993) Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118** 47-59.

Schmitt, R.M., Bruyns, E.and Snodgrass, H.R. (1991) Hematopoietic development of embryonic stem cells *in vitro*: cytokine and receptor gene expression. *Genes. Dev.* **5** 728-740.

Scholer, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. and Gruss, P. (1990a) New type of POU domain in germ line-specific protein *Oct-4. Nature* **344** 435-439

Scholer, H.R., Dressler, G.R., Balling, R., Rohdewohld, H. and Gruss, P. (1990b) *Oct-4*: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J.* **9** 7: 2185-2195.

Scholer, H.R. (1991) Octamania: the POU factors in murine development. *TIG* **7** 323-329.

Schultz, R.M. (1988) Molecular aspects of mammalian oocyte growth and maturation. In:- *Experimental approaches to mammalian development.*, Cambridge University Press, Cambridge, pp 195-237.

Schwartzberg, P., Goff, S. and Robertson, E.J. (1989) Germ line transmission of a *c-abl* mutation produced by targeted gene disruption in ES cells. *Science* **246** 799-803.

Scott, M.P., Tamkun, J.W. and Hartzell, G.W. (1989) The structure and function of the homeodomain. *Biochem. Biophys. Acta.* **989** 25-48.

Shen, M.M. and Leder, P. (1992) Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **89** 8240-8244.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M. (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* **223** 1296-1299.

Silvers, W.K. (1979) The coat colours of mice: a model for mammalian gene action and interaction. *Springer-Verlag.*

Sive, H.L. (1993) The frog prince-ss: A molecular formula for dorsoventral patterning in *Xenopus. Genes Dev,* **7** 1-12.

Skarnes, W.C., Auerbach, B.A. and Joyner, A.L. (1992) A gene trap approach in mouse embryonic stem cells: The *lacZ* reporter gene is activated by splicing, reflects endogenous gene expression, and its mutagenic in mice. *Gene Dev.* **6** 903-913.

Skarnes, W.C., Moss, J., Hurtley, S.M. and Beddington, R. (1995) Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl. Acad. Sci. USA* **92** 6592-6596.

Slack, J.M.W., Darlington, B.G., Heath, J.K and Godsave, S.F. (1987) Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature* **326** 197-200.

Slack, J.M.W. (1990) Growth factors as inducing agents in early *Xenopus* development. *J. Cell Sci. Suppl.* **13** 119-130.

Slack, J.M.W. and Tannahill, D. (1992) Mechanism of anteroposterior axis specification in vertebrates. Lessons from the amphibians. *Development* **114** 285-302.

Smith, A.G. and Hooper, M.L. (1987) Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of embryonal carcinoma and embryonic stem cells. *Dev. Biol.* **121** 1-9.

Smith, A.G., Heath, K.J., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M. and Rogers, D. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336** 688-690.

Smith, A.G. (1991) Culture and differentiation of embryonic stem cells. *J. Tiss. Cult. Meth.* **13** 89-94.

Smith, A.G. (1992) Mouse embryo stem cells: their identification, propagation and manipulation. *Seminars in cell biology* **3** 385-399.

Smith, J.C., Price, B.M.J., Green, J.B.A., Weigel, D. and Herrmann, B.G. (1991) Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. *Cell* **67** 79-87.

Smith, J. (1995) Angles on activins absence. *Nature* **374** 311-312.

Snow, M.H.L. (1977) Gastrulation in the mouse: growth and regionalization of the epiblast. *J. Embryol. Exp. Morphol.* **42** 293-303.

Snow, M.H.L. and Tam, P.P.L. (1979) Is compensatory growth a complicating factor in mouse teratology? *Nature* **279** 554-557.

Solter, D. and Knowles, B.B. (1978) Monoclonal antibody defining stage-specific mouse embryonic antigen (SSEA-1). *P.N.A.S. USA* **75** 11: 5565-5569.

St Johnston, D. and Nusslein-Volhard, C. (1992) The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68** 201-219.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, I., Gadi, I., Kontgen, F. and Abbondanzo, S.J. (1992) Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* **359** 76-79.

Stewart, C. L., Gadi, I. and Bhatt, H (1994) Stem cells from primordial germ cells renter the germ line. *Dev. Biol.* **161**, 626-628.

Strickland, S. and Mahdavi, V. (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15** 393-403.

Strickland, S., Smith, K.K. and Marotti, K.R. (1980) Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* **21** 347-355.

Tam, P.P.L and Snow, M.H.L. (1981) Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *J. Embryol. Exp. Morphol.* **64** 133-147.

Tam, P.P.L. and Beddington, R.S.P. (1987) The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99** 109-126.

Tam, P.P.L. (1989) Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development* **107** 55-67.

Tam, P.P.L. and Beddington, R.S.P. (1992) Establishment and organisation of germ layers in the gastrulating mouse embryo. *CIBA Found. Symp.* **165** 27-49.

ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K. and Heldin, C.H. (1994) Characterisation of type I receptors for transforming growth factor-beta and activin. *Science* **264** 101-104.

Thomas, P.Q. (1994) Homeobox gene expression in murine embryonic stem cells. *Ph.D Thesis, Department of Biochemistry, University of Adelaide*

Vale, W., Hsueh, C., Rivier, C. and Yu, J. (1990) The inhibin/activin family of hormones and growth factors. In:- *Peptide growth factors and their receptors* II (eds, Sporn, M.B.and Roberts, A.B.) pp 211-248.

van den Eijnden-van Raaij, A.J.M, van Achterberg, T.A.E., van der Kruijssen, C.M.M, Piersma, A.H., Huylebroeck, D., de Laat, S.W. and Mummery C.L. (1991) Differentiation of aggregated murine P19 embryonal carcinoma cells is induced by a novel visceral endoderm-specific FGF-like factor and inhibited by Activin A. *Mech. of Dev.* **33** 157-166.

Verschueren, K., Dewulf, N., Goumans, M-J., Lonnoy, O., Feijen, A., Grimsby, S., Vande Spiegle, K., ten Dijke, P., Morens, A., Vanscheeuwwijck, P., Heldin, C-H., Miyazono, K., Mummery, C., Van Den Eijnden-Van Raaij, J. and Huylebroeck, D. (1995) Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. *Mech. of Dev.* **52** 109-123.

Vidricaire, G., Jardina, K. and McBurney, M.W. (1994) Expression of the *Brachyury* gene during development in differentiating embryonal carcinoma cell cultures. *Development* **120** 115-122.

Wang, J-K., Gao, G and Goldfarb, M. (1994) Fibroblast growth factor receptors have different signalling and mitogenic potentials. *Mol. Cell. Biol.* **14** 181-188.

Wang, R. Clark, R., and Bautch, V.L. (1992) Embryonic stem cell derived cystic embryoid bodies form vascular channels: an *in vitro* model of blood vessel development. *Development* **114** 303-316.

Wartiovaara, J., Leivo, I. and Vaheri, A. (1979) Expression of the cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. *Dev. Biol.* **69** 247-257.

Wilkinson, D.G., Peters, G., Dickson, C. and McMahon, A.P. (1988) Expression of the FGF-related proto-oncogene *int-2* during gastrulation and neurulation in the mouse. *EMBO J.* **7** 3 691-695.

Wilkinson, D.G., Bhatt, S. and McMahon, A.P. (1989) Expression pattern of the FGF-related proto-oncogene *int-2* suggests multiple roles in fetal development. *Development* **105** 131-136.

Wilkinson, D.G., Bhatt, S. and Herrmann, B.G. (1990) Expression pattern of the mouse *T* gene amd its role in mesoderm formation. *Nature* **343** 657-658.

Williams, R.L., Hilton, D.J., Pease, S., Wilson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A. and Gough, N.M. (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336** 684-692.

Wobus, A.M., Wallukat, G. and Hescheler, J. (1991) Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotrophic responses to adrenergic and cholinergic agents and calcium ion channel blockers. *Differentiation* **48** 173-183.

Yamada, G., Kioussi, C., Schubert, F.R., Eto, Y., Chowdhury, K., Pituello, F. and Gruss, P. (1994) Regulated expression of *Brachyury(T)*, *Nkx1.1* and *Pax* genes in embryoid bodies. *Biochem. Biophys. Res. Com.* **199** 2: 552-563.

Yamaguchi, T.P., Harpal, K., Henkemeyer, M and Rossant, J. (1994) *FGFR-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* **8** 3032-3044.

Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P. and Ornitz, D.M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64** 841-848.

Yeom, Y.I., Ha, H-S., Balling, R., Scholer, H. and Artzt, K. (1991) Structure, expression and chromosomal location of the *Oct-4* gene. *Mech. of Dev.***35** 171-179

Yoshida, K., Chambers, I., Nichols, J., Smith, A., Saito, M., Yasukawa, K., Shoyab, M., Taga, T. and Kishimoto, T. (1994). Maintenance of the pluripotential phenotype of embryonic stem cells through direct interaction of gp130 signalling pathways. *Mech. Dev.* **45**, 163-171.

Zhou, X., Sasaki, L., Lowe, L., Hogan, B.L.M. and Kuehn, M.R. (1993) *Nodal* is a novel TGFβ-like gene expressed in the mouse node during gastrulation. *Nature* **361** 543-547.