In vitro Culture of Chicken and Mouse Embryo-Derived Cells

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Doctor of Philosophy

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors Dr. Helen Sang and Dr. Jim McWhir. No part of this thesis has been, or will be, submitted for any other degree, diploma or qualification.

Peter Graham Lodge
November 2003
Acknowledgements

I would like to thank my supervisors, Jim McWhir and Helen Sang for their advice, encouragement and patience during the last three and a half years. Thanks are also due to all the members of both labs who have provided much help and support, often at short notice. Ed Gallagher, in particular, closely supervised much of the work described here and deserves special thanks. I would also like to thank Mike Clinton’s lab for much helpful advice (and reagents) and the many others at Roslin Institute who helped throughout the last few years.

At the University of Edinburgh, I would like to thank Austin Smith, Alexander Medvinsky and Evelyn Telfer for supervising the project and Edward Duvall for helping to characterise tumours.

Finally I would like to thank my parents and my wife for providing mental, physical, financial, technical, artistic and moral support and for helping me to keep everything in perspective.
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### Abbreviations

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<table>
<thead>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ASC</td>
<td>adult stem cell</td>
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<td>ALV</td>
<td>avian leukosis virus</td>
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<td>bp</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>CNTF</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SOS</td>
<td>son of sevenless</td>
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<tr>
<td>SH</td>
<td>src homology</td>
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<tr>
<td>SSEA</td>
<td>stage-specific embryonic antigen</td>
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<tr>
<td>TNAP</td>
<td>tissue non-specific alkaline phosphatase</td>
</tr>
<tr>
<td>TEG</td>
<td>trypsin/EGTA solution</td>
</tr>
<tr>
<td>TE</td>
<td>tris/EDTA</td>
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<tr>
<td>Tris</td>
<td>tris (hydroxymethylaminomethane)</td>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
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<td>μl</td>
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Abstract

Mouse embryonic stem (mES) cells can be maintained in vitro without loss of pluripotency in the presence of leukaemia inhibitory factor (LIF). Germline competent mES cells can be genetically modified in vitro and used to make transgenic mice via chimaeras. Germline competent chicken ES (chES) cells would be a powerful tool for the production of transgenic chickens. ES cells have been isolated from primates and mice but attempts from other species have been unsuccessful. Novel ES cell isolation strategies have been tested using inbred mouse strains. Using standard techniques, mES cells cannot be isolated from CBA strain embryos whereas they can be isolated from 129Sv strain embryos. A vital function of LIF in mES cells is activation of signal transducer and activator of transcription 3 (STAT3). LIF also activates the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway that appears to promote differentiation. LIF is a member of the interleukin-6 (IL-6) family of cytokines that includes ciliary neurotrophic factor (CNTF). Manipulation of IL-6 family signals is a new approach to mES cell isolation that may be applied to development of methods for chES cell isolation.

Prior to investigation of new approaches, standard mES cell isolation was performed. chES cells were not isolated in conditions adapted from standard mES cell isolation. A new approach involving the manipulation of LIF-mediated signals was evaluated in mES cell isolation experiments. The drug PD98059 inhibits the MEK/ERK
pathway by preventing phosphorylation of MEKI. ES cell isolation frequency from strain 129Sv embryos in the presence of 25μM PD98059 and 500 U/ml LIF showed an increase that approached significance (p=0.059) however, CBA ES cells could not be isolated in the same conditions. The drug U0126 blocks the MEK/ERK pathway by directly inhibiting both phosphorylated MEKI and MEKII. CBA ES cells were isolated at a frequency of 22.3% in medium containing 2μM U0126 and 2x10³ U/ml LIF. Increased STAT3 activation by 2x10³ U/ml LIF was hypothesised to have facilitated CBA ES cell isolation. CBA ES cells expressed Oct4 and differentiated into the three germ layers in vitro via embryoid bodies and in vivo in teratomas. Contribution of CBA ES cells in chimaeras is under investigation. In chicken cells, U0126 inhibited the MEK/ERK pathway and 10ng/ml CNTF stimulated STAT3 phosphorylation. chES cells were not isolated in the presence of these supplements.
Chapter 1 Introduction

1.1 General Introduction

Developing reliable and efficient systems for the production of transgenic livestock animals, including poultry, has proved a major challenge. Success in mouse transgenic technology has not been repeated in livestock. The range of potential applications in the biomedical and biotechnology industries has however ensured continued efforts towards this objective. Suggested applications of transgenic technology are the production of pharmaceutical proteins or organs suitable for xenotransplantation into humans (Clark et al., 1987; Dooldeniya & Warrens, 2003).

The use of transgenic livestock animals as bioreactors to produce pharmaceutical proteins of commercial importance is now an established field. An animal capable of producing significant amounts of easily harvested protein following the introduction of cloned gene constructs is required. Protein production in transgenic livestock animals overcomes problems associated with prokaryotic processing of eukaryotic proteins (Williams et al., 1982). The lactating mammary gland of transgenic ungulates was proposed as a bioreactor for protein production (Clark et al., 1987). Cloned gene products expressed from mammary gland-specific promoters can be harvested from the lactating mammary gland of transgenic ungulates. This approach has now been successfully applied in a range of species including goats (Denman et al., 1991), sheep (Wright et al., 1991) and pigs (Velander et al., 1992).

There are features of poultry and eggs that present an attractive alternative to ungulates for the production of pharmaceutical proteins. Poultry have a shorter
generation interval than ungulates and the isolation of protein from eggs can be automated to a great extent. Laying hens produce one egg per day that typically contains 3.5-4 grams of protein. The majority of this protein is encoded by a single gene: ovalbumin. Ovalbumin-derived sequences could potentially be used to direct transgene expression resulting in a high yield of transgenic protein per egg. A transgenic bird could potentially lay over 300, naturally pathogen free, eggs per year. Additional benefits such as automated egg collection and existing protein fractionation technology make poultry very attractive as protein bioreactors. Transgenic proteins are subject to species-specific post-translational modifications by the host cell. There is evidence that post-translational protein modification in chickens show high similarity to human protein modification. A comparison of thirteen species-specific modifications of immunoglobulins found chicken and human glycosylation had the greatest similarity (Raju et al., 2000). However, the potential advantages of the chicken as an organism for transgenic protein production are offset by the difficulties in reliable production of transgenic birds.

Creating transgenic animals requires the introduction of cloned gene constructs into an animal, usually at a very early embryonic stage of development. There are three commonly used methods of achieving this: 1) introduction of cloned gene constructs directly to oocytes or embryos; 2) introduction of cells carrying cloned gene constructs into embryos and 3) introduction of nuclei containing cloned gene constructs into embryos.
Gene targeting uses deoxyribonucleic acid (DNA) constructs flanked by large regions of sequence with homology to endogenous sequence. These sequences allow homologous recombination (HR) with chromosomal DNA. The frequency of HR events is often low and drug selection is used to isolate correctly targeted cells. Mouse embryonic stem (ES) cells can be genetically modified in this way without losing pluripotency. Modified ES cells can be introduced to early embryos where they contribute to development of all cell lineages in the developing mouse. These "chimaeric" mice contain some cells with the parental-derived genotype and some with the ES cell-derived genotype. Chimaeras containing ES cell-derived germ cells may produce heterozygous offspring when mated with wild type animals. Gene targeting technology in mice has been largely used to study gene function by abolishing the function of endogenous genes (knock-outs).

Transgenic birds have been produced by a variety of methods but to date only transgene addition has been possible. Additive transgenics can deliver DNA sequences encoding pharmaceutical proteins and tissue-specific promoters, however expression levels are unpredictable for a variety of reasons (see 1.3.1.2). Cloned gene constructs integrate at random sites, often in multiple copy arrays, that may be "silenced" by methylation in the host cell (Mehtali et al., 1990; Cedar et al., 1988). Reliable transgene expression can be achieved if DNA integration is directed by HR to a site where it is controlled by an endogenous promoter.

There are currently two strategies that may be capable of delivering targeted modifications to endogenous chicken genes. If chicken ES cells were isolated they
could be modified by HR and used to produce transgenic birds. Alternatively ES or somatic cells could be modified by HR prior to their use as nuclear donors in nuclear transfer (NT). There are major technical obstacles to be overcome in the development of either of these approaches.

Although murine ES cells are readily available, there is limited evidence of ES cell isolation from other species. Following the isolation of murine ES cells (Evans & Kaufman, 1981; Martin, 1981) attempts were made to isolate ES cells from various species (see 1.3.2.2). However, with the possible exception of human ES cells (Thomson et al., 1998), cells with the germline colonising potential of murine ES cells have not been isolated from other species.

There is a further restriction of ES cell isolation to certain inbred strains of mouse (see 1.3.2.2.). Most ES cell lines are derived from the inbred mouse strain 129 whereas ES cells from the CBA strain of mice cannot be isolated using standard procedures. This “strain barrier” to ES cell isolation has made CBA mice an attractive model to test new approaches to ES cell isolation. CBA ES cells have been isolated using novel approaches (McWhir et al., 1996; Brook & Gardner, 1997; Schoonjans et al., 2003; see 1.3.2.2). Despite successfully isolating CBA ES cells, these techniques have not been successfully used to isolate ES cells from other species.

Murine ES cells are dependent upon stimulation of the glycoprotein130 (gp130) transmembrane receptor by leukaemia inhibitory factor (LIF) to maintain their
undifferentiated phenotype. Two important consequences of LIF signalling have been extensively characterised in murine ES cells. One is activation of the signal transduction factor signal transducer and activator of transcription 3 (STAT3) which is essential for maintenance of the ES cell phenotype (Niwa et al., 1998). The other is activation of the mitogen-activated protein kinase kinase/ extracellular regulated kinase (MEK/ERK) pathway which is not required for self-renewal in ES cells (Burdon et al., 1999). A balance between these signals appears to allow propagation of murine ES cells. ES cells also require endogenous activity of at least two transcription factors: oct4 and nanog (Niwa et al., 2000; Mitsui et al., 2003; Chambers et al., 2003). Oct4 maintains ES cells in combination with gp130 stimulation of STAT3 whereas nanog expression can maintain ES cells in the absence of STAT3 activity.

The manipulation of these cell signalling pathways may provide an alternative approach to ES cell isolation from CBA embryos. Many of the molecules involved in LIF signalling are conserved between chicken and mice. If these can be demonstrated to have functional equivalence then approaches used to isolate CBA ES cells may be applied to ES cell isolation from chicken embryos.

1.2 Early development

Production of transgenic animals frequently involves the manipulation of oocytes, early zygotes, embryos and primordial germ cells (PGCs). Ovulation, fertilisation and early embryonic events in mouse and chicken development are summarised here.
1.2.1 Mouse development

Pre-implantation mouse development following ovulation is summarised in figure 1.1. Ovulation occurs during the dark cycle and oocytes are fertilised at approximately 0.5 days post coitus (dpc). The zygote undergoes several rounds of cell division until the first differentiation events occur with compaction of the morula at 3dpc. Cells on the outer surface of the embryo contribute to extraembryonic lineages and those inside the embryo give rise to embryonic cell types. By 3.5dpc the embryo (now termed blastocyst) consists of the inner cell mass (ICM), surrounded by an outer layer of trophectoderm cells, and a fluid filled cavity, the blastocoel. Embryos can be easily recovered for manipulation during these stages.

PGCs are the precursors of germ cells in the embryo. In the mouse PGC fate is not determined until the beginning of gastrulation. Prior to gastrulation, epiblast cells still have the choice whether or not to adopt a germ cell fate (Tam & Zhou, 1996). Germ cell fate is induced in proximal epiblast cells by interaction with extra-embryonic ectoderm leading to expression of \textit{fragilis} and \textit{stella} (Saitou et al., 2002). PGCs become identifiable by the expression of an alkaline phosphatase isozyme, tissue non-specific alkaline phosphatase (TNAP) shortly after this stage. It is possible to trace the migration of PGCs and determine their numbers using TNAP activity as a marker (figure 1.2.). On the first day of gastrulation approximately 50-100 PGCs form a clump at the base of the developing allantois posterior to the primitive streak (fig. 1.2a). From here they disperse and the majority become associated with the endoderm by 8dpc (fig. 1.2b). By 10.5dpc the PGC population has started to translocate from the hindgut to the genital ridges (fig. 1.2c-e) where
they start to express the germ cell-specific marker *mouse vasa homologue (Mvh)* (Fujiwara *et al.*, 1994). The population of genital ridge PGCs consists of about 26000 cells by 13.5dpc (Tam & Snow, 1981). At this stage PGCs enter either mitotic arrest in the testis or meiosis in the ovary.

![Early mouse embryonic development](image)

**Figure 1.1 Early mouse embryonic development** Following ovulation the ovum enters the infundibulum and is fertilised in the ampulla. Totipotent embryonic cells divide symmetrically until the morula compacts around 3dpc. The first differentiation events occur with blastocyst formation at 3.5dpc. Adapted from Hogan, Constantini & Lacy (1986).
Following fertilisation the embryonic genome undergoes extensive demethylation which is substantially complete by the time the pre-implantation blastocyst is formed (approximately 3.5dpc). The genome is differentially re-methylated after implantation (Monk et al., 1987), potentially shaping cell lineage specification (Reik et al., 2001). Methylation of the genome in PGCs persists for longer than in other cell types and erasure is not complete until they have colonised genital ridges by 13-14dpc. Methylation is also implicated in modification of gamete-specific imprinted genes (Li et al., 1993). Imprinted genes are exempt from the global demethylation that occurs prior to implantation (Tremblay et al., 1997; Shibata et al., 1998).
Imprinted genes are eventually de-methylated in PGCs by the time they have colonised the genital ridge. The germ cell genome is then re-marked in a sex-specific pattern during gametogenesis.

1.2.2 Chick development

The development of a chicken embryo from fertilisation to oviposition occurs in the oviduct as shown in figure 1.3.

A laying hen typically ovulates once each day. Shortly after ovulation fertilisation occurs in the infundibulum of the oviduct. The ovum progresses through the oviduct...
and rapid cell division begins approximately three hours post fertilisation. The albumen is laid down in the magnum during the next four to five hours and the shell membrane is synthesised in the isthmus. Over the next 19-20 hours the eggshell is laid down over the membrane in the shell gland until the egg is laid 20-24 hours after fertilisation. The early stages of chick development have been comprehensively characterised (Eyal-Giladi and Kochav, 1976). Development was divided into fourteen morphologically discrete stages denoted by a Roman numeral. Frequent reference will be made to the stage X blastoderm. This is the approximate embryonic stage of the freshly laid egg. Subsequent to stage XIV, embryonic development through to hatch has been described in 24 stages, denoted by Arabic numerals (Hamburger and Hamilton, 1951).

Chicken PGCs first become identifiable by morphology in the germinal crescent (fig. 1.4a). From here they are carried passively through the lateral extraembryonic and the intra-embryonic circulation until proximal to the germinal ridge (fig. 1.4b; Swift, 1914; Meyer, 1964). PGCs leave the blood vessels by active migration and travel into the genital ridges (fig. 1.4c). PGCs can only be identified by their morphology when they reach the extraembryonic region known as the germinal crescent. Characterisation of the chicken vasa homologue (Cvh) has made it possible to trace the germ lineage from fertilisation to the formation of mature germ cells (Tsunekawa et al., 2001). CVH protein is maternally inherited by cells that give rise to the germ line. Using immunocytochemistry, PGCs could be identified at every developmental stage. This demonstrated that germ cell fate is maternally specified and not induced
by cell-cell interactions as occurs in mouse PGCs. Homologues of *vasa* are expressed in the germ cells of many other species (see 1.5.4).

Figure 1.4 Chick PGC development  
a) PGCs (red circles) are present in the area pellucida of the stage X blastoderm, b) PGCs are identifiable by morphology in the anterior germinal crescent approximately 18 hours after the egg is laid, c & d) between 24 and 48 hours PGCs enter the extraembryonic vascular system and passively migrate until they reach the embryonic gonad, e) the final stage of migration occurs by as PGCs actively colonise the genital ridge. Adapted from Nieuwkoop and Sutasurya, 1979.

The role of methylation in transcriptional regulation during chick development is unclear. Although methylation is associated with gene silencing in mice, there are reports supporting (Razin *et al.*, 2000) and opposing (Wolfi *et al.*, 1991) a similar
role for methylation in chickens. In addition, an imprinting mechanism has not been identified in chickens (Nolan et al., 2001).

1.3 Transgenic animals

There are several methods used to produce transgenic animals. The three most common strategies are: 1) transduction, transfection or DNA microinjection of early embryos and oocytes, 2) transduction or transfection of pluripotent cells prior to reintroduction to early embryos and 3) transduction or transfection of cells prior to NT.

1.3.1 Direct manipulation of embryos and oocytes

The introduction of genetic modifications at the single cell stage has several benefits for transgenic animal production. If cloned gene constructs integrate into the host genome before DNA replication then they are transmitted to every cell. Founder animals are produced without the time-consuming requirement to mate chimaeric animals (see 1.3.2). The range of genetic modifications is however restricted compared to in vitro cell-based manipulations. In most laboratory species, embryos are infrequently available in high numbers in contrast to cultured cells. In addition, rare events such as HR cannot be identified by drug selection as it is not practical to apply selection to embryos transfected with cloned gene constructs.

1.3.1.1 Methods for the genetic manipulation of mice

It has been possible to create transgenic mammals for more than 25 years. Transgenic mice were first created by transflecting mouse embryos with Moloney
leukaemia virus (Jaenisch, 1976). The virus integrated into the host genome and was transmitted to offspring. Although this breakthrough established the field of animal transgenics, the use of replication competent viruses has too many safety considerations for broad application.

Injection of DNA constructs into the mouse pronucleus has been used to create transgenic mice (Gordon et al., 1980). Fertilised oocytes containing unfused male and female pronuclei were dissected from the oviduct and the cumulus cells were enzymatically removed. Eggs were secured with a holding pipette and DNA was injected directly into the male pronucleus. Injected eggs were then transferred to the oviduct of a recipient foster mother where they continued to develop normally. Pronuclear injection in mice has a success rate of about 15% of oocytes surviving through to birth (Brinster et al., 1985; Page et al., 1995). Chromosomal integration of injected DNA is frequent and typically 10—25% of surviving pups are transgenic (Gordon & Ruddle, 1981; Constantini & Lacy, 1981; Brinster et al., 1985). However, a disadvantage to pronuclear injection is the unpredictable expression of foreign sequences following integration of injected DNA. The site of transgene integration and copy number is random and can influence tissue specificity and the overall level of expression. Transgene constructs also tend to integrate as multiple copy tandem arrays that are susceptible to methylation (Mehtali et al., 1990). Methylation of DNA is known to suppress transcription (Cedar, 1988). A further complication is position effect variegation where heterochromatic factors lead to mosaic transgene expression.
1.3.1.2 Methods for the genetic manipulation of chickens

Transgenic chickens were first created by retroviral-mediated gene transfer into the stage X blastoderm (Salter et al., 1986). A “window” was made in the shell of a freshly laid egg and replication competent avian leukosis virus (ALV) was injected into the subgerminal cavity. The window was sealed and the egg incubated through to hatch. Mosaic birds carrying the randomly integrated virus were produced and transmitted the transgene through the germline to produce hemizygous G1 offspring. This technique is of limited usefulness as transgenic birds carry and produce live virus. To overcome this problem replication-defective retrovirus was produced for subgerminal injection (Bosselman et al., 1989). Approximately 38% of injected embryos hatched and the transgene was detected in the semen of 8% of hatched male birds. Male birds carrying the transgene in semen transmitted vector DNA to 2-8% of offspring. Transmission of vector DNA from G1 offspring was not described. Germline transmission of the bacterial β-lactamase gene introduced by ALV has been demonstrated (Harvey et al., 2002a & b). Approximately 23% of injected embryos survived to hatch and vector DNA was detected in the semen of approximately 5% of hatched roosters. Expression of β-lactamase was detected at stable levels (1.9-6.7μg/ml) in the egg whites from G2 birds derived from a single transgenic rooster for more than a year. A disadvantage of retrovirus-mediated transgenesis is that only relatively small lengths of DNA (4-5kb) can be packaged.

DNA injection into the germinal disc of a fertilised ovum has also been used to produce transgenic birds (Love et al., 1994; Naito et al., 1994a). Injected eggs were subsequently cultured in surrogate shells through to hatching using an ex vivo culture
system (Perry, 1988). A disadvantage of this system is that DNA cannot be directly introduced to pronuclei as the cloudy cytoplasm prevents visualisation in ovo. The procedure is also complicated by polyspermic fertilisation which prevents positive identification of either the female or contributing male pronuclei (Perry, 1987). Consequently, foreign DNA can only be injected into the germinal disc cytoplasm of the zygote and tends to be transiently expressed. As incubation continues DNA is detected in a decreasing proportion of embryos (Perry et al., 1991). Germline transmission from a transgenic cockerel obtained by this technique has been demonstrated (Love et al., 1994). The efficiency of the technique is low with less than 1% of injected ova yielding transgenic founders. This approach does have the significant advantage over retrovirus-based strategies that much larger DNA constructs (>10kb) can be introduced. DNA constructs incorporating sequences from the Drosophila transposable element mariner have been injected subgerminally to produce transgenic birds (Sherman et al., 1998). Mariner encodes a transposase which directs its own transposition. Higher frequencies of genomic integration and germline transmission were observed when mariner was used.

A variety of approaches have been taken to introduce DNA into chicken embryos at later stages of development. Transient expression of injected DNA was observed following subgerminal injection of liposome-DNA complexes into the stage X blastoderm (Rosenblum & Chen, 1995). Liposome-DNA complexes have been injected directly into the marginal veins of embryos at stages 11-15 (Watanabe et al., 1994). Migratory PGCs are circulating at this stage and two-three days after injection transgene expression was detected in gonadal PGCs. However, only short-
term expression was reported. Germinal crescent PGCs have been transfected by bombardment with DNA-coated microparticles (Li et al., 1995). Transgene DNA was detected in the sperm of founder males and transmitted to offspring. However, vector DNA was undetectable as G1 chicks grew older suggesting it had persisted episomally. Transient expression of transgenes has also been observed following electroporation of stage 11-12 embryos (Muramatsu et al., 1997).

Sperm has been proposed as a vehicle for the delivery of cloned gene constructs to oocytes. Sperm-mediated gene transfer is an attractive approach for several reasons. During fertilisation the biological function of spermatozoa is to deliver DNA into the ovum. They would therefore appear to be ideal vectors for the delivery of transgenes. Incubation of sperm cells with plasmid DNA prior to artificial insemination has yielded transgenic mice (Lavitrano et al., 1989). However, an attempt to reproduce this approach in mice and apply it to chickens has been unsuccessful (Gavora et al., 1991). Lipofection and electroporation have both been used to increase the uptake of DNA by sperm cells. Approximately 60% of sperm retained plasmid DNA following electroporation, however DNA entry was coincident with a loss in fertility (Nakanishi & Iritani, 1993). Lipofection of sperm with exogenous DNA does not appear to reduce fertility and transfected sperm successfully contribute to transgenic offspring (Rottmann et al., 1992; Nakanishi & Iritani, 1993). Vector DNA was detectable at decreasing levels in offspring indicating episomal persistence. Transgenes have been detected by PCR in founders and two subsequent generations following sperm-mediated gene-transfer (Squires &
However the genes did not appear to have stably integrated chromosomally as a Mendelian segregation pattern was not observed.

PGCs can be used to generate chimaeric chickens by direct transfer to a recipient bird. PGC chimaeras were first produced by injection of turkey germinal crescent PGCs into the vasculature of recipient chick embryos (Reynaud, 1969). PGCs marked with a retrovirus have been used to produce transgenic chimaeras (Simkiss et al., 1989). DNA from donor PGCs was detected in the gonads of approximately 36% of embryos which were sacrificed after 17 days. A similar strategy was used to produce live offspring, however they did not show germline transmission of donor PGC-derived feather colour markers (Petitte et al., 1991). Depletion of endogenous migratory PGCs in recipient birds by busulphan treatment (Vick et al., 1993a) and blood removal (Naito et al., 1994b) prior to PGC transfer successfully overcame this problem resulting in germline chimaeric birds. These experiments demonstrated that PGCs retain the ability to migrate and develop normally in a recipient bird after removal and transfer from a donor. Exogenous DNA sequences have been transfected into PGCs by a variety of methods including lipofection (Naito et al., 1998), electroporation (Hong et al., 1998b) and using replication defective retroviruses (Vick et al., 1993b). However, episomal transgene expression has been a common feature of these studies and germline transmission has only been observed following viral transduction (Vick et al., 1993b).
1.3.2 Pluripotent Stem Cells

Pluripotency is defined as the capacity to give rise to all embryonic cell lineages including the germline. Stem cells with this property can be manipulated in vitro and returned to the early embryo where they participate in normal development.

1.3.2.1 Embryonal carcinoma cells

Murine embryonal carcinoma (EC) cells were isolated from spontaneously occurring germ cell tumours termed teratocarcinomas (Kleinsmith & Pierce, 1964). Spontaneous germ cell tumours are particularly prevalent in some inbred strains of mice. Testicular tumours are observed at elevated frequency from strain 129 mice (1% in most substrains, Stevens, 1973). A high incidence of ovarian tumours is observed in strain LT mice (Damjanov et al., 1975) which have a common origin with strain 129 mice. Testicular teratocarcinomas can be experimentally induced by grafting male genital ridges to ectopic locations such as the kidney capsule. Teratocarcinomas can also be induced by grafting early embryos into ectopic locations. Embryo-derived teratocarcinomas can be derived from most inbred strains but testicular teratocarcinomas are restricted to certain strains e.g. strain 129.

Germ cell tumours can be broadly grouped in to two types: benign teratomas which are composed of a wide variety of differentiated tissue types and malignant teratocarcinomas which contain an additional undifferentiated EC cell population. Undifferentiated EC cell lines can be isolated from teratocarcinomas and propagated indefinitely in culture. The progenitors of EC cells originate in the epiblast (Diwan & Stevens, 1976) and the ability to differentiate is retained by EC cells during in
vitro proliferation. Human EC cells have been isolated from spontaneously occurring human germ cell tumours and maintained in culture (Andrews, 1988). Like murine EC cells, human EC cells retain the capacity to differentiate in vitro.

When feeder cell-dependent murine EC cells were grown in suspension in the absence of feeder cells they formed aggregates. The cells on the outer surface of these aggregates frequently differentiated into endoderm and the centrally located cells differentiated into a variety of cell types (Martin & Evans, 1975a; Martin et al., 1977). Differentiated EC cell aggregates are referred to as embryoid bodies (EBs) as they share some morphological similarities with 6dpc embryos. However, there are important differences such as the presence of parietal endoderm cells in EBs which is not present in the 6dpc embryo. The differentiation capacity of EC cells has also been characterised by reintroduction of EC cells into recipient mice. EC cells differentiate in vivo to give rise to teratomas containing differentiated cell types. Teratomas show improved growth when recipient mice with a reduced immune capability are used as hosts (Damjanov et al., 1982). The developmental potential of murine EC cell lines has also been studied by injecting murine EC cells into blastocysts (Brinster, 1974). Injected blastocysts were transferred to the oviduct of recipient foster mothers where they continued to develop. Murine EC cells were incorporated into the developing embryo and contributed to a range of cell lineages. Inbred mouse strains are homozygous at loci controlling coat colour. By selecting blastocysts of a different strain to donor cells, chimaerism can be observed by the contribution of murine EC cells to coat colour. Murine EC cell lines contribute to a range of differentiated cell types (reviewed by Martin, 1983) and there is one report
of contribution to the germline (Stewart & Mintz, 1981). EC cells typically have an abnormal karyotype, which is likely to prevent progression through meiosis to produce mature, functional gametes (Smith, 2001).

Early murine EC cell lines were often maintained by feeder cells and this co-culture was observed to have a positive effect on maintenance of an undifferentiated phenotype (Rosenthal et al., 1970; Evans, 1972). Murine EC cell lines with a broader differentiation potential were isolated in the presence of feeder cells (Martin & Evans 1975b, Martin et al., 1977). This suggested that undefined factors with a positive effect on multipotency were being produced by feeder cells.

1.3.2.2 Embryonic stem cells

The isolation of epiblast-derived EC cells suggested that pluripotent cells could be isolated directly from preimplantation mouse embryos. In 1981 cells resembling EC cells were derived from pre-implantation blastocysts (Evans & Kauthian, 1981; Martin, 1981).

The protocol developed by Evans & Kaufman, (1981) involved the collection of delayed implantation blastocysts. Delayed implantation was achieved by removal of maternal ovaries at 2.5dpc followed by subcutaneous injection of progesterone. Delayed implantation results in expansion of epiblast cells in vivo. Blastocysts were recovered and cultured on layers of mitotically inactivated feeder cells. After successive passages, feeder cell-dependent cell lines with an undifferentiated morphology were isolated. Feeder cells appeared to provide factors which
maintained ES cells in the undifferentiated state. Unlike EC cells, these cell lines were largely karyotypically normal and unlike EC cells commonly contained a Y chromosome. There appears to be selective pressure against XX ES cells in vitro and female ES cells frequently carry X chromosome abnormalities (Robertson et al., 1983; Rastan & Robertson, 1985). ES cells differentiated in vivo to form teratocarcinomas when injected into syngeneic mice. In vitro, ES cells formed EBs (see 1.3.2.1) when grown in suspension and subsequently differentiated into ectodermal cells.

Martin, (1981) used 3.5dpc blastocysts in ES cell isolation experiments. Blastocysts were treated with an antibody capable of binding all cells. However the tight gap junctions between trophectoderm cells on the outer surface prevented antibody access to the centrally located ICM cells. After washing, embryos were treated with complement which caused lysis of the antibody-bound trophectoderm cells. The removal of trophectoderm cells by this method is termed immunosurgery (Solter & Knowles, 1975). Isolated ICMs were cultured on a layer of fibroblast feeder cells and grown in medium that was conditioned by an EC cell line. This conditioned medium was hypothesised to contain factors that promoted the expansion of undifferentiated cells. ES cell lines were derived from the ICMs of normal embryos and dependency on conditioned medium was lost after five passages. Higher numbers of blastocysts were required as starting material than by Evans & Kaufman (1981), possibly because of the smaller population of undifferentiated cells in normal blastocysts compared to delayed implantation blastocysts. The ES cell lines were found to have a high capacity for differentiation when injected into athymic mice.
where they formed teratocarcinomas containing representative cells of the three germ layers. In the absence of feeder cells ES cells formed EBs which produced a variety of differentiated cell types.

Provided certain conditions are met ES cells can be maintained in culture indefinitely without any obvious change in phenotype. This is called self-renewal and is one of the defining properties of ES cells. For self-renewal ES cells are dependent on the cytokine LIF, (Smith et al., 1988; Williams et al., 1988; see 1.5.1.1.). ES cells can be cultured indefinitely in the presence of LIF.

The nuclei of murine ES cells frequently contain multiple, prominent nucleoli and characteristically are large relative to cytoplasmic volume (Abbondanzo et al., 1993). Murine ES cells express early embryonic cell surface markers: stage-specific embryonic antigen-1 (SSEA-1; Solter & Knowles, 1978) and stage-specific embryonic antigen-7 (ECMA-7; Kemler et al., 1981). Murine ES cells also have high endogenous TNAP activity which is lost during differentiation (Wobus et al., 1984). During early embryogenesis the genome-wide pattern of methylation observed in adult cells is largely lost by the blastocyst stage (Monk et al., 1987). ES cells also show this lack of genomic methylation that is increased during differentiation (Weng et al., 1995). However, ES cells maintain methylation patterns associated with imprinted genes (Labosky et al., 1994).

The differentiation potential of ES cells can be studied in vitro or in vivo. ES cells grown in suspension in the absence of LIF form EBs (Evans & Kaufman, 1981;
Martin 1981). When EBs were allowed to attach to tissue culture grade plastic they differentiated to form a variety of cell types. Cell lineages were then identified by morphology or by immunostaining with ectoderm specific antibodies (Evans & Kaufman, 1981).

The differentiation potential of murine ES cells has also been investigated in vivo. Injection of ES cells into immuno-compromised mice resulted in the formation of teratocarcinomas (Evans & Kaufman, 1981; Martin, 1981). The cell-lineages contained within these tumors give an indication of the differentiation potential of the ES cell line. When injected into blastocysts, ES cells contributed to the developing embryo (summarised in figure 1.5; Bradley et al., 1984). ES cells were isolated from cultured blastocysts (fig. 1.5a & b). Blastocysts were collected at 3.5dpc and secured with a holding pipette. ES cells were injected between the trophectoderm cell junctions into the blastocoel cavity with a fine glass needle (fig. 1.5c). Injected cells then incorporated into the ICM. Blastocysts were transferred to the uterus of a pseudo-pregnant female (fig. 1.5d) where embryonic development proceeded. Pseudo-pregnant female mice were physiologically prepared as blastocyst recipients by mating with vasectomised male mice. During embryonic development, ES cells contributed to the three germ layers and crucially the germline. By mating chimaeric male mice (fig. 1.5e), ES cell derived offspring, identifiable by coat colour, were derived (fig. 1.5f).
Figure 1.5 Production of mice using ES cells

a) & b) ES cell lines are derived from harvested blastocysts, c) ES cells are injected into blastocysts from a different inbred strain, represented in grey, d) reconstituted blastocysts are transferred to pseudopregnant female mice of another strain (white), e) chimaeric offspring are identifiable by coat colour, f) chimaeric mice may produce entirely ES cell-derived offspring if ES cells are germline competent.
An alternative method of producing chimaeric mice from ES cells is the aggregation technique (Wood et al.; 1993). Morulae were recovered from pregnant mice at 2.5dpc and the zona pellucida was removed by incubation in acid Tyrodes buffer. Small clumps of ES cells were overlaid on the morulae and the mixed morula/ES cell culture was allowed to aggregate overnight in vitro. The aggregated embryo formed a blastocyst that was transferred to a surrogate female the following day. The aggregation technique is less demanding than blastocyst injection in terms of expertise and equipment but as embryos spend 24 hours in vitro a more rigorous tissue culture regime is required.

Tetraploid rescue is a modified version of the aggregation technique which in some instances can give rise to 100% ES cell derived mice (Nagy et al., 1993). A brief electrical pulse is applied to a two-cell embryo which fuses, resulting in the formation of a tetraploid one-cell embryo. A tetraploid embryo is capable of preimplantation development and can be used in aggregation at the morula stage. In aggregated embryos the tetraploid-derived component contributes almost exclusively to extraembryonic tissues and the ES cell-derived component gives rise to the majority of the embryo proper (Nagy et al., 1990).

It is possible to genetically alter ES cells without the loss of pluripotency. Strategies used to achieve this can be broadly divided into two: random and targeted integration. DNA constructs that integrate at random sites are subject to the same inconsistent expression patterns observed with pronuclear microinjection. This approach only allows the addition of transgenes. Modification of existing genes
requires the use of gene targeting technology. Gene targeting constructs include regions of homology to the endogenous target locus that direct integration of the construct by HR. In practice, constructs also integrate randomly and a selective method is required to identify correctly targeted cells. A popular strategy is to use a promoterless antibiotic resistance gene such as neomycin phosphotransferase (neo) which is expressed from an endogenous promoter following HR and confers resistance to geneticin in mammalian cells. This approach is only appropriate for genes expressed in ES cells. Where the target gene is not expressed, the likelihood of correctly identifying a targeted clone can be increased by the use of positive-negative selection (Mansour et al., 1988; figure 1.6). In addition to selecting for a homologous integration event using the neo gene, selection was also applied against random integration. This was achieved by the inclusion of a negatively selectable marker (HSVtk) outside the region of homology. HSVtk confers sensitivity to the drug ganciclovir. Both selectable markers have active promoters, polyadenylation sites and the appropriate sequences to direct expression following integration. Following HR the presence of the neo gene allowed cells to be enriched in geneticin (fig 1.6a). However the neo gene was also retained by cells that had undergone random integration (fig. 1.6b). Random integrants also contained the tk gene and were depleted by ganciclovir selection. In practice, positive-negative selection rarely enriches the frequency of targeted clones by more than 10-fold (e.g. Mombaerts et al., 1991). Clones of antibiotic resistant cells are expanded and analysed by Southern blot to identify cells carrying desired modifications. A modified ES cell clone can be used to create transgenic mice via chimaeric intermediates. This
technique has been widely used to create “knockout” mice where gene function is studied following ablation of one or both copies of a gene.

Figure 1.6 The positive negative selection strategy. The targeting vector contains a positive selectable marker, neomycin (neo, orange), within the region of homology. A negative selectable marker, thymidine kinase (tk, green), is placed out with the region of homology, a) following HR, neo is integrated and tk is lost. Cells expressing neo are resistant to geneticin, b) following random integration the neo and tk genes will usually be integrated. Cells that express tk are sensitive to ganciclovir. Selection in geneticin (positive) and ganciclovir (negative) will enrich for cells that have undergone HR (a) and deplete cells that have undergone random integration. Adapted from Hooper (1992).
Knockout mice have been used to study gene function in a wide range of medical conditions including cancer (e.g. Donehower et al., 1992), immune disorders (e.g. Liao et al., 1991) and inherited genetic conditions (e.g. cystic fibrosis, Dorin et al., 1992). Other applications of knockout mice include characterisation of gene function in development (e.g. Nichols et al., 1998).

Using standard protocols (Robertson, 1987), ES cells can only be readily isolated from a limited number of mouse strains. ES cells have been isolated from strain C57BL/6 (Ledermann & Burki, 1991), BalbC (Kawase et al., 1994) and DBA (Roach et al., 1995), however, the vast majority of ES cell lines in common use are strain 129-derived. Many EC cell lines are also derived from strain 129 (Martin, 1983) which has a high incidence of spontaneous testicular teratocarcinoma (see 1.3.2.1.). Other mouse strains e.g. CBA are refractory to ES cell isolation using standard techniques. ES cells have been isolated from blastocysts with an 87.5% CBA-derived genotype using antibiotic selection to remove differentiating cells (McWhir et al., 1996). Transgenic mice carrying a DNA construct consisting of the \textit{neo} gene driven by the \textit{oct4} promoter (Octneo) were created by pronuclear injection. Octneo mice had a 50% CBA-derived genetic background. Following successive breeding steps transgenic Octneo embryos with an 87.5% CBA derived genetic background were derived. Octneo embryos were collected at the blastocyst stage and ES cell isolation was performed in the presence of G418. The \textit{oct4} promoter is expressed in undifferentiated cells (Pesce et al., 1998; see 1.5.2.) and is tightly downregulated following differentiation. Under selective ablation conditions, non-
ES progenitors were killed by antibiotic selection and G418-resistant ES cells proliferated.

ES cell isolation from CBA blastocysts was also achieved by the microsurgical removal of differentiating cells (Brook & Gardner, 1997). By removing primitive endoderm from delayed implantation strain 129 blastocysts it was demonstrated that ES cells are derived from the epiblast compartment of the embryo. Microsurgical removal of non-epiblast cells increased the frequency of ES cell isolation to 100%, demonstrating the negative influence of non-ES cell progenitors in strain 129 mice. ES cell lines were isolated from microsurgically isolated epiblasts from several mouse strains including CBA.

Schoonjans et al. (2003), reported ES cell isolation from various strains of mouse blastocyst including CBA/CaOla. Rabbit fibroblast cells transfected with rabbit genomic LIF were used to condition medium for early ES cell cultures. Using conditioned medium, ES cell lines were derived at high (>40%) frequency from seven different mouse strains. The CBA/CaOla mouse strain has not however been fully characterised and the contribution of Ola to this inbred background has not previously been characterised in ES cell isolation experiments.

It is interesting that two of the reports of CBA ES cell isolation describe the removal of non-ES cell progenitors (McWhir et al., 1996; Brook & Gardner et al., 1997). These non-ES cell progenitors may exert a negative effect on ES cell self-renewal by cell-cell signalling. Despite being used to successfully isolate CBA ES cells neither
of these approaches has been used to isolate non-murine ES cells (e.g. Buehr et al., 2003).

Isolation of murine ES cells stimulated researchers to attempt isolation from other rodents and rabbits. Cell lines with an undifferentiated morphology were isolated from Syrian hamster blastocysts (Doetschman et al., 1988). These cell lines formed EBs in suspension culture and differentiated into embryonic ectoderm and myocardium in vitro. The in vivo differentiation potential of these cells was not described. Rats are a popular laboratory animal with many applications in the fields of neuroscience and psychology. Rat ES cells would greatly enhance the range of available applications. The isolation of rat ES cells has previously been reported (Iannaccone et al., 1994), however this report was later retracted (Iannaccone et al., 1997). Other attempts to isolate rat ES cells have also been unsuccessful (Ouhibi et al., 1995). This is possibly a consequence of rapid Oct4 downregulation observed in primary cultures derived from rat ICMs (Buehr et al., 2003). Blastocyst-derived cell lines have been established from the rabbit and differentiate via EBs into the three germ layers (Graves & Moreadith, 1993). Rabbit ICM-derived cells were injected into blastocysts to yield coat colour chimaeric offspring but germline transmission was not observed (Schoonjans et al., 1996).

Fish species e.g. zebrafish are increasingly popular organisms for the study of developmental biology. They have a short generation interval and produce large numbers of embryos which also makes them ideal candidates for transgenic applications. Consequently ES cell isolation has been attempted from a variety of
fish species. Cell lines derived from medaka fish embryos showed many of the characteristics of mouse ES cells including high TNAP activity and the ability to differentiate in vitro following EB formation (Hong et al., 1996). These cell lines contributed to the three germ layers in chimaeras but colonisation of the germline was not shown (Hong et al., 1998b). Cells derived from zebrafish embryos also exhibit TNAP activity and can differentiate in vitro to form neural cell types (Sun et al., 1995). Contribution to the germline in chimaeras by primary embryonic cells co-cultured with a trout spleen feeder cell line has been demonstrated (Ma et al., 2001).

Expression of the germ cell marker vasa in embryonic cell cultures correlates with germline competence and appears to be maintained by co-culture with the trout feeder cells. Contribution to the germline in chimaeras following long term culture was not described.

The commercial importance of the livestock industry and the potential for xenotransplantation and pharmaceutical protein production has led to attempted ES cell isolation from ungulates. Cells with an ES-like morphology have been isolated from pig embryos at early (Evans et al., 1990; Notarianni et al., 1990) and at later stages of development (Strojek et al., 1990). Porcine embryo-derived cell lines are capable of differentiation in vitro (Evans et al., 1990; Notarianni et al., 1990) and generate teratocarcinomas in vivo (Hochereaudereviers and Perreau, 1993). Contribution of undifferentiated porcine cell lines to chimaeras has been demonstrated, however no germline contribution was observed (Wheeler, 1994).

Cells with a similar morphology to murine ES cells have been isolated from bovine morulae and blastocysts (Saito et al., 1992; Stice et al., 1996). These cells were
observed to differentiate in vitro and contributed to embryonic development in vivo when used as donors in NT experiments. No embryos developed to term. There have also been reports of ES-like cells derived from pre-implantation caprine embryos (Meinnecke-Tillmann & Meinecke, 1996) and equine embryos (Saito et al., 2002).

Phenotypic consequences of genetic abnormalities have been extensively studied in mice. This has been largely possible because of the range of genomic manipulations through gene targeting technology. Primates would provide a phylogenetically closer model of human disease and this has encouraged efforts to isolate primate ES cells. Thomson et al., (1995) isolated ES cells capable of continual, feeder cell-dependent culture from the rhesus monkey. These cells strongly expressed TNAP and the cell surface markers SSEA-3 and SSEA-4. Rhesus monkey ES cells were capable of differentiating into trophoblast and endoderm in vitro and when injected into severe combined immunodeficient (SCID) mice gave rise to tumours containing the three germ layers. ES cell lines with very similar features to rhesus monkey were isolated from the common marmoset by the same group (Thomson et al., 1996). Contribution of primate ES cell lines in chimaeras has not been reported.

The relative ease with which primate stem cell lines were derived led to attempted ES cell isolation from human blastocysts. Thomson et al., (1998) reported the isolation of human ES cells which had high levels of telomerase activity and carried many of the markers expressed by primate ES cell lines. These cells grew
continuously in a feeder dependent manner and spontaneously differentiated into endoderm and trophoblast in the absence of feeders or when overconfluent.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell markers</th>
<th>Differentiation</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>SSEA-1, ECMA-7, SSEA-3, TNAP</td>
<td>in vitro, tumors, chimaeras, germline</td>
</tr>
<tr>
<td>Rabbit</td>
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<td>Chicken</td>
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<td>in vitro, chimaeras, germline</td>
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<tr>
<td>Zebrafish</td>
<td>TNAP</td>
<td>in vitro, Chimaeras, Germline</td>
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Table 1.1 Properties of embryo-derived cells
Embryo-derived cells with similar properties to mouse ES cell lines have been isolated from several species. All of these cells are TNAP positive. The capacity for differentiation of these cells is measured by increasingly stringent assays. In vitro differentiation has been observed in all species listed as has in vivo differentiation in chimaeras. Germline transmission following long term culture has only been demonstrated in murine ES cells.
Human ES cells formed derivatives of the three germ layers in tumours when injected into SCID mice. Human ES cells grown in suspension culture form EBs (Itskovitz-Eldor et al., 2000). EBs differentiated in vitro to express molecular markers of the three germ layers as detected by reverse transcription polymerase chain reaction (RT-PCR). Human ES cells are dependent on either feeder cells or conditioned medium for the maintenance of an undifferentiated phenotype, however the factors responsible have not yet been identified. The range of applications proposed for these cells is enormous including disease models, tissue repair, cell transplantation and drug discovery.

The properties of embryo-derived cells from a range of species is summarised in table 1.

1.3.2.3 Embryonic germ cells

A third pluripotent cell type can be derived when murine PGCs are cultured in the presence of a cocktail of growth factors. Murine PGCs de-differentiate in vitro to become pluripotent cells, called embryonic germ (EG) cells. EG cells share many of the features of ES cells including the ability to contribute to the germline in chimaeras.

PGCs from a male genital ridge, particularly in strain 129 mice, have the capacity to form teratocarcinomas in ectopic locations. The isolation of EC cells from teratocarcinomas (Kleinsmith & Pierce, 1964) suggested undifferentiated cells could be derived directly from PGCs. Although PGCs survive in culture they have a highly
differentiated morphology and have limited proliferation potential. It was demonstrated that if PGCs were obtained before midgestation and grown in the presence of basic fibroblast growth factor (bFGF), stem cell factor (SCF) and LIF they could be induced to proliferate (Matsui et al., 1992; Resnick et al., 1992). These cells were termed EG cells and had many similarities to ES cells such as endogenous TNAP and SSEA-1 expression (Matsui et al., 1992; Resnick et al., 1992). EG cells retain the capacity to differentiate in vitro via EBs and in vivo, forming tumours in immunodeficient mice. EG cells also contribute to chimaeras following blastocyst injection (Matsui et al., 1992). EG cells isolated from 8.5dpc PGCs can form functional gametes that are transmitted by chimaeras (Labosky et al., 1994; Stewart et al., 1994).

The major difference between murine ES and EG cells is their imprinting status. Imprinting is the process by which sex-specific genes are repressed and is associated with methylation of certain DNA sequences. In ES cells and EG cells derived from 8-8.5dpc PGCs the parentally derived imprint is maintained but in EG cell lines derived from genital ridge PGCs (approximately 13dpc) the imprint has been lost. EG cell lines that maintain the parental imprint contribute to the germline whereas germline transmission from EG cell lines derived form genital ridge PGCs has not been observed (Labosky et al., 1994). This suggests an essential role for the parental imprint in gametogenesis from pluripotent stem cells.

EG cells have been isolated from F1 hybrid mice (Matsui et al., 1992; Resnick et al., 1992) and C57BL/6 mice (Labosky et al., 1994). As with ES cell isolation, there
appears to be a strain barrier to EG cell isolation from CBA mice that can be overcome by the selective ablation method (Gallagher et al., 2003). Genital ridges were dissected from transgenic Octneo CBA mice and cultured in the presence of geneticin. Oct4 expressing PGCs, and subsequently EG cells, survived. Using this approach EG cells with a 94% CBA-derived genetic background were derived.

Cells with similar features to murine EG cells have been identified following culture of PGCs from pigs, chickens and humans. Porcine PGC-derived cells express TNAP activity, differentiate into a range of cell types in vitro and contribute to chimaeras following injection into blastocysts (Shim et al., 1997; Piedrahita et al., 1998). Putative EG cells have also been isolated from chickens (Park & Han, 2000). These cells are glycogen rich, express SSEA-1, and are capable of differentiation in vitro and in vivo (see 1.4.2.). Shamblott et al. (1998) isolated human EG cells from genital ridges after five to nine weeks of embryonic development. Human EG cells express TNAP and similar surface antigens to human ES cells. In addition they express SSEA-1, expressed by mouse but not human ES cells. Human EG cells retain the capacity to differentiate into the three germ layers in vitro via EB intermediates (Shamblott et al., 2001).

1.3.2.4 Adult stem cells

Although the existence of repopulating stem cells in adult animals has long been suspected, it is only recently that these cells have been characterised in detail. A common feature of adult stem cells (ASCs) is that they appear to be located in “niches”. From these “niches” ASCs produce daughter cells that migrate and divide
to restock populations of cells that have been depleted. Evidence from *Drosophila* suggests that niche cells regulate the temporal and spatial aspects of ASC division (Kiger et al., 2000; Tran et al., 2000).

ES cells are relatively easy to grow and study *in vitro* as a consequence of their unlimited growth potential. This has not been the case with ASCs which have proved difficult to propagate as pure populations in culture. ES cells depend on gp130 signalling for maintenance (see 1.5.1) and identification of equivalent ASC regulatory signals will greatly aid their characterisation.

Much ASC characterisation has used repopulation assays following the purification of tissue-specific populations. Populations of marked cells are purified on the basis of cell surface markers and introduced into a compromised host animal. The host animal is then examined for contributing marked cells following *in vivo* differentiation. This type of analysis has a number of weaknesses. Most importantly: 1) donor cells are not always clonally derived and 2) contributing cells are not assayed for functionality. More sophisticated analyses are beginning to address these criticisms.

ASCs that have been characterised include haematopoietic stem cells (HSCs; Spangrude et al., 1988; Osawa et al., 1996), neural stem cells (NSCs; McKay, 1997; Doetsch et al., 1999), mesenchymal stem cells (MSCs; Prockop, 1997; Pittinger et al., 1999) and epidermal stem cells (ESC.s; Watt, 2001). Although less clearly
defined than ES cells these ASCs have the ability to self-renew, are multipotent and can differentiate into functional derivatives in vitro.

ASCs were previously believed to be lineage-restricted. If mammalian development is viewed as a hierarchy (figure 1.7), starting with the zygote and ending with terminally differentiated cells, ASCs were assumed to have developmental
equivalence to partially differentiated cells. The assumption that ASCs cannot produce cells of different lineages has recently been seriously challenged. For example, cells of the ectodermal lineage have been derived from mesodermal HSCs (Eglitis & Mezey, 1997; Mezey et al., 2000) and transplantation of HSCs has restored tissue specific dystrophin expression in a mouse model of Duchenne’s muscular dystrophy (Gussoni et al. 1999). However, neither of these studies used clonally isolated cells or demonstrated HSC self-renewal. These criticisms have been partially addressed by serially transplanting single HSCs to derive haematopoietic and epithelial cells (Krause et al., 2001), and haematopoietic and endothelial cells (Grant et al., 2002).

Other stem cell populations have demonstrated developmental plasticity. NSCs (ectoderm derived) maintained in vitro can reconstitute the haematopoietic lineage (Bjornson et al., 1999) and hepatocytes (endoderm derived) can be derived from cells with a mesodermal origin following environmental damage (Peterson et al., 1999). Neither of these studies used clonally isolated cells for transplantation. Clarke et al. (2000) introduced murine NSCs into early chick and mouse embryos and found contribution to all three germ layers. Donor NSCs were derived from single neurospheres which are believed to be clonal, however this was not shown conclusively.

Multipotent adult progenitor cells (MAPCs) have recently been described and, if these results can be repeated, answer many of the criticisms of ASC research. MAPCs were originally isolated from rodent or human mesenchymal stem cell
cultures (Reyes et al., 2001; Reyes et al., 2002; Jiang et al., 2002a). These cells can be maintained for many generations *in vitro* and differentiate into the three germ layers *in vitro* or *in vivo* following single cell cloning. MAPCs have also been isolated from murine muscle and brain (Jiang et al., 2002b). However, the isolation of MAPCs has only been described from one laboratory.

An alternative explanation for the developmentally plastic properties of ASCs is spontaneous cell fusion. When murine somatic neural cells are co-cultured with murine ES cells spontaneous fusion occurs at a low level to generate hybrid cells (Ying et al., 2002; Pells et al., 2002). These cells were tetraploid and had a highly undifferentiated phenotype. Hybrid cells differentiated into the three germ layers *in vitro* and contributed to chimaeras following blastocyst injection. Spontaneous fusion between ES cells and bone marrow derived somatic cells has also been observed (Terada et al., 2002). These hybrid tetraploid cells were also highly undifferentiated and contributed to the three germ layers *in vitro* and in teratocarcinomas *in vivo*. Cell fusion has been observed *in vivo* (Wang et al., 2003; Vassilopoulos et al., 2003). In these experiments bone marrow-derived hepatocytes were found to be the result of cell fusion following liver repopulation.

The apparent developmental plasticity of ASCs suggests applications in the derivation of histocompatible cells for therapy or possibly organ culture. ES cell research is restricted in many countries because of ethical considerations and ASCs offer a promising alternative. The application of ASCs to the field of transgenic animals has not been explored. The applications of stem cells in transgenic animal
production are largely dependent on the germline properties of ES cells and there is no evidence for germline competency in ASCs. However, as ASCs are relatively undifferentiated, they may have applications in NT experiments, as undifferentiated nuclear donor cells appear to support the development of cloned animals at higher efficiency than terminally differentiated cells (Hochdelinger and Jaenisch, 2002b).

1.3.3 Nuclear transfer

The transfer of diploid somatic nuclei from genetically modified cells can also be used to produce transgenic animals. This approach has several parallels with stem cell-based approaches. Somatic cells are grown in culture where they can be manipulated either by transgene addition or by gene targeting. Modified cells are then used to provide nuclei for transfer.

Mammalian NT was first achieved in sheep by transplanting a blastomere nucleus into an unfertilised mature oocyte (Willadsen, 1986). Ovine NT was demonstrated using cultured embryo-derived cells as nuclear donors (Campbell et al., 1996). Donor cells were maintained and expanded in vitro and expressed markers of the differentiated state prior to NT. Successful cloning from a variety of differentiated cell lines was achieved by Wilmut et al., (1997). This report included the cloning of a sheep from an adult mammary epithelial cell line (figure 1.8). A cell line was isolated from the mammary gland of a Finn Dorset ewe and grown in low concentrations of serum to induce quiescence. Ova were removed from the ovaries of a Scottish blackface ewe and enucleated. By placing a diploid somatic cell adjacent to the enucleated oocyte and applying a brief electrical current the oocyte
and somatic cell membranes were fused. This allowed the diploid nucleus to enter and activated the oocyte by simulating fertilisation. Embryos created this way were maintained *ex vivo* until the morula stage before being introduced to a surrogate host where normal development proceeded.

**Figure 1.8 Mammalian cloning by NT** A primary cell line was isolated from the mammary gland of a Finn Dorset ewe. These cells were induced to enter quiescence before being fused to enucleated oocytes from a Scottish blackface ewe. After *ex vivo* culture, reconstituted embryos were returned to surrogate females where normal development proceeded.
For normal development following NT, the cell cycle of the donor nucleus must be synchronised with the oocyte. The cell cycle is controlled largely by the large family of cyclin-dependent kinases (CDKs). CDKs regulate transition through the cell cycle by forming complexes with cyclins and cyclin-dependent kinase inhibitors. Maturation promoting factor (MPF) is a CDK/cyclin complex of Cdc2 and cyclin B present at high levels in the meiotic oocyte. Following NT, MPF induces breakdown of the nuclear membrane and premature chromosome condensation (PCC). Subsequently, nuclear reformation and DNA re-replication occurs before cell division commences. Donor nuclei in G₁ or G₀ have a diploid chromosome complement (2n) and may produce cells capable of normal development (figure 1.9). Donor nuclei in S phase or G2 phase have a chromosome complement between 2-4n and result in an abnormal chromosome complement following DNA re-replication (figure 1.9). Additionally, the chromosomes of donor nuclei are substantially damaged by PCC (Schwartz et al., 1971; Collas et al., 1992).

When nuclei donor cells are starved of serum in vitro they exit the cell cycle and enter a state known as quiescence (G₀). Cells in quiescence have low levels of cyclins and CDKs and aberrations in hybrid cells are reduced when quiescent cells are used as donors. The induction of quiescence in nuclei prior to transfer was an important breakthrough in sheep cloning. Quiescence was believed to make the donor cell genome more responsive to reprogramming factors present in the ooplasm. During normal embryonic sheep development the zygotic genome is not activated until the 8-cell stage (Crosby et al., 1988). Following NT the donor nucleus is believed to be reprogrammed to a pluripotent state during this period.
Figure 1.9 Cell cycle of donor nuclei in NT

a) The cell cycle is divided into mitosis (M), G₁, S and G₂ phases. At the beginning of G₁ cells may leave the cell cycle and enter a state of quiescence (G₀). Cells in G₀ or G₁ have a diploid chromosome complement (2n), b) MPF induces breakdown of the nuclear membrane and premature chromosome condensation in donor nuclei. After nuclear reformation, DNA is re-replicated prior to cell division. In the case of donor nuclei in S phase the DNA is substantially damaged by MPF and only partial re-replication is possible. Only 2n donor nuclei (green) will have the correct chromosome complement following DNA re-replication.
If the nuclear donor cell can be modified, then NT provides an alternative method of introducing modifications to whole organisms. Transgenic sheep have been produced by transferring the nuclei of cells that had been genetically manipulated in culture (Schnieke et al. 1997). Primary fibroblasts were transfected with a human factor IX construct *in vitro* by lipofection. These cells were subsequently used as donors to produce transgenic sheep by NT. Sheep have also been produced by NT following gene-targeting of an endogenous locus (McCreath et al., 2000).

An advantage of NT over cell transfer is that one less breeding step is required as chimaeras are not created as intermediates. This is particularly relevant to some livestock species with long generation times. However, the technique has a low success rate and abnormalities in reconstituted embryos are common (Sinclair et al., 2000). Gene-targeting requires powerful strategies to identify desired clones due to the low frequency of HR in somatic cells compared to ES cells (Sedivy & Dutriaux, 1999). Somatic cell NT has been successfully performed in a variety of species including cattle (Cibelli et al., 1998), goats (Baguisi et al., 1999) and mice (Wakayama et al., 1998). NT has not been demonstrated in chickens.

1.4 **Pluripotent chicken cells**

Pluripotent chicken cells would provide a resource for introducing precise modifications to the chicken genome. Cells at two developmental stages are good candidates for the isolation of pluripotent cell lines: blastodermal cells and PGCs. Teratocarcinoma-derived murine EC cells were characterised in detail prior to murine ES cell isolation. Techniques were developed which gave investigators an
insight into the derivation and maintenance of undifferentiated cell lines. Spontaneous germ cell tumours have been reported at low frequency in chickens (Reviewed by Campbell, 1969) but unlike mammalian tumours they are always benign. These teratomas are typically composed of a mass of cells derived from the three germ layers however there are no reports of an undifferentiated component that can be subcultured to yield EC cells. ASCs have only recently been characterised in the mouse and there are no reports of functionally equivalent cells in chickens. There is no evidence of contribution to the germline from ASCs which would severely restrict their applications in transgenic animal production (see 1.3.2.4).

1.4.1 Blastodermal cells

By the time the egg is laid the stage X blastoderm consists of a disc of about 60,000 undifferentiated cells (figure 1.10). The blastoderm is visible on the surface of the yolk beneath the vitelline membrane which encloses the entire yolk (fig. 1.10a & b). When viewed under magnification, the area pellucida and the area opaca of the blastoderm are visible (fig. 1.10c). Cells of the area opaca are so called because of the opaque appearance given to them by contact with the yolk. Cells of the area pellucida appear clear because they are separated from the yolk by fluid secreted into the subgerminal cavity (fig. 1.10d).

Chicken blastodermal cell (CBC) embryo chimaeras were first generated by the transfer of clumps of CBCs between embryos (Marzullo, 1970). Live chimaeric offspring were produced by injecting disaggregated CBCs into the subgerminal cavity of host embryos (Petitte et al., 1990). Chimaeric offspring and germline
Figure 1.10 The stage X chick blastoderm  a) Top view of a freshly laid egg with a portion of shell removed. The stage X blastoderm is visible as a pale disc on the surface of the yolk, b) schematic representation of yolk and blastoderm, c) schematic representation of enlarged stage X blastoderm. The cells of the area pellucida (central, white) are separated from the yolk by the subgerminal cavity and appear clear. Cells of the area opaca (peripheral, cream) are in contact with the yolk and appear opaque, d) Schematic representation of stage X blastoderm cross section showing the subgerminal cavity which is filled with fluid secreted from blastodermal cells. At stage X the blastoderm is approximately 6mm in diameter.
transmission were identified by bird-strain specific feather pigmentation. Irradiation of the host embryo retards development of endogenous CBCs and confers an advantage on donor cell proliferation at early developmental stages. Irradiation of the recipient embryo increased the level of germline contribution of donor cells (Etches et al., 1993) and has yielded animals with a 100% donor-derived germline contribution (Carsience et al., 1993).

The capacity of CBCs to re-enter normal development when injected into the subgerminal cavity stimulated research into chicken ES cell isolation from the stage X blastoderm. Etches et al., (1996) described culture of stage X CBCs for periods of up to 48 hours without loss of germline competence. Cells were maintained in the presence of primary mouse fibroblasts and LIF, bFGF and SCF. Pain et al., (1996) maintained CBCs for over 160 days or 35 passages. These cells retained the capacity to colonise somatic tissues but failed to colonise the germline following more than seven days in vitro. This report comprehensively characterised CBCs isolated from the stage X blastoderm and grown with or without feeder cells. These CBCs had many similar characteristics to mouse ES cells were described, including TNAP activity, retinoic acid-induced differentiation and expression of epitopes recognised by ECMA-7, SSEA-3 and SSEA-1 (table 1). High telomerase activity was also recorded, although telomerase is widely expressed in chicken tissues (Venkatesan, 1998). TNAP expression appeared to be maintained by the presence of LIF and other interleukin-6 (IL-6) family cytokines. CBCs proliferated more rapidly in the presence of LIF; this effect was more marked in cultures maintained beyond seven days. Proliferation of TNAP-positive colonies was supported by a combination of
LIF, bFGF and SCF. Growth in the presence of STO feeder cells, which provide undefined growth factors, also enhanced the growth of TNAP positive colonies. These data suggest the requirement for a combination of factors for TNAP positive CBC proliferation. CBCs were routinely cultured in medium supplemented with LIF, SCF, bFGF, interleukin-11 (IL-11) and insulin-like growth factor 1(IGF-1). CBCs could be induced \textit{in vitro} to form EB-like structures that differentiated into cells representative of the three germ layers. CBCs were also injected into the sub-germinal cavity of irradiated embryos to produce chimaeric chickens. Germline chimerism was observed from donor cells maintained \textit{in vitro} for less than one week. A criticism of the work of Pain \textit{et al.} (1996) is their failure to clonally isolate cell lines. Consequently it remains a possibility that a mixed population of cells within CBC cultures is responsible for contribution to different cell-lineages. Single cell cloning of CBCs prior to chimaera production would address this challenge. CBCs can be modified at low efficiency by liposome-mediated transgenesis (Pain \textit{et al.}, 1999).

The characterisation of a chicken homologue of \textit{Drosophila vasa} (Cvh; Tsunekawa \textit{et al.}, 2000) strongly suggests germline potential is restricted to a subpopulation of blastodermal cells. Germ cell fate appears to be determined by the incorporation of maternally derived cytoplasmic factors, including CVH protein, at early cleavage stages. This supports the hypothesis that stage X blastodermal cells are already lineage-restricted. If \textit{Cvh} expression is required for germline transmission then only a small subpopulation of stage X blastodermal cells may ultimately yield germline
competent ES cells. Therefore the *vasa* positive blastoderm subpopulation may be the best starting material in chicken ES cell isolation experiments.

1.4.2 Primordial germ cells

Pluripotent mouse EG cells were derived from cultured PGCs. Cultured chicken PGCs may therefore be another potential source of pluripotent cell lines. It is possible to isolate PGCs from chickens at any stage during their migration from the germinal crescent to the developing gonad. When transferred between birds, PGCs can differentiate into functional gametes (Reynaud, 1969).

Conditions used in mouse EG cell isolation have been observed to promote chick PGC proliferation during the first 48 hours *in vitro*. Proliferation of blastoderm-derived PGCs increased fivefold in the presence of STO feeder cells (Petitte & Karagenc, 1996). PGC proliferation increased by up to twofold in medium supplemented with ciliary neurotrophic factor (CNTF) or SCF (Karagenc and Petitte, 2000). CNTF is a growth factor with overlapping functions to LIF (see 1.5.1). The third factor required in murine EG isolation, bFGF, was not found to have an effect on PGC proliferation. The effect of STO feeder cells and growth factors on long term culture of chick PGCs was not described in these experiments.

The derivation of EG cells from chicken PGCs was reported by Park & Han (2000). These cells were isolated and grown in the same growth factors used routinely to culture CBCs by Pain *et al.*, (1996). Early passage PGC-derived cells were shown to differentiate *in vitro* into the 3 germ layers from EBs. They also contributed to
chimaeras in vivo as evidenced by feather colour. No contribution to the germline in chimaeras was observed. However, the cells used in these differentiation experiments were from early passages and no differentiation was reported from established long-term cell cultures.

1.5 Maintaining pluripotency

Pluripotent stem cells are maintained as a developmentally static population. They have an unlimited capacity to proliferate without expressing markers indicative of differentiation. Mouse ES cells are maintained exogenously by IL-6 cytokines and by endogenous activity of the oct4 gene.

1.5.1 Cytokine signalling in mouse ES cells

Signalling through the gp130 receptor is required to maintain pluripotent stem cells for prolonged periods of time both in vitro and in vivo.

1.5.1.1 Leukaemia inhibitory factor

The propagation of pluripotent stem cells in vitro is only possible under certain conditions. Mouse ES cells were originally cultured in the presence of feeder cells. Feeder cells were necessary for self-renewal of ES cells and in their absence ES cells differentiated. Self-renewal of ES cells could also be achieved through the use of conditioned medium suggesting the presence of soluble factors. The buffalo rat liver (BRL) cell line was used to condition culture medium capable of supporting ES cells (Smith & Hooper, 1987). Smith et al., (1988) identified the cytokine LIF as the factor in conditioned medium responsible for ES cell maintenance. Williams et al.
(1988) purified LIF from the human bladder carcinoma cell line 5637 which is also capable of supporting ES cells. Supplementation with purified LIF has also been shown to support the isolation of ES cells in the absence of feeder cells (Pease et al., 1990; Nichols et al., 1990).

LIF is a member of the IL-6 family of cytokines and signals through the gp130 receptor subunit. The IL-6 family of cytokines include IL-6, LIF, IL-11, cardiotrophin 1 (CT-1), oncostatin M (OSM) and CNTF. IL-6 cytokines signal through receptor complexes containing one or two gp130 subunits (figure 1.11). Transmission through the common gp130 receptor subunit results in overlapping biological effects of IL-6 family cytokines. LIF, OSM, CNTF and CT-1 have all been observed to promote ES cell self-renewal (Smith et al., 1988; Rose et al., 1993; Conover et al., 1993; Pennica et al., 1995). IL-11 and IL-6 cannot directly support ES cell self-renewal as their specific receptor-subunits (IL-11r & IL-6r respectively) are not expressed by ES cells. When soluble IL-6r is provided exogenously with IL-6, ES cell propagation is supported (Yoshida et al., 1994).

Stimulation of gp130 by IL-6 cytokines is required for ES cell propagation in vitro and has a role in maintaining epiblast cells in vivo. LIF KO mice survive into adulthood although homozygous females are sterile (Stewart et al., 1992). Signalling through the LIF receptor (LIFr) is also dispensable for maintenance of the epiblast in vivo, as the severe developmental abnormalities observed in LIFr KO mouse embryos appear to occur after implantation (Ware et al., 1995). The possibility that epiblast cells are maintained in vivo by other IL-6 cytokines was eliminated by the
observation that gp130 KO mouse embryos develop normally until midgestation (Yoshida et al., 1996). LIF signalling through gp130 therefore appears to be dispensable for maintenance of the epiblast during normal development.

Figure 1.11 IL-6 cytokines signal through receptor complexes containing the gp130 subunit. IL-6 and IL-11 bind a complex of two gp130 (red) and one cytokine specific subunit (IL-6r and IL-11r respectively; light blue). LIF binds a heterodimer of LIF receptor (grey) and gp130. CT-1 binds a heterodimer of gp130 and an uncharacterised subunit (green). CNTF binds a receptor complex of gp130, LIF receptor and CNTF receptor (CNTFr; yellow). OSM mediates signals through heterodimers of gp130 and the OSM receptor (OSMr, purple).

During periods of environmental stress embryogenesis can be arrested by delayed embryo implantation in certain mammals. This process is termed embryonic diapause. Mouse embryos lacking gp130 do not survive if implantation is delayed, strongly suggesting an in vivo role for gp130 signalling in the maintenance of epiblast cells during the process of embryonic diapause (Nichols et al., 2001).
1.5.1.2 Signal transducer and activator of transcription 3

The intracellular domain of gp130 is associated with janus kinase (JAK) non-receptor tyrosine kinases. Stimulation of gp130 by IL-6 cytokines leads to dimerisation of receptor subunits. Dimerisation brings receptor-associated JAKs into close proximity which leads to their activation by phosphorylation at catalytically active sites (figure 1.12). Upon receptor dimerisation several tyrosine residues in the cytoplasmic tail of gp130 are phosphorylated. This creates Src-homology-2 (SH-2) docking sites which activate STAT factors which also contain SH-2 domains. Phosphorylated tyrosine residues on the cytoplasmic tail of gp130 also create binding sites for the tyrosine phosphatase SHP-2. SHP-2 binding can initiate the cascade that results in the other major downstream event in murine ES cells: MEK/ERK activation.

Seven mammalian STAT genes have been identified. With one exception (STAT4) they are ubiquitously expressed and activity is predominantly regulated by tyrosine and serine phosphorylation. STAT proteins can be activated by a variety of receptors and certain subsets of STATs tend to be activated by the same class of receptor complexes. For example IL-6 family cytokines activate STAT3 or STAT1 by stimulating gp130 receptor complexes.

STATs are conserved proteins of 750-850 amino acids and contain various conserved domains. The N-terminus contains a tetramerization domain and a leucine-zipper-like domain. A DNA-binding domain is located centrally and an SH-3-like, an SH-2
domain and a transactivation domain are located at the C-terminus. The SH-2 domain plays a pivotal role in STAT activation and is specific to each STAT protein.

Following ligand-induced dimerisation of receptor subunits (fig. 1.12a & b) gp130 is tyrosine phosphorylated (fig. 1.12c). STATs are then recruited to active JAK/gp130 complexes via the SH-2 domain (fig. 1.12d). Tyrosine kinases then phosphorylate tyrosine residues (Y705 on STAT3) proximal to the SH-2 domain (fig. 1.12e; Wen et al., 1995). Following tyrosine phosphorylation, STAT3 disengages (fig. 1.12f) and either forms homodimers or heterodimerises with STAT1 (fig. 1.12g). STAT dimers are then serine phosphorylated (fig. 1.12h) before translocating to the nucleus where they act as transcription factors through a DNA-binding domain (fig. 1.12i).

The most abundant STAT factor in murine ES cells is STAT3. It is activated via ligands of the IL-6 cytokine family and the majority of STAT dimers in ES cells are STAT3 homodimers (Burdon et al., 1999). If STAT3-binding tyrosine residues within gp130 are substituted with phenylalanine then STAT3 activation is abolished and ES cells cannot self-renew (Niwa et al., 1998). The pivotal role of STAT3 in ES self-renewal was further supported by preventing STAT3 homodimerisation. ES cells stably expressing a STAT3 mutant incapable of forming homodimers were unable to self-renew (Niwa et al., 1998).
Figure 1.12 Activation of STAT3 by interleukin 6

a) gp130 receptor subunits (black bars) exist as monomers when unbound, b) interleukin 6 (IL-6; red circle) binding induces dimerisation of gp130 receptor and subsequent phosphorylation (blue circle) of receptor-associated JAKs (dark green), c) activated JAKs phosphorylate tyrosine residues on the cytoplasmic domain of gp130, d) phosphorylated gp130 recruits STAT3 (yellow) via the SH-2 domain which subsequently becomes phosphorylated, e) STAT3 becomes tyrosine phosphorylated and disengages from gp130 (f), before dimerising in the cytoplasm (g), h) following serine phosphorylation, STAT3 dimers translocate to the nucleus where they act on DNA (light green) as transcription factors (i).
STAT3 KO embryos are unable to progress through gastrulation and die around 7.5dpc (Takeda et al., 1997). STAT3 activity therefore appears to be dispensable for proliferation of epiblast cells in vivo. An alternative factor, capable of maintaining ES cells in the absence of gp130 signalling and STAT3 activity has been described (Dani et al., 1998) and may support epiblast cell proliferation in vivo.

1.5.1.3 The MEK/ERK Pathway

Activation of the MEK/ERK pathway is another consequence of gp130 stimulation in ES cells (figure 1.13). In contrast to STAT3 activation, a pathway is initiated when gp130 binds the cytosolic protein tyrosine phosphatase SHP-2. SHP-2 contains two SH-2 domains through which it is recruited to gp130. Following recruitment, SHP-2 is phosphorylated creating a binding site for growth factor receptor bound protein 2 (GRB2). GRB2 recruits son of sevenless homologue (SOS) and thereby activates the Ras oncogene (Ras). Activation of Ras initiates the MEK/ERK signalling pathway and can be induced by alternative receptors e.g. the epidermal growth factor receptor. The MEK/ERK pathway can also be stimulated through gp130 by growth factor receptor bound protein 2-associated protein 1 (Gab1) activation of Ras.

Unlike STAT3 activation, MEK/ERK stimulation is not required for ES cell self-renewal (Burdon et al., 1999). This was demonstrated by the stable introduction of a mutated gp130 receptor, unable to engage SHP-2, into D027 ES cells. In D027 ES cells, the endogenous activity of both copies of the lif gene had previously been ablated by HR (Dani et al., 1998). D027 ES cells containing the mutant receptor,
prolonged STAT3 activation and enhanced self-renewal was observed, suggesting a negative role for MEK/ERK activation in ES cell self-renewal. ES cells also showed enhanced self-renewal when cultured in the presence of the MEK/ERK pathway inhibitor PD98059.

Figure 1.13 Activation of the MEK/ERK pathway by LIF. LIF (yellow circle) binds the LIFr/gp130 receptor complex which brings JAKs (red) together. Activated JAKs subsequently activate SHP-2. This leads to activation of Ras via GRB2 or Gab1. Ras can also be activated by other receptors (?). Active Ras initiates a cascade of signals which leads to activation of ERK1 and 2 by MEKI and II.
ES cells cultured in medium supplemented with PD98059 showed enhanced self-renewal and reduced dependency on IL-6 cytokines. This contrasts with many somatic cell types where stimulation of the MEK/ERK pathway through Ras is required for cell cycle progression through G1/S (Pages et al., 1993). However, ES cells have features of their cell cycle which distinguish them from many somatic cell types. They have a shortened G1 phase and prior to G1 entry dephosphorylation of the retinoblastoma protein does not occur (Savatier et al., 1994). Savatier et al., (1996) showed that ES cells have low levels of cyclin D1 which supports the hypothesis that an alternative control mechanism of G1/S transition exists in ES cells.

Although MEK/ERK activation appears to be redundant in ES cell self-renewal, it is likely to be important in ES cell differentiation. ES cells which overexpress catalytically inactive SHP-2 are compromised in their ability to activate MEK/ERK and to differentiate in vitro (Burdon et al., 1999). Mutant mouse embryos carrying a mutated SHP-2 (Saxton et al., 1997) die between days 8.5 and 10.5 of embryonic development. Post mortem analysis of embryos revealed a failure of gastrulation and defects of the embryonic and extra-embryonic mesoderm. Mouse embryos mutant for GRB2 cannot form egg cylinders but GRB2 function is dispensable for ES cells (Cheng et al., 1998). This supports a role for MEK/ERK activity in ES cell differentiation.
1.5.1.4 Phosphatidylinositol-3-OH kinase signalling

Stimulation of gp130 also leads to phosphatidylinositol-3-OH kinase (PI3K) signalling through SHP-2. PI3K promotes proliferation and survival of murine ES cells by regulating cyclin D1 levels. Cyclin D1 is present at low levels compared to differentiating cells and may not be required for G1/S transition in murine ES cells (Savatier et al., 1996). Although PI3K can be activated by gp130 stimulation, PI3K appears to maintain cyclin D1 levels independently of cytokine stimulation in ES cells (Jirmanova et al., 2002).

1.5.2 ES cell maintenance by transcription factors

ES cells require gp130 stimulation by IL-6 cytokines (e.g. LIF) to activate STAT3 and maintain self-renewal. A further requirement is activity of the transcription factors *oct4* and *nanog*.

1.5.2.1 Oct4

*Oct4* is a member of the highly conserved POU family of transcription factors. These transcription factors derive their names from the octamer recognition sequence through which they bind DNA. Oct transcription factors influence various aspects of murine development e.g. Oct2 regulates in B cell maturation and Oct6 regulates in postnatal nerve fibre myelinization (Strubin et al., 1995; Jaegle et al., 1996).

*Oct4* is present in the undifferentiated cells of the early embryo (Pesce et al., 1998). Maternally-derived *oct4* messenger ribonucleic acid (mRNA) and OCT4 protein, present in the unfertilised oocyte, are gradually replaced with embryonic OCT4
protein following the onset of zygotic transcription. Oct4 is expressed in early blastomeres before becoming restricted to the ICM after the earliest embryonic differentiation events. Subsequently oct4 expression is observed in the epiblast prior to the onset of gastrulation. Expression is downregulated until restricted to PGCs by day 9 of embryonic development (Rosner et al., 1990; Scholer et al., 1990b; Yeom et al., 1996). Expression of oct4 is also observed in EC, ES and EG cells (Pesce et al., 1998). Oct4 expression is required for formation of pluripotent stem cells and their in vivo precursors (Nichols et al., 1998). Mouse embryos homozygous for a non-functional copy of oct4 fail to develop a pluripotent ICM compartment in the blastocyst. ES and EC cell lines cannot be isolated from oct4 knockout embryos. Niwa et al. (2000) showed that the level of oct4 expression is critical in determining early differentiation events from ES cells. Overexpression of oct4 caused ES cells to differentiate into primitive endoderm or mesoderm while reduced expression levels resulted in the formation of trophectoderm. ES cells were previously believed to be incapable of differentiation into trophectoderm suggesting a key role for oct4 in the control of early differentiation. Only when a specific level of Oct4 was maintained were ES cells capable of self-renewal.

Sequences upstream of the Oct4 gene incorporate a promoter and two enhancer elements directing tissue specificity (Scholer, 1989; Okazawa et al., 1991). The promoter is located within 250bp of the transcription initiation sites. Expression in the embryonic ectoderm and EC cells is directed by the proximal enhancer which is located approximately 1.2kb upstream (Yeom, et al., 1996). The distal enhancer element is approximately 2kb upstream and directs expression in ES precursors, post
implantation germ cells in vivo and in ES and EG cells in vitro. A 1.9kb fragment of upstream sequences of the Oct4 promoter incorporating the promoter and proximal enhancer element was used to drive an antibiotic resistance gene (neo) in the selective ablation approach (see 1.3.2.2.). The success of this approach in isolating ES cells from CBA mice suggested a similar approach might work in ES cell isolation from other species. Although murine oct4 and its promoter have been extensively characterised (Ovitt & Scholer, 1998) identification of genes with homology to oct4 from other species has been hindered by the high level of conservation in octamer-binding POU factors. Oct4-related POU-domain genes have been identified in Xenopus (Whitfield et al., 1993), rat (Andersen et al., 1993), cynomolgus monkey (Heikinheimo et al., 1995) and sea urchin (Char et al., 1994). Buehr et al. (2002) attempted ES cell isolation from rat embryos using a selective ablation approach however, transgene expression from the oct4 promoter was found to be tightly downregulated in vitro. An extensive investigation to identify chicken oct4 was unable to identify a homologue (Soodeen-Karamath and Gibbins, 2001). The failure to identify a functional homologue of oct4 has prevented an oct4 promoter-based selective ablation approach being used in chicken ES cell isolation.

1.5.2.2 Nanog

Although STAT3 activation by LIF is required to maintain murine ES cells, it is dispensable in cells of the ICM and epiblast. In addition, human ES cells do not respond to gp130 stimulation, suggesting the existence of alternative mechanisms that maintain pluripotent cell types. The transcription factor nanog is expressed in
pluripotent cells of the early mouse embryo and mouse ES cells and operates independently of gp130 (Chambers et al., 2003; Mitsui et al., 2003).

Mitsui et al. (2003) showed that nanog expression is required to prevent differentiation of murine ES cells. Nanog expression is independent of gp130/STAT3 in ES cells and nanog null embryos failed to derive an epiblast compartment. Embryos and ES cells differentiated into parietal endoderm and endoderm respectively suggesting a role for nanog in suppressing endoderm differentiation. Chambers et al. (2003), demonstrated expression of nanog in early mouse embryos, mouse PGCs and murine ES cells. Nanog maintained ES cells independently of LIF/STAT3 but not in the absence of oct4.

1.5.3 Cytokine signalling in chicken cells

Although a homologue of murine LIF has not been identified in chickens, it was shown to stimulate proliferation of CBCs (Pain et al., 1996). This may be due to the apparent functional redundancy between LIF and other members of the IL-6 family. For example CNTF can maintain mouse ES cells in the absence of LIF and this cytokine has been demonstrated to direct signalling in chick neurons (e.g. Geissen et al., 1998). Gp130 has not been cloned in the chick but there is evidence supporting a biological function. Antisense gp130 RNA expressed in chick neural cells substantially reduced IL-6 cytokine mediated signalling (Geissen et al., 1998) and increased growth of chick neurons was observed following the introduction of exogenous gp130 (Clarke et al., 1993). Chick growth promoting activity receptor alpha (GPARα, a receptor subunit capable of supporting CNTF signalling in chick
neurons has also been identified (Heller et al., 1995). Reports of multipotent cell culture in chickens have routinely used IL-6 family cytokines in addition to stem cell factor and bFGF (Pain et al., 1996; Park & Han, 2001). Proliferation of cells from early cultures has been supported either by directly supplementing with cytokines or by the use of feeder cells.

CNTF activates STAT3 in chickens (Wishingrad et al., 1997). It is unknown if STAT3 has a role in the maintenance of undifferentiated chicken cells. The differentiation response that accompanies MEK/ERK activation in mice is also observed in chicken neurons (Wu & Howard, 2001), but the role of IL-6 cytokines in MEK/ERK activation is unknown.

1.5.4 Genetic control of pluripotency in chicken cells

A chicken homologue of murine Oct4 has not been identified despite an intensive search (Soodeen-Karamath & Gibbins, 2001). Three genes have been identified in putative chicken ES cells (Acloque et al., 2001) which are downregulated following differentiation, but the function of these genes has not been determined.

In Drosophila, vasa function is required for normal development of pole cells, the precursors of germ cells. VASA protein is associated with the germ plasm and subsequent to cleavage expression becomes restricted to the future germ cells. Drosophila VASA is an adenosine triphosphate (ATP)-dependent RNA helicase of the DEAD-box protein family. It is widely conserved, invariably showing an association with the germline (Saffman and Lasko, 1999). Expression of the mouse

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Vasa homologue Mvh only begins after PGCs have colonised the embryonic gonad and it appears that gonadal somatic cells induce expression (Toyooka et al., 2000).

A chicken homologue of vasa has been identified (Cvh; Tsunekawa et al., 2000) and shows a similar expression pattern to Drosophila vasa. In Drosophila, VASA is maternally derived as part of a cytoplasmic structure termed the germ plasm. The germ plasm is a mitochondria-rich substance that is inherited asymmetrically by progenitors of germ cells. In chickens, cvh expression appears to follow the same germ cell-specific pattern as in Drosophila. CVH can be detected in early cleavage embryos, and PGCs can be identified by the presence of CVH throughout early development.

Homologues of vasa have been identified in many species including mouse (Fujiwara et al., 1994), human (Castrillon et al., 2000), Xenopus (Komiya et al., 1994) and zebrafish (Yoon et al., 1997). Zebrafish vasa is inherited in a pattern similar to chickens and segregates into future germ cells during early cell divisions. Germ-line chimeras have been produced from cultures of zebrafish embryo cells (Ma et al., 2001) using a layer of spleen feeder cells. Embryo cells were cultured for more than 30 days during which time vasa was expressed and differentiation inhibited. In the absence of feeder layers embryo cells ceased to express vasa and failed to contribute to the germline in chimeras. These data, when combined with the widely conserved role in germ cell determination, suggest germline contribution in non-mammalian chimaeras may be linked to the expression of vasa. As the
segregation of CVH in the early chick embryo closely resembles the zebrafish expression pattern, *Cvh* expression is likely to be important in germline contribution in chick chimaeras. This contrasts with the expression of the mouse *vasa* homologue *mvh*. *Mvh* expression in murine PGCs is only established upon colonisation of the genital ridges around 10.5dpc (Fujiwara *et al.*, 1994).

1.6 **Summary**

ES cell based systems offer several advantages for the production of transgenic animals. Precise genomic modifications can be introduced to ES cells *in vitro* and identified using antibiotic selection. Modified ES cells can then be used to create whole organisms via chimaeric intermediates. The establishment of a chicken ES system has three requirements 1) isolation and culture of stable pluripotent cell lines, 2) a selectable method of transfecting cells with foreign genes and 3) germline contribution after introduction of clonally-derived cells into a recipient embryo.

ES cell-based systems are now widely used to study gene function in transgenic mice. However attempts to isolate ES cells from other species have been largely unsuccessful (with the exception of primates) representing a significant challenge to broader application of gene targeting technology. An alternative method of introducing targeted modifications, NT, is technically demanding.

Most commonly used ES cell lines have been derived from inbred strain 129 mice. ES cell lines from the CBA inbred strain cannot be isolated using standard procedures. CBA mice have therefore been used in experiments to evaluate new
approaches to ES cell isolation (McWhir et al., 1996; Brook & Gardner, 1997; Schoonjans et al., 2003). The selective ablation approach (McWhir et al., 1996) is unsuitable for use in chicken embryos because a functional homologue of murine Oct4 has not been isolated. The use of microsurgery to isolate epiblasts and subsequently CBA ES cell lines (Brook & Gardner, 1997) is difficult to apply to chicken embryos because morphologically indistinguishable cells have different developmental capacities (see 1.4.1). Although these two approaches are impractical for chicken ES cell isolation, CBA mice remain useful for testing novel ES cell isolation strategies.

Mouse ES cells have three basic requirements that allow them to be propagated in vitro: expression of the transcription factors oct4 and nanog and stimulation of the gp130 receptor by IL-6 family cytokines. These requirements are necessary to maintain ES cells. ES cell isolation strategies designed to exploit these requirements may have applicability to other species. A selective ablation strategy (McWhir et al. 1996) is hindered by failure to isolate a chicken homologue of Oct4 (Soodeen-Karamath & Gibbins, 2001). The gp130 receptor activates STAT3 and the MEK/ERK pathway. Manipulation of the intensity of these signals may provide new routes to murine ES cell isolation. If CBA ES cells can be isolated by manipulated cell signalling, then the approach may be applicable to chicken ES cell isolation.
1.7 Aim and Objectives

The aim of this thesis was to develop stem cell-based methods for the production of transgenic chickens.

The objectives of this thesis were as follows,

1) To learn murine ES cell isolation by standard procedures

2) To explant and culture chicken embryo-derived cells and chicken PGCs

3) To determine if downregulation of the MEK/ERK pathway increased the frequency of murine ES cell isolation

4) To determine if downregulation of the MEK/ERK pathway and upregulation of gp130 signalling facilitated murine strain CBA ES cell isolation

5) To evaluate downregulation of the MEK/ERK pathway and upregulation of STAT3 in chicken ES cell isolation
Chapter 2 Materials and methods

All chemicals used were of analar grade and purchased from Sigma or BDH unless stated otherwise. Plasticware was purchased from Nunc, Greiner, Iwaki or Eppendorf unless stated otherwise.

2.1 Centrifugation

Centrifugation of microcentrifuge tubes was performed in an IEC Micromax RF (Hybaid) refrigerated benchtop centrifuge. Centrifugation of Nalgene 50ml tubes (React Scientific) was performed in a J2-21 M/E refrigerated centrifuge (Beckman) using a JA-20 rotor.

2.2 General materials

Tris/EDTA (TE)
10mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.4.

LB-medium
1% Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco), 0.125M sodium chloride (NaCl).

LB-Agar
1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.125M NaCl and 1.5% agar (Difco)
**SOC-broth**

2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10mM NaCl, 2.5mM potassium chloride (KCl), 20mM Mg\(^{2+}\) stock (1M MgCl\(_2\) 6H\(_2\)O/1M MgSO\(_4\) 7H\(_2\)O) and 20mM glucose (Fisons).

**Genomic DNA re-suspension solution**

400mM Tris-HCl (pH 8.0), 60mM EDTA, 150mM NaCl.

**Type III DNA loading buffer (6x)**

0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.

**10X TBE buffer (Ambion)**

0.89M Tris-HCl, 0.89M borate, 0.02M EDTA, pH 8.0.

**PBS (Oxoid)**

0.16M NaCl, 0.003M KCl, 0.008M disodium hydrogen phosphate and 0.001M potassium dihydrogen phosphate in distilled water. Supplied in tablet form. Reconstituted in distilled water (dH\(_2\)O) and autoclaved prior to use.

**2X sodium dodecyl sulfate loading buffer**

100mM Tris-HCl pH 6.8, 4% sodium dodecyl sulphate (SDS), 0.2% bromophenol blue, 20% glycerol, 200mM dithiothreitol (DTT; Biorad).
10X Tris-glycine running buffer
250mM Tris-HCl, 2.5M glycine, 1% SDS, pH 8.3.

Western transfer buffer
0.5M Tris-HCl, 3.84M glycine pH adjusted to 8.3 with concentrated hydrochloric acid (HCl). 20% methanol added to 1X buffer before use.

Tris-buffered saline (TBS)
0.02M Tris base, 0.137M NaCl pH adjusted to 7.6 with HCl.

Blocking solution (immunoblotting)
5% skimmed milk powder (Marvel), 0.1% polyoxyethylene sorbitan monolaurate (Tween-20) in TBS.

TBST wash solution (immunoblotting)
0.1% Tween-20 in TBS.

Western stripping solution
100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7.

Blocking solution (immunochemistry)
5% heat-inactivated donkey serum (Biopharm), 0.05-0.1% Tween-20 in PBS.
2.3 **Cell culture materials**

All materials purchased were of cell culture grade and were purchased from Sigma, Invitrogen or BDH unless stated otherwise.

2.3.1 **Murine ES cell maintenance medium**

Glasgow’s minimum essential medium (GMEM) supplemented with 5% foetal calf serum (FCS; Globepharm), 5% newborn calf serum (NCS; Globepharm), 0.1mM non-essential amino acids (NEAA), 0.1mM β-mercaptoethanol, 1mM sodium pyruvate, 2mM L-glutamine and 500U/ml recombinant murine leukaemia inhibitory factor (LIF).

2.3.2 **Murine ES cell isolation medium**

GMEM supplemented with 20% FCS, 0.1mM NEAA, 0.1mM β-mercaptoethanol, 1mM sodium pyruvate, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 500U/ml LIF.

2.3.3 **STO/m220 medium**

Dulbecco’s modified eagles medium (DMEM) supplemented with 5% FCS, 5% NCS, 0.1mM NEAA and 2mM L-glutamine.

2.3.4 **Chicken embryonic fibroblast medium**

Chicken embryonic fibroblasts (CEFs) were cultured in DMEM supplemented with 10% FCS, 100U/ml penicillin and 100μg/ml streptomycin.
2.3.5 Chicken blastoderm cell medium

Chapter 3

GMEM, supplemented with 10% FCS, 2% chicken serum (Globepharm), 20ng/ml conalbumin, 0.1mM NEAA, 2mM L-glutamine, 10mM hepes, 0.2mM β-mercaptoethanol, 1mM sodium pyruvate, 1μM each dATP, dGTP, dCTP and dTTP (Abgene) and 100U/ml Penicillin with 100μg/ml Streptomycin. CBC medium was further supplemented with 500U/ml mLIF; 10ng/ml recombinant human basic fibroblast growth factor (bFGF); 20ng/ml recombinant human insulin growth factor I (hIGF-1); 20ng/ml recombinant human stem cell factor (SCF) and 10ng/ml recombinant human interleukin 11 (IL-11) as described in chapter 3.

Chapter 7

GMEM, supplemented with 10% FCS, 0.1mM NEAA, 2mM L-glutamine, 0.2mM β-mercaptoethanol, 1mM sodium pyruvate, 100U/ml penicillin and 100μg/ml streptomycin. CBC medium was further supplemented with 500U/ml mLIF, 10ng/ml bFGF, 20ng/ml hIGF-1, 20ng/ml SCF and 20ng/ml recombinant human ciliary neurotrophic factor (CNTF) as described in chapter 7. 10% charcoal treated foetal calf serum (Globepharm) was used in 7.2.5.

2.3.6 Chicken PGC medium

GMEM; supplemented with 10% FCS, 2% chicken serum, 20ng/ml conalbumin, 0.1mM NEAA, 10mM hepes, 0.2mM β-mercaptoethanol, 1mM sodium pyruvate, 1μM each dATP, dGTP, dCTP and dTTP, 100U/ml penicillin with 100μg/ml streptomycin, 500U/ml mLIF, 10ng/ml bFGF; and 20ng/ml SCF.
2.3.7  **Cardiogenesis differentiation medium**

DMEM supplemented with 20% FCS, 50μM β-mercaptoethanol, 0.1mM NEAA and 2mM L-glutamine.

2.3.8  **Neurogenesis differentiation medium**

DMEM supplemented with 20% FCS, 50μM β-mercaptoethanol, 10μg/ml human transferrin, 0.2% bovine serum albumin, 10^{-7} M retinoic acid, 0.1mM NEAA and 2mM L-glutamine.

2.3.9  **Trypsin/EGTA (TEG) solution**

92.7mM NaCl, 0.845mM di-sodium hydrogen orthophosphate (Na₂HPO₄), 1.58mM potassium dihydrogen orthophosphate (KH₂PO₄), 4.46mM KCl, 5mM D-glucose (Fisons), 22.28mM Tris-HCl, 0.0009% phenol red, 0.25% trypsin, 1.05mM ethylenebis(oxyethylenenitrito)tetraacetic acid (EGTA) and 0.000105% polyvinyl alcohol.

2.3.10  **Pronase solution**

0.025% Pronase (Roche), 92.7mM NaCl, 0.845mM Na₂HPO₄, 1.58mM KH₂PO₄, 4.46mM KCl, 5mM D-glucose, 22.28mM Tris-HCl, 0.0009% phenol red, 1.05mM EDTA and 0.000105% polyvinyl alcohol.
2.3.11  **EGTA cell dissociation solution**

92.7mM NaCl, 0.845mM Na₂HPO₄, 1.58mM KH₂PO₄, 4.46mM KCl, 5mM D-glucose, 22.28mM Tris-HCl, 0.0009% phenol red, 1.05mM EGTA and 0.000105% polyvinyl alcohol.

2.3.12  **EDTA cell dissociation solution**

92.7mM NaCl, 0.845mM Na₂HPO₄, 1.58mM KH₂PO₄, 4.46mM KCl, 5mM D-glucose, 22.28mM Tris-HCl, 0.0009% phenol red, 1.05mM EDTA and 0.000105% polyvinyl alcohol.

2.3.13  **Freezing mix (2X)**

60% complete medium, 20% FCS, and 20% dimethyl sulfoxide (DMSO).

2.3.14  **Paraformaldehyde solution**

4% paraformaldehyde (PFA) was dissolved in PBS and pH adjusted to 7.4 with sodium hydroxide (NaOH).

2.4  **Animal methods**

Blastocyst injection and embryo transfer was performed by J. McWhir according to published protocols (Robertson, 1987).

2.4.1  **Mouse blastocyst collection**

Natural matings were set up between stud male and oestrus selected female mice. Females were sacrificed by cervical dislocation on day 3.5 post coitus, and uterine
horns were removed. Uterine horns were flushed with ES cell isolation medium buffered with 20mM hepes. Blastocysts were washed in fresh ES cell isolation medium and counted under a dissecting microscope in a still air hood.

2.4.2 Generation and recovery of tumours from SCID mice

SCID mice (Harlan) were maintained in a pathogen free environment. ES cells were trypsinised (see 2.5.3) and approximately 1x10^7 cells were resuspended in 200μl of PBS containing 100U/ml penicillin and 100μg/ml streptomycin. 200μl of ES cell suspension was injected into the skeletal muscle of the hind leg of each SCID mouse (injection of ES cells was performed by J. McWhir). Tumours became visible as lumps beneath the surface of the skin approximately one month later and the animal was sacrificed and the tumour dissected. Tumours were washed in PBS before being weighed and photographed. Tumours were then fixed in 4% PFA solution at 4°C for several days prior to embedding in paraffin wax.

2.4.3 CEF preparation

Freshly laid, fertilised eggs were incubated, with rocking, at 37°C for 10 days. The eggshells were broken by tapping above the air space, membranes were cut and the embryo was decapitated with scissors (performed by C. Mather). The carcass was removed to a plastic dish where wings, legs and viscera were discarded. The carcass was then moved into 30mls of 0.05% TEG solution in a 50ml polypropylene tube. The carcass was finely minced with scissors, vortexed for 2 minutes and incubated at room temperature for 5 minutes. After vortexing for a further minute the suspension was left to settle for 10-15 minutes. Approximately 90% of the supernatant was
removed and divided between three T75 flasks and the total volume of medium was made up to 20mls/flask. The remaining 10% contained larger aggregates of cells and tissue and was discarded. After 6 hours, medium was aspirated and replaced with fresh CEF medium.

2.4.4 Chicken gonad dissection

Freshly laid eggs were incubated, with rocking, at 37°C for 5 days. The eggshell was broken and the embryo decapitated and transferred into a petri dish. After washing with PBS, membranes and viscera were removed to reveal the gonadal mesenephros (GM). For cell culture (see 3.2.6), the genital ridges were then dissected using 2 pairs of forceps. Genital ridges were transferred to chicken primordial germ cell medium in a 1.5m1 microcentrifuge tube (one ridge per tube) and vortexed briefly. Genital ridges were further dissociated by passing through a 23G needle (Terumo) prior to plating at a density of one ridge per well in 24-well plates.

For histological analysis (see 3.2.5), the whole GM was excised with a scalpel and washed three times in PBS. The GM was fixed in 4% PFA on ice for 20 minutes and washed three further times in PBS. Fixed GM sections were sucrose saturated by incubation in 15% sucrose solution overnight at 4°C. The following day sucrose saturated GM were embedded in 15% sucrose/7.5% gelatin cubes and frozen in isopentane at -65°C. Cubes were stored for a minimum of 24 hours at -80°C before cutting into 10μm sections on a cryostat (Bright, OTF5000) and stored on glass slides prior to staining for TNAP activity.
2.4.5 Chicken blastoderm dissection

Hair loop method

Freshly laid, fertilised eggs were broken into a petri dish containing PBS. The egg whites were removed and the vitelline membrane was peeled back to reveal the embryo. The developmental stage of embryos was judged according to Eyal-Giladi and Kochav, (1976) and only embryos of stages IX-XI were dissected. A loop of hair attached to the end of a short wooden stick was used to excise the blastoderm. The blastoderm was then stored in a microcentrifuge tube containing chicken blastoderm cell (CBC) medium. In chapter three, embryos were dissected, washed twice in CBC medium without growth factors, passed through a 23G needle and centrifuged to collect the cells. CBCs were seeded at a density of four embryos/well in 24-well plates in CBC medium containing growth factors. In chapter seven, embryos were vortexed briefly and seeded one embryo/well in 12-well plates in CBC medium containing growth factors (see 7.2).

Intact blastoderm dissection

Intact blastoderms were dissected for immunochemical analysis (see 3.2.2). Freshly laid eggs were collected and a section of the vitelline membrane and the blastoderm were dissected from the yolk. The vitelline membrane was carefully peeled back to reveal the upper surface of the blastoderm. The developmental stage of the blastoderm was determined under a dissecting microscope. Only stage X embryos were used in subsequent analysis. Blastoderms were removed from the yolk using fine forceps and washed in PBS by pipetting up and down with a wide bore plastic pipette. Intact blastoderms were then washed a further three times by gentle
agitation in a petri dish containing PBS. Blastoderms were fixed in 4% PFA solution at 4°C for 30 minutes and stored at -20°C in 100% methanol.

2.5 Cell Culture Methods

2.5.1 Freezing cells

Cells were washed once with PBS before incubation in TEG solution until observed to detach from the surface of plasticware. Cells were washed with culture medium to inactivate trypsin, centrifuged at 1000g, resuspended in fresh medium (half of the final volume for freezing) and put on ice. The same volume of pre-cooled 2X freezing mix was added slowly to the resuspended cells and gently mixed. This suspension was aliquoted into pre-cooled 1.5ml cryotubes (1ml suspension/tube) and stored overnight at -80°C. After 24 hours cryotubes were quickly moved to -150°C or into liquid nitrogen for long term storage.

2.5.2 Resuscitation of cells

Frozen vials of cells were removed from liquid nitrogen or -150°C freezer and placed in a water bath at 37°C. After thawing, cells were resuspended in 5mls of medium at 37°C and centrifuged at 1000g for 5 minutes before being plated out in fresh medium.

2.5.3 Maintenance of murine ES cells

ES cells were maintained at 37°C in a gassed incubator with a 5% CO₂ atmosphere. All plasticware was coated with gelatin at least half an hour before use. Culture medium was replaced daily and cells were passaged when they reached 80-90%
confluency, usually every 2-3 days. Medium was changed on cells approximately 2 hours prior to passage. Cells were passaged by washing once with PBS before incubation in TEG solution. During incubation cells were observed to become rounder and lose their tight connections with other ES cells and the plastic surface. ES cells detached from the plastic surface by gently agitating the flask after approximately one minute incubation in TEG. Cells were then washed with medium to inactivate trypsin and centrifuged at 1000g for 5 minutes. ES cells were resuspended as a single cell suspension in fresh ES cell medium before being plated out in freshly prepared flasks.

2.5.4 Passage of CBCs

CBCs were passaged in TEG solution, Pronase EDTA solution, EDTA solution or EGTA solution. Alternatively, CBCs were passaged mechanically. Using a fine glass mouth pipette, CBC colonies were removed from the surface of the multi-well plate and transferred to a small drop of dissociation solution or fresh medium for mechanical dissociation. Colonies were incubated for 1-2 minutes in dissociation solution and disaggregated by gently passing up and down the fine glass pipette. The resulting cell suspension was then replated onto freshly prepared feeder cell layers. The CBC line derived in section 7.2.6 was passaged mechanically by passing up and down through a p1000 pipette tip in culture medium prior to re-plating.

2.5.5 Preparation of feeder cells

STO feeder cells were used extensively throughout this thesis. CEFs and m220 feeder cells were used in chapter 3. Mitotically active feeder cells were grown to 70-
80% confluency in T180 flasks. Medium was aspirated from cells and replaced with 15mls of fresh medium supplemented with 10μg/ml Mitomycin C. Feeder cells were incubated for three and a half hours at 37°C. After incubation, cells were washed three times with PBS, trypsinised and centrifuged at 1000g for five minutes. Five vials of feeder cells were then frozen down from each T180 flask. To resuscitate, vials of inactivated STO feeder cells were thawed in water at 37°C and resuspended in STO medium. Cells were plated in multi-well plates to form an almost confluent monolayer for use the following day.

2.5.6 Mouse ES cell isolation

Blastocysts were collected as described in 2.4.1 and plated at a density of one embryo/well onto feeder layers of mitotically inactivated STO cells in 24-well plates (figure 2.1b). Plates were incubated and approximately 5-6 days later, when judged to have developed sufficiently, embryonic explants were removed using a narrow glass mouth pipette (figure 2.1c). Explants were transferred to a droplet of TEG under mineral oil in a petri dish and incubated for 2-3 minutes to allow enzymatic dissociation. After incubation, explants were mechanically dissociated by pipetting up and down once and resuspended cells were inoculated onto freshly prepared feeder cells in a 24-well plate. Cultures were monitored daily for the presence of colonies of cells with an ES cell-like phenotype. When ES-like colonies were identified, their development was followed closely until they were judged to be of a sufficient size for further passage. If colonies were present singly then they were removed with a glass pipette into a drop of TEG under mineral oil. Colonies were incubated for approximately one minute in TEG before dissociation and seeding onto
Trophectoderm cell — Inner cell mass — Blastocoel cavity

a) Blastocysts collected at 3.5dpc.

b) Blastocysts plated on feeder cells.

c) Explant picked after 5-6 days and dissociated.

d) ES-like colonies subcultured 6-7 days later.

e) Step d) repeated.

f) Pure ES cell line established.

Figure 2.1 Schematic representation of murine ES cell isolation

a) Blastocysts were collected from fertilised female mice on 3.5dpc, b) Blastocysts were cultured on a layer of inactivated STO feeder cells (blue) in multi-well plates and dissociated after 5-6 days (c), d &e) colonies with an ES-like morphology (pink) were successively subcultured from mixed embryo-derived cultures containing both ES-like colonies and colonies of differentiated cells (green), f) pure ES cell lines were subsequently established.

fresh feeder cells (figure 2.1d). If multiple colonies were present then 200μl of TEG solution was added to the well and, after approximately 2 minutes incubation, all colonies were removed with a narrow glass pipette and seeded directly onto fresh
feeder cells and treated as a single cell line. After the 3rd passage (figure 2.1e), cells were moved onto gelatin-coated 24-well plates and passaged the same way as standard murine ES cells (Fig 2.1f; see 2.5.3). Cells were moved into increasingly larger vessels at subsequent passages and stored as frozen stocks at –150°C.

2.5.7 Fixation and permeabilisation of cultured cells
Medium was aspirated from cultured cells and the cell surface was washed 3 times with PBS. 4% PFA was added to the cells and vessels were incubated for 15 minutes at 4°C. Fixed cells were washed 3 times in PBS to remove residual PFA. Fixed cells were then incubated with 100% methanol for one minute to permeabilise before washing twice in PBS and storage for up to one week at 4°C in PBS.

2.5.8 Lipofection of ES cells
ES cells were transfected using the effectene lipofection kit (Qiagen). Twenty-four hours prior to transfection murine ES cells were seeded at a density of approximately 1x10^5/well in 6-well plates. Cells were incubated overnight and reached approximately 40-50% confluency the following day. 0.4μg of plasmid DNA was diluted in buffer EC (Qiagen) to a total volume of 100μl. 3.2μl of enhancer (Qiagen) was added, and the samples were briefly vortexed. Samples were pulsed in a centrifuge and incubated for five minutes at room temperature. 10μl of effectene reagent (Qiagen) was added, samples were mixed by pipetting up and down and then incubated for 10 minutes at room temperature. Medium was aspirated from wells and cells were washed once with PBS before adding 1.6ml ES cell medium. 0.6ml ES cell medium was added to DNA-liposome complexes that were mixed by
pipetting before being added drop-wise onto the cells. Cells were incubated at 37°C. Eight hours after addition, medium containing transfection complexes were aspirated, cells were washed with PBS and 3ml medium was replaced. Cells were incubated and gene expression was assayed 24-48 hours later.

2.5.9 Preparation of embryoid bodies

Approximately $1 \times 10^7$ ES cells were seeded as a single cell suspension in ES cell medium on 100mm bacterial grade plastic culture dishes without gelatin. ES cell medium was replaced with LIF-free ES cell medium after 24-48 hours when small aggregates of ES cells had formed. Over the next week EBs started to differentiate, typically showing an outer layer of endoderm-like cells. EBs were washed every second day to prevent attachment to plastic and medium was changed as required. One week after seeding, EBs were plated in gelatin-coated chamber slides in neuronal or cardiac differentiation medium and maintained until visibly differentiated.

2.5.10 Preparation of chromosome spreads

ES cells were prepared in a T75 flask at approximately 50-70% confluency. ES cell medium was replaced with ES cell medium containing 100ng/μl colcemid which arrests cells in metaphase by disrupting the cytoskeleton. After one hour, cells were passaged, centrifuged and resuspended by gentle agitation. 6ml chilled hypotonic solution (0.075M KCl) was added dropwise, taking care to avoid formation of cell clumps. The tube containing resuspended cells was inverted to mix and incubated at room temperature for ten minutes. Following centrifugation (1000g; 5 minutes),
supernatant was aspirated and cells resuspended by gentle agitation. Fresh fixative was prepared (Methanol: Glacial acetic acid 3: 1) and chilled on ice. Cells were resuspended by dropwise addition of 6mls chilled fixative, inverted once and incubated at room temperature for 5 minutes. Cells were centrifuged (1000g; 5 minutes), supernatant aspirated and fixation repeated two further times. Following the final centrifugation the cell pellet was resuspended in 1ml fixative. Cell suspension was dropped onto clean glass slides from 18-24 inches height and allowed to air dry. To improve visualisation, slides were incubated for five minutes in 2% Giemsa stain, washed in tap water and air dried.

2.6 **Bacterial Methods**

2.6.1 **Transformation of competent bacteria**

Ultracompetent cells (Epicurian Coli XL10-Gold, Stratagene; JM109, Promega) were allowed to thaw slowly on ice. 100μl of cells were aliquoted into 2 pre-cooled 1.5ml microcentrifuge tubes. 5μl of a ligation mix or plasmid DNA was added to one tube and 5μl dH2O was added to the other control tube. Tubes were incubated on ice for 20 minutes before being heat shocked at 42°C for 45 seconds. Tubes were transferred to ice for 2 minutes before 900μl of pre-warmed SOC or LB broth was added. Tubes were incubated at 37°C for 1 hour with shaking before being plated out on 2 plates each of LB agar containing appropriate antibiotics.

2.6.2 **Small scale preparation of plasmid DNA**

DNA was isolated using the Wizard Plus SV Minipreps system (Promega). A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to
inoculate 3ml of LB medium (with antibiotic) in a 15ml polypropylene tube. This culture was incubated for 12-16 hours at 37°C with shaking at 250rpm. Cultures were harvested by centrifugation (10000g; 5 minutes) and the supernatant was discarded. Cells were resuspended in 250μl of cell resuspension solution and transferred to a 1.5ml microcentrifuge tube. 250μl of cell lysis solution was added and mixed by inversion four times. Tubes were incubated at room temperature for approximately 5 minutes until the suspension was observed to clear. 10μl alkaline protease solution was added and mixed by inversion four times, the tubes were then incubated for 5 minutes at room temperature. 350μl of neutralization solution was added and the tubes were inverted four times before centrifugation (14000g; 10 minutes; room temperature). The supernatant (cleared lysate) was transferred to spin column in a collection tube and centrifuged (14000g; 1 minute; room temperature). The flowthrough was discarded and the column was washed with 750μl of column wash solution by centrifugation (14000g; 1 minute; RT). Washing was then repeated using 250μl of column wash solution. (14000g; 2 minutes; room temperature). DNA was eluted by the addition of 100μl dH₂O and centrifugation (14000g; 1 minute; room temperature).

2.6.3 Large scale preparation of plasmid DNA

DNA was isolated using the Qiagen Plasmid Maxi Kit (Qiagen). A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to inoculate a 3ml starter culture of LB medium (with antibiotic) in a 15ml polypropylene tube. This culture was incubated for 8 hours at 37°C with shaking at 250rpm. This starter
culture was then inoculated into 100ml selective LB medium and grown for 12-16 hours at 37°C with shaking at 250rpm.

Cultures were harvested by centrifugation (6000g; 15 minutes; 4°C) and the supernatant was discarded. Cells were resuspended in 10ml of buffer P1. 10mls of buffer P2 (heated to 37°C) was added and mixed by inversion four times and tubes were incubated at room temperature for approximately 5 minutes. 10mls of chilled (4°C) buffer P3 was added and the tubes were inverted four times before incubation on ice for 15 minutes. Samples were centrifuged (20000g; 30 minutes; 4°C) and the supernatant collected and filtered through 3MM paper (Whatman). A QIAGEN-tip 500 was equilibrated with 10ml of buffer QBT and the filtered supernatant allowed to enter the QIAGEN-tip by gravity. The QIAGEN-tip was then washed twice (30ml buffer QC) and the DNA eluted with 15ml buffer QF. 0.7 volumes of room temperature isopropanol were added to precipitate the DNA. The tube was mixed and centrifuged (15000g; 30 minutes; 4°C) and the supernatant discarded. The pellet was then washed with 5ml room temperature 70% ethanol and centrifuged (15000g; 10 minutes). After air-drying, the pellet was dissolved in 500µl-1ml TE or dH₂O.

2.7 Molecular biology methods I: DNA

2.7.1 Agarose gel electrophoresis

Agarose powder was dissolved in 1X TBE by heating in a microwave and allowed to cool. Ethidium bromide was added to a final concentration of 0.5µg/ml before pouring the gel using a horizontal electrophoresis gel kit (Anachem) and inserting gel combs. DNA samples were mixed with 6X Type III loading dye and loaded into the
slots, 2 slots were reserved for loading molecular weight markers (Roche). An electrical current was applied at between 30 and 150volts. Following electrophoresis, DNA was visualised using UV light.

2.7.2 Preparation of genomic DNA from cultured cells

Cells were prepared to reach 80-90% confluency in one well of a 24-well plate. Cells were washed twice in PBS and incubated with 100μl TEG until observed to detach from the culture surface. Cells were resuspended in medium (1ml) and centrifuged in a 1.5ml microcentrifuge tube at 1000g for 5 minutes. Cell pellets were resuspended by briefly vortexing in 340μl genomic DNA (gDNA) re-suspension solution (see 2.2). RNase A was added (20μg) and cells were incubated for 20 minutes at 37°C. Samples were then incubated for a further 20 minutes at 65°C. Samples were regularly inverted during these incubation steps. Chloroform (580μl, chilled on dry ice) was added and the samples were rotary mixed for 20 minutes. Samples were centrifuged at 12000g for 20 minutes and the supernatant was removed to a fresh microcentrifuge tube. A further 580μl chilled chloroform was added and the step repeated. Two volumes of absolute ethanol (750μl) were added to precipitate the DNA and the tubes were inverted and centrifuged at 12000g for 10 minutes. The supernatant was removed and pellets were washed in 500μl of 70% ethanol and centrifuged at 12000g for 5 minutes. Supernatants were removed and DNA pellets were dried for two minutes at 45°C on a thermocycler (Biometra, TRIO-Thermoblock). DNA pellets were resuspended overnight at 4°C in 60μl of dH2O.
2.7.3 Polymerase chain reaction

For microsatellite analysis, genomic DNA was used as template in PCR. Complementary DNA (cDNA) template was used as template in reverse transcription PCR (RT-PCR). During preparation of the reaction mixture all reagents were kept on ice. In a total reaction volume of 20µl or 50µl the following components were added: enzyme buffer (Roche; final concentration: 1X), 200µM dNTP (Abgene), 200nM each forward and reverse primers (MWG-Biotech), 2 or 5 units TaqDNA polymerase (Roche) and 10-100ng of DNA template. The remainder of the reaction volume was made up with autoclaved dH₂O. The reaction mix was overlaid with mineral oil in a 0.5ml microcentrifuge tube to prevent evaporation. PCR reactions were placed in a thermocycler and cycled through the temperatures specified in table 2.1. Microsatellite PCR primers used in chapter 5 were obtained from Resgen (sequence unavailable), sequences of other primers are listed in table 2.1.

In chapter 6, transcriptional activity of AFP and oct4 was detected by RT-PCR. RT-PCR was performed using 1µl of a 1:10 dilution of cDNA (see 2.8.2) as template in a 50µl reaction. Other conditions were as described above. Oct4 and Alpha-fetoprotein (AFP) primers used in chapter 6 were obtained from MWG-biotech.
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<td></td>
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<td>94°C 2 min</td>
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<tr>
<td></td>
<td></td>
<td>72°C 5 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Primer sequences  Primer names are listed in column one. Primer sequences are listed in column two. Cycling conditions and chapters used are listed in columns three and four respectively.

* sequence information of microsatellite primers obtained from Resgen is unavailable.

### 2.7.4 Restriction enzyme digestion

Restriction enzymes were obtained from Roche. Plasmid DNA was digested with five units of restriction enzyme for each μg of DNA in a microcentrifuge tube. Tubes were incubated for at 37°C for one hour or until digest was observed to have reached completion by agarose gel electrophoresis.
2.8 Molecular biology methods II: RNA

2.8.1 RNA isolation from tissues/cells

All equipment was autoclaved twice or thoroughly cleaned with RNase Zap before use. Unless stated otherwise, samples were kept on ice throughout the procedure. Tissue or cells were placed in a plastic universal (Bibby Sterilin) with approximately 2mls RNAzo1B reagent (AMS Biotechnology)/100mg of tissue or cells. Material was then thoroughly homogenised using a polytron PT 2100 tool previously cleaned with 0.2M NaOH and 2 washes of double autoclaved distilled water. Homogenised material was placed on ice and 1/10 volumes of chloroform was added before shaking vigorously for 15 seconds. The sample was incubated on ice for 5 minutes and centrifuged at 12000g for 15 minutes at 4°C. After centrifugation the upper aqueous phase was transferred to new tube, being careful to avoid protein contamination by accidental carry over of the interphase. One volume of cold isopropanol was added to the sample which was mixed well and incubated for 15 minutes at 4°C. The sample was then centrifuged (12000g; 15 minutes; 4°C), the supernatant removed and the pellet washed with 75% ethanol. The washed pellet was centrifuged (7500g; 8 minutes; 4°C) and dried either on ice (15 minutes) or by incubating in a heat block (Driblock DB-1; Techne) for 2 minutes at 45°C before solubilisation in double autoclaved dH2O. To avoid degradation RNA was stored as an ethanol precipitate. 1/10 volumes of 3M sodium acetate (NaOAc) was added to solubilised RNA then two and a half volumes of absolute ethanol before mixing and storage at -20°C.
2.8.2 cDNA synthesis

cDNA was prepared using the 1st strand cDNA synthesis kit (Amersham Pharmacia). RNA was precipitated, quantitated and diluted in double autoclaved dH2O to a final concentration of 0.625µg/µl. 5µg of RNA in 8µl was denatured (65°C; 10 minutes) and added to 5µl of bulk first strand mix, 1µl 200mM DTT and 1µl 0.2µg/µl NotI-d(T)18 primer. The reaction was allowed to proceed for 1 hour at 37°C before inactivation of the enzyme at 90°C for 10 minutes. cDNA was aliquoted and stored at -20°C.

2.9 Protein methods

2.9.1 Protein isolation from cultured cells

Cells were washed once in ice cold PBS and 2X SDS loading buffer was added (100µl to 1 well of a six well plate). Cells were removed from plasticware using a cell scraper and lysates transferred to a qiashredder column (Qiagen) on ice. Genomic DNA was disrupted by centrifugation of the shredder column (13000g; 2minutes). Samples were then boiled for 5 minutes to allow SDS-protein binding, aliquoted and stored at -80°C.

2.9.2 SDS polyacrylamide gel electrophoresis

Protein lysates in loading buffer (10-20µl) were loaded onto a precast Tris-glycine gel (Invitrogen) using the Xcell surelock mini-cell apparatus (Invitrogen). Using 1X Tris-glycine running buffer, the samples were separated by electrophoresis at 90-100V for two hours.
2.9.3 Western transfer of proteins to PVDF membrane

Using the Xcell surelock blotting module (Invitrogen) a sandwich was constructed consisting of two blotting pads, one piece 3MM paper, polyvinylidene difluoride membrane (PVDF; Hybond-P, Amersham Pharmacia), Tris-glycine gel, one further piece 3MM paper and two more blotting pads. All components were pre-wetted in western transfer buffer except the hydrophobic PVDF membrane which was pre-wetted in methanol prior to transfer buffer. The sandwich was placed in the blot module and transfer allowed to proceed for 1 hour 30 minutes at 100mA. The module was disassembled and the PVDF membrane allowed to air dry before either being stored at 4°C or immunoblotted.

2.10 Immunological methods

2.10.1 Antibodies

Antibodies were used in immunoblotting (IB) and immunochemistry (IC) in chapters three to seven. Primary antibody dilutions, chapters and suppliers are listed in table 2.2.

2.10.2 Antibody probing of PVDF membrane-bound proteins

PVDF membrane was re-hydrated in methanol for 10 seconds. The membrane was then blocked in freshly prepared blocking solution for 2 hours at room temperature with agitation. Primary antibody was mixed at the appropriate dilution (see table 2.2) in fresh blocking solution and incubated overnight at 4°C with agitation. The membrane was then washed 6 times in wash solution (10 minutes each wash; room temperature). HRP-conjugated secondary antibody was mixed at the appropriate
dilution (see table 2.3) in freshly prepared blocking solution and incubated for 2 hours at room temperature with agitation. The membrane was then washed 6 times in wash solution (10 minutes each wash; room temperature). Membranes were then exposed to pre-mixed ECL western reagents (Amersham Pharmacia), wrapped in saranwrap (Saran) and exposed to film (Biomax MS; Kodak) for 15 seconds-5 minutes. Film was developed in a Konica SRX-101A X-ograph machine.

<table>
<thead>
<tr>
<th>1) Antibody</th>
<th>2) Dilution (IB)</th>
<th>3) Dilution (IC)</th>
<th>4) Chapters</th>
<th>5) Supplier</th>
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<td>3, 6, 7</td>
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<td>6</td>
<td>Sigma</td>
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<tr>
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<td>BD</td>
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Table 2.2 Primary antibodies  Name of primary antibody is listed in first column. Dilution of antibody used in immunoblotting (IB) and immunochemistry (IC) detailed in columns two and three respectively. Chapters in which antibodies were used (column four). Antibody suppliers listed in column five, DSHB: Developmental Studies Hybndoma Bank, CST: Cell signalling technology, BD: Becton Dickinson and company biosciences, RD: Research diagnostics.

2.10.3 Removal of bound antibodies from PVDF membrane

PVDF membranes were washed twice in PBS (5 minutes, room temperature). Membranes were incubated in western stripping solution for 30 minutes at 50°C with occasional agitation and washed twice in PBS (5 minutes, room temperature).
<table>
<thead>
<tr>
<th>1) Antibody</th>
<th>2) Dilution (IB)</th>
<th>3) Dilution (IC)</th>
<th>4) Chapters</th>
<th>5) Supplier</th>
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</thead>
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<td>-</td>
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<td>Vector</td>
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Table 2.3 Secondary antibodies Name of secondary antibody is listed in first column. Dilution of antibody used in immunoblotting (IB) and immunocytochemistry (IC) detailed in columns two and three respectively. Chapters in which antibodies were used (column four) and antibody suppliers are listed in column five, CST: Cell signalling technology.

2.10.4 Immunochemistry

Slides of fixed cells were incubated in blocking solution for 1 hour at room temperature. Slides were then incubated with the primary antibody at the appropriate dilution (see table 2.2) in blocking solution overnight at 4°C. Unbound primary antibody was removed by washing 3 times in PBST (20 minutes; room temperature). Slides were then incubated with the secondary antibody at the appropriate dilution (see table 2.3) in blocking solution for 1 hour at room temperature. Unbound secondary antibody was removed by washing 3 times in PBS (20 minutes; room temperature) and slides were mounted. For fluorescent applications slides were mounted in vectashield mounting medium containing propidium iodide (PI; vector labs.) or 4',6-Diamidino-2-phenylindole (DAPI; vector labs).

Prior to immunostaining fixed blastoderms were incubated in blocking solution for 2-3 hours. Blastoderms were then incubated in blocking solution containing the SSEA-1 antibody overnight at 4°C. The following day embryos were washed three times for 20 minutes in PBS. A secondary antibody conjugated to fluorescein isothiocyanate (FITC) was then applied and incubated for one hour at room
temperature in the dark. Secondary antibody was removed by washing three times for 20 minutes in PBST. Embryos were incubated with 1µg/ml Hoechst (H33258) in PBS for 2 minutes to counterstain nuclei and embryos were mounted in mowiol (Fluka).

Paraffin wax embedded tissues were cut into 10µm sections using a rotary microtome HM325 (Microm, Germany). Sections were fixed to polylysine slides (BDH) by baking at 55°C overnight. Paraffin was removed by incubating sections in xylene for five minutes (three times). Slides were re-hydrated by incubation in absolute ethanol for five minutes (twice) followed by incubation in 95% ethanol for five minutes (twice). Slides were washed in dH2O for five minutes (twice) and then in PBS for five minutes. To unmask antigens, slides were incubated in 10mM trisodium citrate (pH 6.0) for 10 minutes at 100°C. Slides were then washed in dH2O for five minutes (three times) and PBS for five minutes. Slides were incubated in blocking solution for 1 hour and then incubated in primary antibody overnight at 4°C. Slides were washed for 20 minutes in PBST (three times) and incubated with AP-conjugated secondary antibody for 1 hour. Slides were washed for 20 minutes in PBST (three times) and incubated in AP staining solution until a blue colour was observed to develop. Slides were counterstained by incubating in nuclear fast red (Vector labs) for 10 minutes and washing in PBS for 2 minutes. After counterstaining, slides were mounted in aquamount.
2.11 **Histological methods**

2.11.1 **Alkaline phosphatase activity**

Staining was performed using the Vector blue alkaline phosphatase substrate kit III or IV (Vector laboratories). Reagents 1,2 and 3 were sequentially added to 100mM Tris-HCl pH 8.2 with mixing at each stage. Staining solution was applied to fixed cells and incubated for at least 25 minutes before washing twice in PBS. Slides were counterstained with nuclear fast red (see 2.10.4) and mounted in aquamount.

2.11.2 **Haematoxylin/eosin stain**

Paraffin wax embedded tissues were cut into 10μm sections using a rotary microtome. Sections were fixed to polylysine slides by baking at 55°C overnight. Paraffin was removed by incubating sections in xylene for five minutes (twice). Sections were dehydrated through 70% ethanol, 80% ethanol, 90% ethanol, 100% ethanol and tap water. Haematoxylin staining was performed for five minutes and slides were washed for one minute in tap water. Sections were then stained with aqueous eosin solution for two minutes and washed for 30 seconds in tap water. Stained sections were then re-hydrated through 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol. Slides were then incubated in xylene for five minutes (twice) to clear residual alcohol. After drying out sections were mounted in DPX mounting solution.

2.11.3 **Oil red O stain**

A working solution was prepared by adding three parts oil red O stock solution to two parts dH₂O and mixing well. After ten minutes the solution was filtered using a
syringe and a 0.2μm filter and added to fixed cells. After ten minutes cells were washed twice in PBS and photographed the same day.
Chapter 3 Murine ES cell isolation and in vitro culture of chicken embryo-derived cells

3.1 Introduction

Production of transgenic animals using an ES cell-based system has only been exemplified in the mouse. Therefore, a thorough understanding of murine ES cell isolation is desirable prior to attempted isolation of chicken ES cell lines.

3.1.1 Murine ES cell isolation

Established protocols for the isolation of murine ES cells are widely used (Robertson, 1987) and have incorporated several changes since the technique was first described (Evans & Kaufman, 1981; Martin, 1981; see 1.3.2.2 and 2.5.6). An adaptation of the protocol described by Robertson (1987) is employed in this chapter. Blastocysts are cultured on layers of feeder cells and enzymatically disaggregated. Subsequently, colonies of undifferentiated cells are sequentially passaged until pure lines of ES cells are derived.

Although murine ES cell isolation is a relatively straightforward procedure, the timing of early passages is critical. Cultures must be monitored daily and colonies passaged when judged to have reached an appropriate size. If colonies of ES cell precursors are passaged when they are too small there may be insufficient cells to establish new colonies. However, if colonies are allowed to grow for too long then ES cell precursors may start to irreversibly differentiate. Therefore a delicate balance between colony expansion and differentiation must be reached.
3.1.2 Culture of explanted chicken embryos and genital ridges

The stage X blastoderm is used as starting material in the ES cell isolation experiments described in 3.2.3 and 3.2.4. The chick embryo is easily accessible at this stage and contains cells capable of contributing to both somatic and germ lineages in chimaeras (Petitte et al., 1990; Pain et al., 1996).

It may also be possible to derive pluripotent cells from chicken PGCs. Mouse EG cells derived from 8.0-8.5dpc PGCs contribute to the germline in chimaeras (Labosky et al., 1994; Stewart et al., 1994). Cultured PGCs from the stage 28 chick genital ridge are capable of contributing to somatic lineages in chimaeras (Park & Han, 2000) and could potentially be used to derive chick EG cells.

The SSEA-1 carbohydrate epitope is expressed in the early mouse embryo and on mouse ES and EG cells (Solter & Knowles, 1978; Matsui et al., 1992). Expression of SSEA-1 is also observed in PGCs of the stage X blastoderm and CBCs in vitro (Karagenc et al., 1996; Pain et al., 1996). Expression of SSEA-1 is downregulated in CBCs following retinoic acid-induced differentiation (Pain et al., 1996). Therefore SSEA-1 was used as a marker of undifferentiated CBCs in the following experiments. SSEA-1 expression by CBCs maintained for longer than 5 days in vitro has not been described.

High TNAP activity is indicative of both undifferentiated cells and PGCs in both the mouse and the chicken (see 1.3.2.2 and 1.4.). In the following experiments, TNAP expression was used to identify PGCs and PGC-derived cells.
3.1.3 **Aims of chapter 3**

1) to become familiar with the techniques involved in murine ES cell isolation and to isolate murine ES cells

2) to isolate pluripotent cell lines from CBCs and chicken PGCs using protocols adapted from mouse ES cell isolation
3.2 Results

3.2.1 Murine ES cell isolation

To become familiar with ES cell isolation techniques, murine ES cell isolation was performed. A total of 163 blastocysts were collected from strain 129 mice and seeded at a density of one blastocyst per well on layers of mitotically inactivated STO feeder cells in 24-well plates. After 5-7 days, blastocyst explants (figure 3.1a) were passaged and replated onto freshly prepared feeder cells. Passage one (P1) cultures were closely monitored for the appearance of colonies of undifferentiated cells. Colonies of undifferentiated cells (figure 3.1b) were observed in 14 of 163 blastocyst P1 cultures. These colonies were dissociated in TEG and replated on fresh feeder cells. Colonies of ES-like cells were observed in three out of fourteen passage two (P2) cultures. During successive subculture of these three ES-like cell lines, two lines differentiated irreversibly and ceased proliferation. One line of ES cells (figure 3.1c) from 163 blastocysts was expanded and stored at -150°C.

3.2.2 SSEA-1 expression in the stage X chicken blastoderm

SSEA-1 expression was intended to identify undifferentiated cells in future chicken ES cell isolation experiments. Expression of SSEA-1 in the intact stage X blastoderm was examined to determine if the epitope could be detected, and to estimate the number of SSEA-1 positive cells present. Six stage X blastoderms were dissected and SSEA-1 expression was detected using an SSEA-1-specific antibody. A secondary antibody conjugated to FITC allowed SSEA-1 positive cells to be visualised (green; figure 3.2a).
Figure 3.1 Murine ES cell isolation  a) Morphology of attached blastocyst on STO feeder cells after five days, b) colony with typical ES cell morphology on STO feeder cells following first passage, c) established ES cell line on gelatin-coated plastic. Scale bars: 100μm.
Figure 3.2 SSEA-1 is expressed by centrally located stage X blastoderm cells  Whole chick blastoderms were immunostained with an SSEA-1 antibody (green) and counterstained with Hoechst (blue); area opaca (ao) and area pellucida (ap) are indicated. a) SSEA-1 positive cells are present in the central portion of the blastoderm, b) all blastodermal cells are visible under UV light, c) fluorescence is not observed following incubation with secondary antibody alone, d) the same view under U.V. light. Scale bars: 1mm.

A similar distribution of SSEA-1 positive cells was observed in all six blastoderms tested. In each case approximately 20-30 SSEA-1 positive cells were located within the area pellucida (ap) of the blastoderm. The morphology of these cells did not differ detectably from other blastodermal cells. A representative blastoderm stained with the SSEA-1 antibody is shown in figure 3.2 (a & b). Following incubation with
secondary antibody alone, no fluorescence was observed in any of six control blastoderms (figure 3.2c & d). Twenty to thirty SSEA-1 positive cells were located within the central portion of the stage X blastoderm.

3.2.3 Culture of CBCs

The stage X blastoderm was used as starting material in chicken ES cell isolation experiments. The procedure for collection of CBCs is represented schematically in figure 3.3 (see also 2.4.5). Freshly laid eggs were collected (figure 3.3a) and stage X blastoderms were dissected using a hair loop (figure 3.3b). Blastoderms were pooled in groups of four in a microcentrifuge tube in CBC medium without growth factors and a single-cell suspension was generated by passing through a 23G needle (figure 3.3c & d). Cells were then plated out at a density of four blastoderms per well in 24-well plates coated with gelatin or containing feeder cells (figure 3.3e). Primary cultures were maintained in vitro and monitored a) for the presence of undifferentiated cells and b) for proliferation of any cell types at three and seven days. Undifferentiated cells derived from chicken, human and mouse embryos have relatively large nuclei and grow in tightly packed colonies of small cells (Pain et al., 1996; Thomson et al., 1998; Evans & Kaufman, 1981; fig3.4a). When mouse or human ES cells are allowed to differentiate, they grow in less tightly packed colonies, assume a flatter morphology and a relatively larger area of the cell is occupied by the cytoplasm (fig 3.4b). Cultures were monitored for the presence of cells with the morphological characteristics associated with undifferentiated cells.
Figure 3.3 Strategy for dissection and dissociation of CBCs  a) Freshly laid eggs are collected, b) the stage X blastoderm is visible as a pale disc on the surface of the yolk, c) blastoderms were dissected from the yolk, using a hair loop, and pooled in microcentrifuge tubes, d) CBCs were washed and dissociated by passing through a 23G needle, e) dissociated cells were plated onto a layer of freshly prepared STO feeder cells (light blue).
Murine ES cells can be isolated in medium supplemented with LIF (Pease et al., 1990; Nichols et al., 1990). To determine if murine LIF could be used to isolate chicken ES cells, 192 stage X blastoderms were plated on gelatin in CBC medium supplemented with 500U/ml murine LIF. An equal number of embryos were cultured in control medium without LIF. CBCs were observed to undergo a brief period of growth during the first 48 hours in vitro. Subsequently, they appeared to differentiate as judged by a change in cell morphology. Typically, CBCs became flattened and were sparsely distributed. Cells with an undifferentiated phenotype were not observed in any cultures. Although proliferation occurred after three days in vitro, no growth was observable after seven days.
Figure 3.5 CBCs in vitro  a) CBC colony after 24 hours culture in medium supplemented with LIF on STO feeder cells, b) high magnification view of CBC colony after 24 hours culture in medium supplemented with LIF on m220 feeder cells, c) CBC colony after 24 hours culture in medium supplemented with LIF, bFGF, SCF, IL-11 and IGF-1 on STO feeder cells, d) high magnification of passage 3 CBC colony after two weeks culture in medium supplemented with LIF, bFGF, SCF, IL-11 and IGF-1 on STO feeder cells. Scale bars 100μm.

Murine ES cells are routinely isolated using feeder cells (Robertson, 1987). Therefore CBC growth was compared on three different types of feeder cell: STO, m220 & CEFs. STO feeder cells support murine ES cell precursors and have been used to isolate murine ES cells (Robertson, 1987; see 3.2.1). The m220 feeder cell line expresses the membrane bound form of SCF, a growth factor necessary for murine EG cell isolation (Matsui et al., 1991; Matsui et al., 1992; see 1.3.2.3). CEFs are primary cell lines that have been shown to support murine ES cells (Yang &
Petitte, 1994) and provide chicken-specific growth factors. CBCs were dissected from 96 embryos and plated out on each of the three feeder cell types and gelatin in CBC medium supplemented with LIF. CBC colonies after 24 hours growth on STO and m220 feeder cells are shown in figures 3.5a and b. CBC proliferation during the first 3-4 days was coincident with differentiation and proliferation was not detected by seven days in culture. Cells with an undifferentiated phenotype were not observed (table 3.1). Growth on different types of feeder cell had not facilitated the propagation of CBCs.

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<td>-</td>
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<tr>
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<tr>
<td>CEF</td>
<td>96</td>
<td>-</td>
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Table 3.1 CBC culture on different feeder cell layers in medium supplemented with LIF
CBCs were grown in medium supplemented with 500U/ml LIF on layers of STO, m220 and CEF feeder cells. Cell growth was observed at day 3 but had ceased by day 7. Undifferentiated CBCs (Undiff.) were not observed.

The effect on CBC proliferation of STO feeder cells combined with exogenously provided growth factor supplements was evaluated. Eight replicates of 96 embryos (768 embryos in total) were cultured on either gelatin or STO feeder cells. CBC culture medium was supplemented with a) 500U/ml LIF, b) 10ng/ml bFGF c) 20ng/ml IGF-1 and d) 500U/ml LIF, 10ng/ml bFGF and 20ng/ml IGF-1 in combination. Subsets of these growth factors have previously been used to isolate murine ES cells, murine EG cells and culture CBCs (Pease et al., 1990; Matsui et al., 1992; Pain et al., 1996). Supplementation with these growth factors did not support
proliferation of cells after seven days *in vitro* and cells with an undifferentiated morphology were not observed (table 3.2).

<table>
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</table>

Table 3.2 Culture of CBCs in medium supplemented with growth factor combinations CBCs were grown in medium containing supplements of growth factors. 500U/ml LIF, 10ng/ml bFGF and 20ng/ml IGF-1 were used, alone or in combination, to supplement culture medium. Cell growth was observed at day 3 but had ceased by day 7. Cells with an undifferentiated morphology were not observed.

Pain *et al.*, (1996) described the isolation of undifferentiated CBCs on STO feeder cells in medium containing IGF-1, LIF, bFGF, 20ng/ml SCF and 10ng/ml IL-11. Ninety-six stage X blastoderms were dissected and cultured on mitotically inactivated STO feeder cells in medium containing these growth factors. An equal number of stage X blastoderms were dissected and cultured on STO feeder cells in control medium without growth factors. In the presence of growth factor supplements, colonies of cells appeared after 3-4 days in all cultures (fig. 3.5c). These colonies had a refractile morphology that prevented identification of nuclei (figure 3.5d). Colonies of refractile cells were passaged by disaggregation in a solution of pronase EDTA using a glass pipette. Refractile cells continued to proliferate for 2-3 weeks or three passages (figure 3.5d). CBCs ceased to proliferate after 2-3 weeks in culture.
3.2.4 CBC colonies isolated in 3.2.3 do not express SSEA-1

A high ratio of nucleus: cytoplasm is a phenotypic indicator of undifferentiated cells. Colonies derived from stage X chicken blastoderms in the presence of STO feeder cells and LIF, bFGF, SCF, IGF-1 and IL-11 had a refractile phenotype, preventing estimation of the nucleus: cytoplasm ratio (figure 3.5d). Expression of SSEA-1 is also indicative of undifferentiated cells, therefore SSEA-1 expression was analysed by immunocytochemistry. CBC colonies were probed with an SSEA-1-specific antibody and a FITC-conjugated secondary antibody. SSEA-1 expression was not observed in CBC colonies (figure 3.6a). Positive staining of murine ES cells demonstrated that the antibody dilution and staining conditions were optimised (figure 3.6b). CBC colonies isolated in 3.2.3 did not express a marker of undifferentiated mouse and chicken cells and did not proliferate. It was concluded that these cells were unlikely to give rise to undifferentiated chicken cell lines.

Figure 3.6 Colonies of blastoderm-derived cells are SSEA-1 negative Cells were stained with an SSEA-1-specific antibody (green; nuclei counter-stained with PI: red). a) Blastoderm-derived colony stained with SSEA-1 antibody, b) murine ES cells stained with SSEA-1. Scale bars: 100μm.
3.2.5 **TNAP is expressed by PGCs in chicken genital ridge**

Murine PGCs can give rise to pluripotent EG cells when cultured in the presence of LIF, bFGF and SCF (Matsui et al., 1992; Resnick et al., 1992). Therefore it may also be possible to isolate chicken EG cells from chicken genital ridge PGCs. SSEA-1 expression and high TNAP activity was expected to identify both PGCs and undifferentiated cells in chicken EG isolation experiments. Prior to chicken EG cell isolation experiments, TNAP expression in stage 28 genital ridge PGCs was investigated. Cryosections of chick genital ridges were prepared and stained for endogenous TNAP activity. Clusters of TNAP positive PGCs were observed in the genital ridge (fig. 3.7).

![Figure 3.7 TNAP expression in stage 28 chick genital ridges](image)

**Figure 3.7 TNAP expression in stage 28 chick genital ridges**  a) TNAP expression (dark brown, black arrows) is observed in the area of the genital ridge colonised by PGCs, scale bar: 400μm, b) clusters of TNAP positive PGCs are visible under higher magnification. Scale bar: 100μm.

3.2.6 **Culture of chicken genital ridge PGCs**

Ninety-six genital ridges were dissected from chick embryos following 5.5 days incubation (stage 28). Cells were dissociated and cultured on a layer of STO feeder
Forty-eight dissociated genital ridges were grown in single wells of a 24-well plate in PGC medium supplemented with LIF, bFGF and SCF. The remaining forty-eight genital ridges were grown on STO feeder cells in control PGC medium without growth factors. Colonies of cells with undifferentiated morphology were frequently observed in genital ridge cultures grown in medium supplemented with growth factors (figure 3.8a) but not in control medium. However, colonies either differentiated following passage (figure 3.8b) or were rapidly overgrown by cells with a fibroblastic morphology. TNAP activity was detected in colonies of cells with undifferentiated morphology and was also detected in some cells with fibroblast morphology in early cultures of PGCs (fig. 3.9a). SSEA-1 expression was detected in colonies of undifferentiated cells but not in fibroblast-like cells (fig 3.9b). Expression of both TNAP and SSEA-1 was undetectable in any cell types after 7 days in culture.

Figure 3.8 Chick PGC-derived cells a) Colony of undifferentiated cells after 24 hours in vitro, b) a PGC-derived colony after 72 hours in vitro. Scale bars: 100μm.
To avoid fibroblast overgrowth, colonies of phenotypically undifferentiated cells were passaged after 24-48 hours. Colonies were passaged either mechanically (135) or enzymatically using TEG (56). Following passage, cultures were monitored for appearance of colonies of cells with undifferentiated morphology. Cells with an undifferentiated morphology were frequently observed in cultures passaged mechanically. However, they ceased to proliferate after 48-72 hours and showed changes in morphology associated with differentiation. These included a relatively large area of cytoplasm compared to nucleoli and a flatter, more spread out appearance. Fibroblastic cells frequently persisted in cultures after passage and colonies of undifferentiated cells rapidly became overgrown. It was not possible to completely remove fibroblastic cells even by early passage of PGC colonies after 24-48 hours.

Figure 3.9 Cells derived from stage 28 genital ridges are TNAP and SSEA-1 positive a) Colony of cells derived from e5.5 genital ridge and maintained in culture for 48 hours stains positively for TNAP activity (black). Some background staining is observed in cells with a fibroblast-like phenotype. b) The same colony of cells stained with an SSEA-1-specific antibody (green; nuclei counterstained with PI: red). Cells are grown on a layer of STO feeder cells. Scale bars: 400μm.
3.3 Discussion

3.3.1 Mouse ES cell isolation

One line of murine ES cells was isolated from 163 blastocysts. This frequency is low compared to commonly reported frequencies of 10-30% (Robertson, 1987). Inexperience may have contributed to this low frequency of ES cell isolation.

The first problem was judging when blastocyst explants had reached an optimal size for subculture. It is likely that many of the explants picked were too small and epiblast-derived ES cell precursors did not survive the passage process. If explants are dissociated before the epiblast-derived population has expanded, there may be insufficient cells to establish early ES cell colonies. Conversely, some of the explants may have been allowed to proliferate for too long prior to passage. If epiblast-derived cells are not physically isolated from other cell types, they may receive signals that induce irreversible differentiation. Identifying blastocysts optimal for passage is a skill that improves with experience.

Incomplete dissociation of blastocyst explants may also have contributed to low frequency of isolation. The amount of time explants were incubated in TEG solution may have been sub-optimal. In an attempt to protect explants from enzymatic damage, explants were dissociated following incubation in TEG solution for approximately two to three minutes. Other published protocols (e.g. Robertson, 1987) incubate explants for approximately five minutes. By protecting explants from enzymatic damage, ES cell precursors may not have dissociated sufficiently from neighbouring somatic cells. ES cell precursors in proximity to somatic neighbours in
P1 cultures would have been subjected to signals that may have resulted in a differentiation response.

A third difficulty was identification of ES cell colonies in cultures of dissociated explants (P1s and P2s). These colonies appear only transiently in cultures and have a tendency to differentiate after a period of expansion. If these colonies are to be successfully maintained as undifferentiated cells they must be subcultured during this critical period. Inexperience is likely to have resulted in failure to identify small colonies of undifferentiated cells.

3.3.2  *In vitro culture of explanted stage X chicken blastoderms*

Prior to chicken ES cell isolation experiments, expression of the SSEA-1 epitope was characterised in the stage X blastoderm. SSEA-1 is a marker of undifferentiated and early germline cells in the chick (Pain et al., 1996; Karagenc et al. 1996). Approximately 20-30 SSEA-1 positive cells were located within the central portion of the stage X blastoderm. A series of experiments was then conducted to attempt chicken ES cell isolation from the stage X chicken blastoderm. Undifferentiated cell lines were not derived from the chick blastoderm in any of a wide range of conditions. These conditions included LIF-supplemented medium, three types of feeder cells (table 3.1) and medium supplemented with growth factors previously used to isolate undifferentiated chick cells by Pain et al. (1996).
Murine ES cells depend on the cytokine LIF for self-renewal (Smith et al., 1988; Williams et al., 1988). CBC proliferation was not stimulated by mammalian LIF in early cultures and may only occur in established cultures (Pain et al., 1996). Undifferentiated CBCs may require additional factors during in vitro proliferation prior to developing LIF sensitivity. This is supported by the inclusion of additional growth factors by Pain et al. (1996). Although a chicken homologue of mammalian LIF has not been identified, other cytokines from the interleukin-6 family (IL-6) have been characterised in chickens (e.g. CNTF, Helfand, 1976). Members of the IL-6 family have overlapping functions to LIF and most can support murine ES cell proliferation (see 1.5.1.1.). LIF may support established CBC cultures by signalling through a common receptor subunit. The effect of CNTF on CBC proliferation is explored in chapter 7.

Although it is possible to isolate murine ES cells in the absence of feeder cells using LIF-supplemented medium alone (Pease et al., 1990; Nichols et al., 1990), isolation is more commonly performed in the presence of mitotically inactivated feeder cells (Robertson, 1987). Undifferentiated CBCs did not proliferate in the presence of three types of feeder cell: STO, m220 and CEF (table 3.2). Pain et al. (1996), described the isolation of undifferentiated chicken cell lines in either the presence or absence of STO feeder cells. The presence of feeder cells led to increased proliferation in undifferentiated cells, but was not required for the isolation of undifferentiated CBCs. The failure to isolate CBC lines using feeder cells in the presence of LIF suggests that CBCs may have other requirements, not provided by
these feeder cells. Additional growth factors, not produced by these feeder cells may also be required for CBC propagation.

Pain et al., (1996) isolated undifferentiated chicken cells in the presence of LIF, IL-11, bFGF, SCF and IGF-1. A subset of these growth factors was evaluated for the capacity to support CBC proliferation. LIF, bFGF and IGF-1 were added to culture medium, alone or in combination, in the presence or absence of STO feeder layers. CBCs did not proliferate in any of the conditions evaluated (table 3.3). Pain et al. (1996) did not observe increased proliferation of undifferentiated cells in the presence of LIF, however, the combined and individual effects of bFGF and IGF-1 were not evaluated.

CBC-derived cells with a refractile morphology proliferated for 2-3 weeks (table 3.4). These cells ceased to proliferate after 2-3 weeks in culture and did not display the undifferentiated morphology typical of cultured CBCs (Pain et al., 1996) and murine ES cells. Additionally, they did not express SSEA-1 which is expressed by chicken PGCs and cultured CBCs (Karagenc et al., 1996; Pain et al., 1996). It was concluded that they were unlikely to be progenitors of chicken ES cells. One explanation for the failure to propagate CBCs could potentially be a result of differences in cross-species functionality of avian and murine stem cell factor. Pain et al., (1996) observed that murine SCF enhanced the proliferation of undifferentiated chick cells when used in combination with LIF and bFGF. However, this proliferation was increased further when avian SCF was used,
therefore avian SCF was used to routinely culture CBCs. A further explanation is the depletion of retinoic acid in culture medium using a commercially unavailable anti-retinoic acid monoclonal antibody (ARMA; Pain et al., 1996). ARMA is hypothesised to prevent differentiation by removing active retinoic acid, a potent inducer of differentiation, from cultures. The importance of the ARMA to successful isolation of undifferentiated chicken cells could be evaluated by a systematic analysis of commercially available anti-retinoic acid antibodies. An alternative would be to reduce the influence of retinoic acid by treating serum with charcoal prior to using it as medium supplement. Treating serum with charcoal removes retinoic acid. The use of charcoal treated serum is examined in chapter 7.

Despite intensive efforts and careful monitoring, SSEA-1 positive cell lines could not be isolated from stage X blastoderms. A further difficulty in replicating CBC isolation (Pain et al., 1996) is the incomplete description of the method. The details of the early stages of undifferentiated chick cell isolation are sparse although these have since been elaborated in a patent application (US: 6,500,668). In murine ES cell isolation there are many critical considerations in the early stages of culture (see 3.3.1) and these procedures have been described in detail (e.g. Robertson, 1987). Without a detailed description of CBC isolation from the chick blastoderm it is difficult to replicate precisely the method used.

3.3.3 In vitro culture of explanted stage 28 chicken genital ridges

PGCs from the day 5.5 genital ridge (stage 28) were cultured in attempts to isolate chicken EG cells. TNAP was expressed both in genital ridge PGCs (figure 3.7) and
in cells with an undifferentiated morphology \textit{in vitro}. These undifferentiated cells also expressed SSEA-1 and were presumably derived from PGCs. Proliferation of TNAP positive cells \textit{in vitro} was accompanied by rapid overgrowth of fibroblast-like cells in genital ridge primary cultures. In all cases, PGC cultures either became overgrown with faster growing fibroblasts or phenotypically differentiated following passage and ceased to proliferate.

The isolation of pluripotent chick EG cells has not been described. Cells with some of the properties of murine EG cells were isolated by Park & Han (2000). These cells proliferated slowly, requiring passage every 7-10 days and only very early passage cells contributed to chimaeras. Cells maintained in culture for longer periods were not analysed. Therefore, it seems likely that the cells that contributed to chimaeras were PGCs that were maintained in culture. Chicken PGCs can be stimulated to proliferate by growth factors and feeder cells for over 48 hours \textit{in vitro} (Karagenc & Petitte, 2000). In the experiments described in this chapter, PGC-derived cells initially grew well and expressed markers indicative of both PGCs and undifferentiated cells. Extended periods of culture or passage resulted in a loss of undifferentiated morphology and cessation of growth. This behaviour is also indicative of PGCs and not of de-differentiated EG cells.

The isolation of murine EG cells was achieved when bFGF was used to in addition to SCF and LIF in isolation medium (Matsui \textit{et al.}, 1992; Resnick \textit{et al.}, 1992). In this chapter, chicken PGCs were cultured in the presence of the cytokines used to isolate murine EG cells: LIF, bFGF and murine SCF. Chicken EG cells may require species
specific cytokines such as avian SCF (human SCF was used in the experiments of Park & Han, 2000) or require entirely different cytokines whose biological function remains to be characterised.

PGC-derived undifferentiated cells frequently became overgrown by cells with a fibroblast-like morphology. Although co-culture with fibroblast cells has previously been shown to have a positive effect on murine EC cell culture (Rosenthal et al., 1970; Evans, 1972), differentiated cell types are believed to exert a negative effect on murine ES cells during the early stages of isolation (McWhir et al., 1996). If undifferentiated PGC-derived cells could be physically separated from other cell types, they may proliferate for longer in vitro. Physical separation of undifferentiated cells is believed to have facilitated the isolation of CBA ES cells (McWhir et al., 1996; Brook & Gardner, 1997). During these experiments it was not possible to remove undifferentiated cells from fibroblasts by mechanical separation. There are several methods that could potentially be employed to remove differentiated cells.

Immunosurgery has been used to remove trophectoderm cells from the mouse blastocyst in murine ES cell isolation (Martin, 1981; see 1.3.2.2). This approach was especially effective because cells of the ICM are contained within an external layer of trophectoderm cells which protected them by preventing antibody binding. The morphology of the chick embryo prevents the same strategy being applied to chick PGC isolation as surfaces of all cells are would be exposed.
Selective ablation has been used to isolate murine ES cells (McWhir et al., 1996; see 1.3.2.2.) and could potentially be used to deplete cultures of non-PGCs by antibiotic selection. This strategy would require an efficient method of transgene delivery, an appropriately expressed promoter and a gene that rapidly confers antibiotic resistance during the short period of time PGCs remain viable in culture. Currently only transgene delivery is possible and is either inefficient (Love et al., 1994) or has restricted capacity for cloned gene constructs (Harvey et al., 2002a & b). The failure to identify a gene with homology to murine Oct4 (Soodeen-Karamath and Gibbins, 2001) may be potentially be overcome if a chicken gene with homology to murine nanog (Mitsui et al., 2003; Chambers et al., 2003) is identified.

Antibody-based methods of cell sorting could potentially be used to isolate PGCs from fibroblast cells prior to cell culture. Magnetic or fluorescence activated cell sorting (MACS/FACS) can enrich or deplete a cell population on the basis of antibody expression and both techniques have previously been used to isolate murine PGCs (Abe et al., 1996; Pesce & DeFelici 1995). Blastodermal cells have been sorted by FACS following transfection and subsequently contributed to the germline in chimaeras (Speksnijder et al., 1999). The survival rate of cells in vitro following FACS has not been described and may be compromised as FACS has been associated with damage to cell membranes (Seidl et al., 1999). MACS has been used to isolate viable PGCs from chick and quail (Wentworth et al., 1989; Ono et al., 1999). The survival of these cells in vitro was not described and may also be compromised as cells are subjected to a series of antibody incubations and PBS washes in the absence of growth medium.
If signals from fibroblast-like cells are the main reason undifferentiated PGC-derived cells fail to proliferate then separation by one of the methods mentioned may be a requirement prior to proliferation.

Genital ridge PGCs were used as starting material in these experiments because of the high numbers of PGCs present. PGCs can also be readily obtained from chick embryos at the germinal crescent stage (see fig. 1.4b) and from embryonic blood (migratory PGCs; see fig. 1.4d). Although fewer PGCs are present at these stages they may be easier to separate from other cell types than genital ridge PGCs. Migratory PGCs are not associated with other tissue types and remain viable after sucrose density gradient centrifugation (Tajima et al., 1993).

Murine EG cell can be isolated from PGCs between 8dpc and 13dpc. The embryonic stage at which PGCs are isolated has implications for the capacity of derived EG cells to contribute to the germline. EG cells isolated from 13dpc PGCs do not contribute to the germline (Labosky et al., 1994), possibly as a result of the erasure of the paternal imprint (see 1.3.2.3). The effect on germline transmission of PGCs isolated at different stages could be compared. However, this may not be a problem as there is no evidence of an imprinting mechanism in chickens (Nolan et al., 2001).

3.3.4 Conclusion

The failure to isolate CBC lines may be the result of a strain difference in ES cell isolation. The chickens used in the experiments of Pain et al. (1996) are Barred
Rock and White Leghorn. The strain of bird used in these experiments is Lohmann brown which is also a laying strain. Bird strains are selectively bred for phenotypic characteristics and maintain a high level of genetic variability within a particular strain. In contrast, inbred mouse strains are homozygous at many loci and have both predictable genotypes and phenotypes. The stability of the genetic background of inbred mice results in predictable and consistent phenotypes e.g. ES cell isolation frequency from strain 129 and strain CBA. This predictability does not occur in birds as genetic variation is maintained. Therefore, bird “strain” differences in birds are unlikely to affect ES cell isolation frequency.

Murine ES cells are maintained and isolated by stimulation of the gp130 receptor (Yoshida et al., 1994). The biological function of gp130 signalling appears to be maintenance of the epiblast cell population during the condition known as embryonic diapause (Nichols et al., 2001; see 1.5.1.1). Embryonic diapause occurs when the blastocyst is maintained in a viable condition without implanting. If an embryonic diapause mechanism is required for ES cell isolation this is concerning as there is not an equivalent condition in chickens. Although gp130 signalling usually maintains ES cells, murine ES cells can be maintained in the absence of gp130 stimulation (Dani et al., 1998). ES cells can also be maintained by the transcription factor nanog, bypassing the requirement for gp130 signalling (Chambers et al., 2003). Embryonic diapause does not occur in humans and human ES cells are not dependent on LIF. Isolation of human ES cells (Thomson et al., 1998) provides further evidence that an embryonic diapause-related mechanism is not required for ES cell isolation.
The recent discovery that murine ES cell proliferation is enhanced in the presence of biochemical inhibitors (Burdon et al., 1999; see 1.5.1.3.) suggests another possible strategy. If inhibitors of conserved differentiation pathways can be shown to enhance ES cell isolation from mice they may have applications in chicken ES cell isolation. Murine ES cell isolation using MEK/ERK inhibitors is investigated in chapters four and five.
Chapter 4 Increased murine ES cell isolation frequency by MEK/ERK inhibition with PD98059

4.1 Introduction

Pluripotent CBC lines were not established using the approaches described in chapter 3 and it was decided to explore novel approaches to ES cell isolation in mice. Pluripotent stem cells with proven germline transmission have only ever been isolated from mice (see 1.3.2.2.). Therefore novel approaches were tested using the well-characterised murine ES cell isolation procedure as a model for chicken ES cell isolation.

4.1.1 ES cell isolation from CBA mice

Most murine ES cell lines have been derived from mice of the inbred strain 129. It is not possible to isolate ES cells from the CBA strain using standard protocols (see 1.3.2.2.). Novel ES cell isolation strategies have been tested using CBA blastocysts (McWhir et al., 1996; Brook & Gardner, 1997; Schoonjans et al., 2003). Two strategies that have overcome this "strain barrier" (McWhir et al., 1996; Brook & Gardner, 1997) have involved removal of non-ES cell precursors from early embryo cultures. Signals from non-ES cell precursors are believed to stimulate differentiation of epiblast and ES cells. Strategies that reduce the influence of non-ES cell precursors may inhibit differentiation of ES cells and provide new routes to isolation of CBA ES cells.
4.1.2 Inhibition of the MEK/ERK pathway

Murine ES cells are dependent upon the growth factor LIF for proliferation in the undifferentiated state. LIF stimulates a complex of the LIF receptor and gp130 which leads to activation of the MEK/ERK pathway (figure 4.1; see 1.5.1). The drug PD98059 inhibits the MEK/ERK pathway by preventing phosphorylation of MEKI. Phosphorylation of MEKI leads to activation of ERK1 and 2. PD98059 has been shown to enhance proliferation of ES cells and to inhibit differentiation in embryoid
bodies (Burdon et al., 1999). As inhibition of MEK/ERK has a positive effect on ES cell self-renewal it was hypothesised that it may also enhance ES cell isolation by inhibiting differentiation.

4.1.3 Aims of chapter 4

1) to establish the frequency of ES cell isolation from strain 129 blastocysts in the presence of PD98059

2) to determine if MEK/ERK inhibition facilitated ES cell isolation from CBA strain mice
4.2 Results

4.2.1 Phosphorylation of ERK is inhibited by PD98059 in ES cells

Inhibition of MEK/ERK activity by PD98059 was confirmed in murine ES cells following stimulation with LIF. ES cells derived from both strain 129 (HM1; Magin et al., 1992) and 94% strain CBA (Gallagher et al., 2003; see 1.3.2.2.) were treated with 500U/ml LIF in medium supplemented with 25μM PD98059 or control medium. Cell lysates were analysed by SDS-PAGE and western blot using an antibody specific to phosphorylated ERK (pERK, figure 4.2). In the absence of LIF and PD98059, background ERK phosphorylation occurs, possibly as a result of undefined factors contained in residual serum (lanes 1 and 4). Stimulation with 500U/ml LIF leads to an increase in ERK phosphorylation in both strain 129 and 94% strain CBA ES cells (lanes 2 and 5). ERK phosphorylation is dramatically reduced, but still detectable, in the presence of 25μM PD98059 (lanes 3 and 6). The membrane was stripped and probed with an antibody recognising total ERK protein. This revealed that neither LIF stimulation nor PD98059 treatment affected the total level of ERK protein in cell lysates. PD98059 therefore inhibits LIF-mediated ERK phosphorylation in strain 129 ES cells and 94% strain CBA ES cells, confirming the previously published results of Burdon et al. (1999).
Figure 4.2 PD98059 inhibits ERK phosphorylation in 94% strain CBA and strain 129 ES cells

ES cells derived from strain 129 blastocysts or 94% strain CBA blastocysts were cultured in the presence or absence of 25μM PD98059 before stimulation with 500U/ml LIF. Cell lysates were western blotted and probed with an antibody specific to phosphorylated ERK (pERK). ERK phosphorylation levels increase in response to LIF stimulation. ERK phosphorylation is substantially reduced in the presence 25μM PD98059 in both cell types. PD98059 treatment does not affect the levels of total ERK1 and 2.

4.2.2 Strain 129 blastocysts cultured in the presence of PD98059

Mouse blastocysts were cultured in the presence of PD98059 to establish any observable toxic effects. Sixty-three blastocysts were collected and plated one per well on STO feeder layers in 24-well plates. Blastocysts were cultured in ES cell isolation medium supplemented with PD98059 (0μM, 5μM, 10μM, 15μM, 20μM, 25μM, 30μM, 40μM and 50μM). Working stock solutions of PD98059 were solubilised in 100% DMSO which was added to medium at a final concentration of 0.1%, therefore six control blastocysts were cultured in ES cell isolation medium
supplemented with 0.1% DMSO to assess any effects of this compound. PD98059 has limited stability at 37°C, therefore the culture medium was changed every day in all experiments. No morphological differences were observed between treated explants and controls after 6 days in culture (figure 4.3a & b). Blastocyst explants developed \textit{in vitro} and attached to the feeder cell layer as expected. The explants appeared normal at this stage and culture was continued for ES cell isolation.

![Figure 4.3 Blastocysts from strain 129 mice appear normal in the presence of PD98059](image)

\textbf{Figure 4.3} Blastocysts from strain 129 mice appear normal in the presence of PD98059. Attached strain 129 blastocysts (black arrows) on STO feeder cells after 6 days in culture. a) ES cell isolation medium supplemented with 25µM PD98059, b) control medium. Scale bars: 200µm.

\subsection*{4.2.3 ES cell isolation from strain 129 blastocysts in the presence of PD98059}

To determine if ES cells could be isolated in the presence of PD98059, ES cell isolation was attempted using the sixty-three strain 129 explants used in 4.2.2. Explants were cultured on STO feeder layers in ES cell isolation medium
supplemented with PD98059 (0µM, 5µM, 10µM, 15µM, 20µM, 25µM, 30µM, 40µM and 50µM) and disaggregated after 5 to 7 days. Six ES cell lines were isolated in the presence of PD98059 following successive subculture (table 4.1). The presence of PD98059 did not prevent ES cell isolation from strain 129 blastocysts.

<table>
<thead>
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<th>PD98059 (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>DMSO</th>
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<td>6</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
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</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
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</table>

Table 4.1 ES cell isolation from strain 129 blastocysts in the presence of PD98059

Blastocysts from strain 129 mice were cultured in control medium, medium containing PD98059 (5µM to 50µM) or medium containing DMSO. Six ES cell lines were isolated from blastocysts cultured in the presence of PD98059.

The effect of supplementing culture medium with 25µM PD98059 on the overall frequency of ES cell isolation from strain 129 blastocysts was determined. 25µM PD98059 has previously been observed to enhance ES cell proliferation (Burdon et al., 1999) and three ES cell lines were derived from six blastocysts at this concentration. Therefore this concentration of PD98059 was used in the subsequent ES cell isolation experiment. Eighty-four blastocysts were cultured in control ES cell isolation medium and seventy-four blastocysts in medium supplemented with 25µM PD98059. During the course of ES cell isolation, colonies in medium supplemented with 25µM PD98059 were less prone to spontaneous differentiation and expanded over several days without loss of undifferentiated morphology. ES cell lines were isolated from 20/74 blastocysts (27%) in the presence of PD98059 and 10/84 blastocysts (11%) in control medium (table 4.2). This experiment was not carried out blind. After passage four, ES cell lines were maintained in ES medium.
without PD98059 supplements. Statistical analysis of these data by a generalised linear mixed model test established that supplementation with 25μM PD98059 did not lead to a significant increase in ES cell isolation from strain 129 blastocysts (p=0.059). This value is close to but outwith the conventional 5% significance cut off and therefore the approach requires further validation.

<table>
<thead>
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<th>Strain</th>
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</tr>
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<tbody>
<tr>
<td>PD98059 25μM</td>
<td>+</td>
</tr>
<tr>
<td>Blastocysts</td>
<td>74</td>
</tr>
<tr>
<td>ES cell lines</td>
<td>20 (27%)</td>
</tr>
</tbody>
</table>

Table 4.2 Frequency of strain 129 ES cell isolation in the presence of 25μM PD98059 ES cell lines were isolated from 20/74 blastocysts in the presence of 25μM PD98059. ES cell lines were isolated from 10/84 blastocysts in control ES cell isolation medium. When analysed using a generalised linear mixed model, this increase in ES cell isolation frequency is close to but outwith the conventional 5% significance cut off (p=0.059).

4.2.4 ES cells isolated in the presence of PD98059 are dependent on LIF

Murine ES cells are dependent on LIF for self-renewal in the undifferentiated state. LIF dependency was tested in ES cell lines isolated in the presence of PD98059 to determine if they differentiated normally in the absence of LIF. ES cell cultures were established from a single parental flask in culture medium supplemented with LIF and medium without LIF. Cells were cultured in parallel and after 4-5 days the phenotype of the cells cultured in the absence of LIF had noticeably changed (figure 4.4). These cells appeared flat and occupied more area per cell than ES cells cultured in the presence of LIF. ES cells cultured in the absence of LIF had smaller nuclei.
relative to cytoplasmic area, proliferated slowly and eventually stopped dividing. ES cell lines derived in the presence of PD98059 differentiated in the absence of LIF.

Figure 4.4 ES cell lines derived in the presence of 25μM PD98059 are dependent on LIF. a) ES cells derived in the presence of 25μM PD98059 were cultured in the presence of LIF. ES cells divide rapidly, have large nuclei relative to cytoplasmic area and form tight colonies. b) ES cells derived in the presence of 25μM PD98059 were cultured for four days in the absence of LIF. Cells showed signs of differentiation including slow proliferation, greater relative cytoplasmic area and flat morphology. Scale bars: 100μm.

4.2.5 CBA blastocyst explants cultured in the presence of PD98059

Experiments in section 4.2.3 demonstrated that ES cell lines could be derived from strain 129 blastocysts in the presence of 25μM PD98059 with an increase in frequency close to statistical significance. Based on this observation, an ES cell isolation experiment was conducted to evaluate the potential of PD98059 for ES cell isolation from CBA strain blastocysts. Fifty-five blastocysts were collected from CBA mice and ninety control blastocysts from strain 129 mice. ES cell isolation was attempted from twenty-seven CBA and forty strain 129 blastocysts in ES cell isolation medium supplemented with 25μM PD98059. ES cell isolation was also
attempted from twenty-eight CBA and fifty strain 129 blastocysts in control ES cell isolation medium. CBA blastocysts attached to feeder layers in both conditions and blastocyst explants were passaged 5-7 days later. Cultures were closely monitored but colonies of undifferentiated cells were not observed following passage of CBA blastocysts in either of the media. ES cell lines were isolated from 5/40 (12.5%) strain 129 control blastocysts in the presence of 25μM PD98059 and 2/50 (4%) blastocysts in control medium (table 4.3). ES cell lines could not be isolated from CBA blastocysts in the presence of 25μM PD98059.

<table>
<thead>
<tr>
<th>Strain</th>
<th>129</th>
<th>CBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD98059 25μM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Blastocysts</td>
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<td>27</td>
</tr>
<tr>
<td>ES Lines</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.3 EE cell isolation from CBA and strain 129 blastocysts in the presence of 25μM PD98059. ES cell lines were isolated from 5/40 (12.5%) strain 129 blastocysts in the presence and 2/50 (4%) in the absence of 25μM PD98059. ES cell lines were not derived from CBA blastocysts in either the presence (27 blastocysts) or absence (28 blastocysts) of 25μM PD98059.

4.3 Discussion

4.3.1 Summary of results

Inhibition of the MEK/ERK pathway by PD98059 was confirmed in ES cells derived from strain 129 blastocysts and 94% strain CBA blastocysts (Gallagher et al., 2003) by western blot. LIF stimulation of ES cells of both genetic backgrounds resulted in increased ERK phosphorylation. The presence of 25μM PD98059 substantially, but not completely, abolished ERK phosphorylation following stimulation with LIF in
both cell types (figure 4.2). PD98059 has been used to inhibit the MEK/ERK pathway in other cell types including blood monocytes (Najib et al., 2002), neural cells (Sung et al., 2002) and hepatocytes (Kamakura, 2002). PD98059 was therefore predicted to inhibit MEK/ERK activity in murine blastocyst explants during ES cell isolation experiments.

Strain 129 ES cell lines were isolated at elevated frequency in the presence of PD98059. ES cell lines were isolated from 27% of blastocysts in the presence of 25μM PD98059 and 11.9% of blastocysts in control medium (table 4.2). These ES cell lines had typical ES cell morphology and were dependent on LIF for self-renewal. The presence of 25μM PD98059 did not facilitate ES cell isolation from CBA blastocysts.

### 4.3.2 Increased ES cell isolation frequency from strain 129 blastocysts

ES cell isolation frequency in strain 129 blastocysts increased twofold in the presence of 25μM PD98059. This increase in isolation frequency was close to but outwith the conventional 5% level of statistical significance when analysed using a generalised linear mixed model (p=0.059). The effect of PD98059 on ES cell isolation therefore requires further validation.

MEK/ERK inhibition by PD98059 may inhibit differentiation of ES cell precursors during the early stages of ES cell isolation. The early stages of adipocyte differentiation require ERK activation and ES cell differentiation into adipocytes can
be blocked by PD98059 (Aubert et al., 1999; Bost et al., 2002). Activation of MEK/ERK via the intermediates SHP-2 and Grb2 is also implicated in differentiation of early mouse embryos (see 1.5.1.3). Mouse embryos lacking a functional SHP-2 die during embryonic development (Saxton et al., 1997) and Grb2 is essential for egg cylinder development in mouse embryos but dispensable in murine ES cells (Cheng et al., 1998). Likewise, serum response factor, a downstream target of MEK/ERK signalling is required for normal gastrulation but not ES cell self-renewal (Arsenian et al., 1998). The role of MEK/ERK activation in ES cell differentiation has been extensively characterised by Burdon et al. (1999). ES cells showed enhanced self-renewal when the MEK/ERK pathway was blocked, either using mutated receptors, or biochemically with PD98059. Differentiation in ES cell-derived embryoid bodies was also inhibited when PD98059 was included in the culture medium. Collectively this evidence strongly supports the role of PD98059 as an inhibitor of differentiation in ES cells and their precursors.

An indirect effect of MEK/ERK inhibition may be increased STAT3 signalling. STAT3 activation is essential in murine ES cells (Niwa et al., 1998) but is downregulated by ERK2 (Jain et al., 1998). Inhibition of MEK/ERK results in increased STAT3 activity both in PC12 cells (Bonni et al., 1997) and in astrocytes (Ihara et al., 1997). Inhibition of MEK/ERK may therefore increase ES cell isolation frequency in strain 129 mice by increasing STAT3 activity.

ES cell precursors are prone to differentiate in early isolation cultures (Robertson, 1987), possibly as a consequence of interactions with other cell types. The increase
in ES cell isolation frequency observed from strain 129 blastocysts may be a result of MEK/ERK inhibition in other cell types. Brook & Gardner (1997) achieved ES cell isolation from 100% from strain 129 blastocysts when early epiblasts isolated by microsurgery were used instead of whole blastocysts as starting material. Removal of non-ES cells enhanced ES cell isolation frequency in this report and MEK/ERK inhibition may reduce the influence of these cells by a different mechanism. Activation of the MEK/ERK pathway is associated with progression through G1/S in the cell cycle of many somatic cell types (Pages et al., 1993). Proliferation of somatic cells may be prevented by blocking the MEK/ERK pathway with PD98059. Murine ES cells are unusual in that they do not require MEK/ERK activity for progression through the cell cycle (Jirmanova et al., 2002) and would be expected to proliferate in the presence of PD98059. PD98059 may therefore reduce the population of somatic cells relative to ES cell precursors. A potential consequence of this population reduction in somatic cells would be a reduction in the negative influence on ES cell isolation frequency exerted by these cells.

Colonies of ES cells in PD98059 were frequently observed to have greater stability than ES colonies in control medium. ES cell colonies are prone to differentiate unless they are passaged during normal ES cell isolation (Robertson, 1987). In the presence of 25μM PD98059 colonies of cells maintained an undifferentiated morphology over the course of several days without passage. These colonies appeared to be “insulated” from the spontaneous differentiation usually observed in the course of ES cell isolation. This observation is consistent with either reduced
differentiation in ES cell precursors or reduced influence of somatic cells through cell cycle inhibition.

The presence of PD98059 during the process of ES cell isolation raises questions about possible long term effects on the differentiation capacity of these cells. The ES cells isolated in this chapter remained dependent on LIF but the differentiation potential was not comprehensively characterised. ES cells isolated in the presence of PD98059 have been shown by others to contribute to all somatic cell lineages in chimaeras (Hochedlinger & Jaenisch, 2002a; Rideout et al., 2002). The enhanced self-renewal observed in ES cells during MEK/ERK inhibition (Burdon et al., 1999) appears to be reversible as cells can differentiate normally when removed from the presence of PD98059.

4.3.3 PD98059 did not facilitate ES cell isolation from CBA embryos

ES cells were not isolated from CBA blastocysts in the presence of 25μM PD98059. Downregulation of ERK by PD98059 was demonstrated in 94% strain CBA ES cells. Therefore the level of MEK/ERK activity occurring at 25μM PD98059 was sufficient to prevent CBA ES cell isolation or MEK/ERK activity is not the critical factor that prevented CBA ES cell isolation.

Although PD98059 substantially inhibits ERK phosphorylation, phosphorylation of ERK1 and ERK2 is still detectable (figure 4.2). PD98059 inhibits the MEK/ERK pathway by preventing phosphorylation of MEKI. ERK1 and 2 can also be activated
by MEKII which PD98059 inhibits with a lower affinity. The low level of ERK phosphorylation observed in 25μM PD98059 could be either a consequence of incomplete inhibition of MEKI or of MEKII-mediated ERK phosphorylation. The addition of higher concentrations of PD98059 may achieve greater inhibition of MEKI, however concentrations higher than 50μM have previously been shown to impair ES cell growth (Burdon et al., 1999). Although MEKII is inhibited weakly by PD98059, MEKII activation of ERK1 and ERK2 may escape PD98059-mediated inhibition. This possibility is addressed in experiments performed in chapter 5, which make use of U0126, an inhibitor with equal affinity for ERK1 and ERK2.

CBA ES cells were first isolated by the selective ablation approach (McWhir et al., 1996). This method conferred antibiotic resistance on undifferentiated cells using a neo gene under the control of the oct4 promoter (see 1.3.2.2). Under selective ablation conditions, all somatic cells die in the presence of geneticin. When the MEK/ERK pathway is inhibited with PD98059, it is hypothesised that some somatic cells cease to proliferate. If signals from somatic cells prevent CBA ES cell isolation then selective ablation will more completely prevent these signals than MEK/ERK inhibition. Non-proliferating somatic cells in CBA ES cell isolation experiments using PD98059 may still exert an inhibitory effect on CBA epiblast cells.

Microsurgical removal of somatic cells also allows isolation of CBA ES cells (Brook & Gardner, 1997). The microsurgery approach removed somatic cells from the epiblast mechanically prior to ES cell isolation directly from cultured epiblast. Microsurgery, like selective ablation, is capable of removing 100% of somatic cells.
This contrasts with MEK/ERK inhibition which, in theory, is only capable of preventing somatic cell proliferation.

The isolation of ES cells from several inbred strains, including CBA, using medium conditioned by a rabbit fibroblast feeder cell line transfected with a rabbit genomic LIF construct was recently described (Schoonjans et al., 2003). Although the total numbers of blastocysts cultured was low, ES cell lines were isolated at high efficiency (66% from CBA) and germline transmission was demonstrated from aggregation chimaeras. Unlike the previous reports of CBA ES cell isolation (McWhir et al., 1996; Brook & Gardner, 1997), the influence of somatic cells on ES cell precursors was not directly prevented by somatic cell removal. Rabbit fibroblast conditioned medium would appear to have a either a qualitatively different effect on ES cell precursors to murine LIF or an inhibitory effect on somatic cells. The rabbit fibroblast-conditioned medium was shown to be free of gp130 signalling intermediates of murine or human origin by an ELISA-based assay. However, it is not currently possible to identify rabbit homologues of key signalling intermediates such as IL-6 cytokines and soluble gp130 receptor. In murine ES cells IL-6 cytokines activate STAT3 and the MEK/ERK pathway (see 1.5.1). STAT3 signalling is necessary for murine ES cells (Niwa et al., 1998), but MEK/ERK activity negatively influences ES cell self-renewal (Burdon et al., 1999). The balance between these signals which allows propagation of strain 129 ES cells may not exist in ES cells of other strains. The presence of signalling intermediates of rabbit origin may have altered this balance of signals leading to self-renewal of CBA ES cells.
4.3.4 Conclusion

The increase in ES cell isolation frequency from strain 129 blastocysts supports further investigation of MEK/ERK pathway inhibition in murine and chicken ES cell isolation. If it is assumed that the critical role of MEK/ERK inhibition in murine ES cell isolation is to inhibit differentiation, then MEK/ERK inhibition may be used as part of an alternative strategy. The MEK/ERK pathway is involved in cellular differentiation in chickens (Wu & Howard, 2001) and mice (Rouyez et al., 1997). If inhibition of differentiation is shown to enhance ES cell isolation in mice, it may be hypothesised to enhance ES cell isolation in chickens.

Inhibition of the MEK/ERK pathway may be used as part of a strategy to manipulate other signalling pathways. LIF activation of STAT3 through gp130 is necessary for proliferation of murine ES cells (Niwa et al., 1998). The level of STAT3 activation may be an important factor in ES cell isolation. Activation of MEK/ERK through gp130 is not required by ES cells and may directly downregulate STAT3 activity (Jain et al., 1998). MEK/ERK inhibition may therefore be used to facilitate greater STAT3 activation through gp130 without the differentiation response associated with MEK/ERK. An approach examining the combined effect of increased gp130 stimulation by LIF and potent MEK/ERK inhibition is investigated in chapter 5.

The results reported in this chapter have been published (see Appendix; Gallagher et al., 2003).
Chapter 5  Increased gp130 signalling and inhibition of the MEK/ERK pathway facilitate ES cell isolation from CBA blastocysts

5.1 Introduction

Stimulation of the gp130 receptor in murine ES cells activates at least two signalling pathways. The MEK/ERK pathway is activated through Ras and STAT3 is activated by interaction with gp130 receptor-associated JAKs (see 1.5.1). An alternative inhibitor of the MEK/ERK pathway to PD98059 is U0126. This chapter examines the combined effect of U0126-mediated MEK inhibition and increased STAT3 activation by high levels of LIF on ES cell isolation frequency from CBA strain-derived blastocysts.

5.1.1 The MEK/ERK Inhibitor U0126

In the previous chapter the potential of PD98059 as an enhancer of murine ES cell isolation was investigated. PD98059 strongly inhibits the phosphorylation of MEKI by upstream kinases (figure 5.1a; Alessi et al., 1995). Unphosphorylated MEKI cannot activate the downstream targets: ERK1 and ERK2. However, PD98059-mediated inhibition of the MEK/ERK pathway may be bypassed by MEKII-mediated activation of ERKs 1 and 2. Although PD98059 does have an inhibitory effect on MEKII, its affinity for MEKII is much weaker than for MEKI (Alessi et al., 1995; Dudley et al., 1995).
Figure 5.1 MEK/ERK pathway inhibition by PD98059 and U0126 Ras activates the MEK/ERK pathway through a series of phosphorylation events (indicated by addition of red circles). a) PD98059 inhibits phosphorylation of MEK I (black line) and weakly inhibits phosphorylation of MEK II (broken grey line). PD98059 has no effect on phosphorylated MEKI or II, b) U0126 inhibits phosphorylated MEKI and II with equal affinity, thereby preventing phosphorylation of ERK 1 and 2.
U0126 is a potent MEK/ERK inhibitor that is equally effective against MEKI and MEKII (figure 5.1b; Favata et al., 1998). The mechanism of MEK inhibition by U0126 is different from PD98059 as it directly inhibits the action of phosphorylated MEKI and II (Favata et al., 1998). By inhibiting both MEKI and MEKII the MEK/ERK pathway is blocked more completely.

In the previous chapter, inhibition of MEKI by PD98059 resulted in an increase in ES cell isolation frequency from strain 129 blastocysts that was close to but outwith the conventional 5% significance cut off. Although this approach requires further validation, it was hypothesised that inhibition of MEKII by U0126 may be more effective and extend the applications of this technique to ES cell isolation from refractory CBA strain blastocysts.

5.1.2 LIF-mediated activation of STAT3

ES cells require stimulation of the gp130 receptor to proliferate in an undifferentiated state (Nichols et al., 1994; Yoshida et al., 1994; see 1.5.1). Stimulation of gp130 is frequently achieved by addition of LIF in ES cell culture. LIF stimulation of gp130 results in phosphorylation of STAT3 which is essential for the propagation of ES cells (Niwa et al., 1998). In this chapter the effect of elevated STAT3 phosphorylation levels on CBA ES cell isolation frequency is examined. This was achieved through increased gp130 stimulation using higher concentrations of LIF. However, increased gp130 stimulation also increases MEK/ERK activation levels which have a negative effect on ES cell propagation (Burdon et al., 1999; see 1.5.1.3). Potent MEK/ERK inhibition by U0126 may permit elevated STAT3
activation without the negative effects of increased MEK/ERK activation (figure 5.2).

Figure 5.2 Schematic of CBA ES cell isolation strategy  a) During routine ES cell isolation LIF (red) leads to activation of STAT3 (yellow) and the MEK/ERK pathway (blue), b) a modified ES cell isolation strategy uses culture medium supplemented with a higher concentration of LIF and the MEK inhibitor U0126 (black box). Active STAT3 is amplified in ES cell precursors by increased LIF signalling. A concomitant increase in MEK/ERK activity is prevented by the MEK inhibitor U0126.
5.1.3  **Aim of chapter 5**

1) to determine if increased gp130-mediated signalling in conjunction with MEK/ERK inhibition enables ES cell isolation from CBA mice
5.2 Results

5.2.1 MEK/ERK inhibition by U0126 and STAT3 upregulation in ES cells

STAT3 phosphorylation and MEK/ERK inhibition by U0126 following LIF stimulation was confirmed in strain 129 ES cells before commencing ES cell isolation experiments. Limited availability of embryos prevented the examination of MEK/ERK inhibition in blastocyst explants, therefore ES cells were used as an alternative. Strain 129 ES cells were pre-incubated with U0126 for 24 hours at a range of concentrations (1μM, 2μM, 4μM, 6μM, 8μM, 10μM). Cells were stimulated with 2x10^3 U/ml LIF for 5 minutes and lysates were analysed by SDS-PAGE and western blot (fig. 5.3a). The blot was probed with an antibody recognising the phosphorylated forms of ERK1 and 2 (fig 5.3a: pERK1 & pERK2). High levels of pERK1 and pERK2 were observed in the absence of U0126 following LIF stimulation. ERK phosphorylation by LIF was potently inhibited when U0126 was present. ERK phosphorylation was observed at low levels in 1μM U0126 and was detectable in 2μM U0126, however it could not be detected at concentrations higher than 2μM (figure 5.3a). The membrane was stripped and re-probed with antibody recognising total ERK1 and ERK2. This confirmed that total ERK was unaffected by U0126 and that wells were evenly loaded. U0126 inhibits LIF-mediated ERK phosphorylation in strain 129 ES cells.
ES cells derived from strain 129 blastocysts were stimulated with LIF (50U/ml to 6x10^3 U/ml) and analysed by SDS-PAGE and western blot. The membrane was probed with antibody specific to the phosphorylated form of STAT3 (fig. 5.3b: pSTAT3). Phosphorylation of STAT3 was not detected either in the absence of LIF or at a low concentration (50U/ml). STAT3 phosphorylation was detectable
following the addition of 250U/ml LIF and increased following stimulation with 500U/ml, the concentration of LIF used in routine ES cell culture. STAT3 phosphorylation increased further in higher concentrations of LIF, appearing to reach a plateau above $2 \times 10^3$U/ml (fig 5.3b). The membrane was stripped and re-probed with an antibody recognising total STAT3. This confirmed that total STAT3 levels were unchanged and that wells were evenly loaded. STAT3 activation increased in response to stimulation with LIF at concentrations between 250U/ml and $2 \times 10^3$U/ml.

### 5.2.2 ES cell isolation from CBA blastocysts

To determine if increased gp130 stimulation and MEK/ERK inhibition enabled ES cell isolation from CBA blastocysts, high LIF concentrations and the inhibitor U0126 were evaluated in an ES cell isolation experiment. LIF-mediated ERK phosphorylation was not detected by western blot in the presence of 10μM U0126, therefore 10μM U0126 was initially used to inhibit the MEK/ERK pathway in CBA ES cell isolation experiments. High levels of STAT3 phosphorylation were observed in response to $2 \times 10^3$U/ml LIF in strain 129 ES cells (figure 5.3b). Therefore $2 \times 10^3$U/ml LIF was used to achieve increased STAT3 activation in CBA ES cell isolation experiments.

During preliminary experiments, the presence of 10μM U0126 was observed to have a toxic effect on STO feeder cells. 2μM U0126 did not exhibit toxicity and substantially inhibited ERK phosphorylation (figure 5.3a). 2μM U0126 was therefore used in subsequent CBA ES cell isolation experiments. During preliminary investigations, an ES cell line was derived from a CBA blastocyst in the presence of
2μM U0126 and 2x10³U/ml LIF. To determine the frequency of ES cell isolation, CBA blastocysts were collected and plated at a density of one blastocyst per well on STO feeder cells in 24-well plates. Blastocysts were cultured in medium containing either 2x10³U/ml LIF + 2μM U0126 (53 blastocysts; figure 5.4a); 2x10³U/ml LW (52 blastocysts; figure 5.4b); 500U/ml LIF + 2μM U0126 (54 blastocysts; figure 5.4c) or 2μM U0126 (55 blastocysts; figure 5.4d).

Figure 5.4 CBA blastocyst explant morphology after 6 days in vitro In the presence of 2μM U0126 explants typically developed a peripheral layer of cells with a clearly defined edge (black arrow). a) CBA blastocyst explant grown in 2x10³U/ml LIF + 2μM U0126, b) CBA blastocyst explant in 2x10³U/ml LW, c) CBA blastocyst explant in 500 U/ml LIF + 2μM U0126, layer of peripheral cells marked with black arrow, d) CBA blastocyst explant in 2μM U0126. Scale bars: 100μm.
In the presence of 2μM U0126, blastocyst explants frequently assumed a distinctive morphology, characterised by a rind of endoderm-like cells, with a clearly defined peripheral edge (fig. 5.4a and c). This explant morphology was observed frequently in explants cultured in the presence of U0126, with or without additional LIF.

Figure 5.5 CBA ES cell isolation  
a) CBA blastocyst explant grown in 2μM U0126 and 2×10^3U/ml LIF on STO feeder cells, b) Disaggregated explants gave rise to colonies of undifferentiated cells following enzymatic dissociation, c) Colonies were further subcultured to yield pure colonies of undifferentiated cells, d) Pure lines of putative CBA ES cells were eventually derived. Scale bars: 100μm.

Following enzymatic disaggregation, several of the explants grown in 2×10^3U/ml LIF + 2μM U0126 (e.g. figure. 5.5a) gave rise to round colonies of cells with an ES cell-like morphology (figure 5.5b and c). Successive subculture of these colonies eventually gave rise to pure lines of cells with an ES cell-like morphology (figure...
Twelve putative ES cell lines were isolated from 53 CBA blastocysts cultured in $2 \times 10^3$ U/ml LIF + 2μM U0126 (table 5.1). No cell lines of any phenotype were isolated from blastocysts cultured in the three other treatments. Cell lines were expanded until seventh passage when they were stored at $-150^\circ$C for future analysis, approximately four weeks had elapsed between blastocyst collection and seventh passage. Putative CBA ES cell lines, although unproven for germline transmission, are referred to as CBA ES cell lines for convenience. CBA ES cell lines were identified by a letter followed by a number which referred to the blastocyst from which they were derived. CBA ES cells were routinely cultured in the presence of $2 \times 10^3$ U/ml LIF + 2μM U0126 and maintain an undifferentiated morphology for at least thirty passages. Attempts to reduce the concentrations of either LIF or U0126 resulted in cellular differentiation. CBA ES cell lines were isolated from 22.64% of blastocysts in medium supplemented with $2 \times 10^3$ U/ml LIF + 2μM U0126.

<table>
<thead>
<tr>
<th>Blastocysts</th>
<th>LIF (U/ml)</th>
<th>U0126</th>
<th>ES cell lines</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>$2 \times 10^3$</td>
<td>2μM</td>
<td>12</td>
<td>22.6%</td>
</tr>
<tr>
<td>52</td>
<td>$2 \times 10^3$</td>
<td>-</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>55</td>
<td>-</td>
<td>2μM</td>
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<td>54</td>
<td>500</td>
<td>2μM</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 5.1 Frequency of CBA ES cell isolation in the presence of LIF and U0126 Blastocysts were collected from CBA mice and cultured in medium supplemented with $2 \times 10^3$ U/ml LIF + 2μM U0126, $2 \times 10^3$ U/ml LIF, 2μM U0126 or 500U/ml LIF + 2μM U0126. ES cell lines were isolated from 12/53 (22.6%) blastocysts in medium containing $2 \times 10^3$ U/ml LIF + 2μM U0126. No ES cell lines were derived in the control groups.

A significant component of both the CBA ES cell isolation and monitoring of controls was performed by Dr E. J. Gallagher.
5.2.3 **Confirmation of CBA genetic background by microsatellite analysis**

ES cell lines derived from strain 129 blastocysts are routinely cultured in the tissue culture facility where CBA ES cells were isolated. Polymerase chain reaction (PCR) analyses of polymorphic DNA microsatellites were performed to eliminate the possibility that CBA ES cell lines had arisen by cellular contamination. Genomic DNA was prepared from CBA ES cell lines and control DNA was isolated from strain 129 ES cell lines and CBA-derived primary embryonic fibroblasts (PEFs). Three highly polymorphic microsatellite markers were analysed: D3Mit147 (fig. 5.6a), D4Mit204 (fig. 5.6b) and D9Mit207 (fig. 5.6c). In each case the PCR product size from CBA ES cell lines corresponded to those derived from the CBA PEFs. The PCR product from strain 129 ES cell-derived DNA was different from CBA DNA. This confirmed that the CBA ES cell lines carried the same genomic microsatellite markers as CBA mice at three polymorphic loci.

5.2.4 **U0126 inhibits MEK activation of ERK in CBA ES cells**

ES cells were isolated from CBA blastocysts in the presence of $2 \times 10^3 \text{U/ml LIF}$ and 2$\mu$M U0126. To confirm MEK/ERK inhibition in the conditions used in CBA ES cell isolation, CBA ES cells were incubated with U0126 for four hours at a range of concentrations (0$\mu$M, 1$\mu$M, 2$\mu$M, 4$\mu$M, 6$\mu$M, 8$\mu$M, 10$\mu$M). Serum was removed two hours prior to treatment to reduce background activation of MEK/ERK. Cells were then stimulated with $2 \times 10^3 \text{U/ml LIF}$ in both the continued presence of U0126 and absence of serum for 10 minutes before preparation of lysates on ice. Lysates
were analysed by SDS-PAGE and western blot (fig. 5.7a). This experiment was carried out using two CBA ES cell lines (G4 and L1) and in each case phosphorylation of ERK1 and 2 was partially inhibited by 2μM U0126. Complete inhibition of MEK/ERK was observed in U0126 concentrations higher than 4μM. Western analysis confirmed partial inhibition of MEK/ERK by 2μM U0126 in CBA ES cells.

Figure 5.6 CBA genotype of ES cell lines confirmed by PCR microsatellite analysis: ES cell lines derived from CBA blastocysts were genotyped by PCR at three highly polymorphic loci: (a) D3Mit147, (b) D4Mit204 and (c) D9Mit207. PCR products from CBA ES cells (lanes 4-15) were the same size as PCR products from CBA genomic DNA (lane 2) and differed from strain 129 genomic DNA (lane 3).
Figure 5.7 MEK inhibition by U0126 and STAT3 phosphorylation in strain CBA ES cells a) Cells were cultured in control medium and medium supplemented with U0126 (1μM to 10μM). Cells were stimulated with LW and lysates were blotted and probed with an antibody specific to phosphorylated ERK (pERK). ERK phosphorylation was detected at decreasing levels in the presence of 1μM, 2μM and 4μM U0126. ERK phosphorylation was not detected in the presence of 6μM U0126. U0126 has no effect on the overall level of ERK 1 and 2 as revealed by re-probing with an antibody recognising total ERK, b) ES cells were treated with control medium and medium containing LW (50U/ml to 6x10^3 U/ml). Cell lysates were blotted and probed with antibody specific to the phosphorylated form of STAT3. Phosphorylation of STAT3 (pSTAT3) increased in response to LW stimulation in CBA ES cells. Re-probing with an antibody recognising total STAT3 levels showed that LW stimulation has no effect on the total level of STAT3 in CBA ES cells.

5.2.5 LIF stimulates STAT3 phosphorylation in CBA ES cells

Isolation of CBA ES cells was achieved using 2x10^3 U/ml LIF and 2μM U0126. To confirm increased STAT3 phosphorylation by 2x10^3 U/ml LIF compared to 500U/ml LIF, CBA ES cells were stimulated with a range of concentrations of LIF (50 U/ml
to $6 \times 10^3$ U/ml) and analysed by SDS-PAGE and western blot (fig. 5.7b). STAT3 phosphorylation increased in response to stimulation with higher concentrations of LIF. These results were confirmed in two CBA ES cell lines (G4 and L1). CBA ES cells were isolated in conditions that resulted in increased levels of phosphorylated STAT3.

### 5.2.6 STAT3 phosphorylation in strain 129 and CBA ES cells

To examine the possibility that there is a strain-specific difference in STAT3 activation following LIF stimulation, STAT3 phosphorylation was compared using strain 129 ES cells and CBA ES cells. ES cells were stimulated with LIF (500U/ml, 1000U/ml and 2000U/ml) in the absence of U0126. Cell lysates were analysed by SDS-PAGE and western blot (fig. 5.8).

<table>
<thead>
<tr>
<th>Strain</th>
<th>129</th>
<th>CBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIF (U/ml)</td>
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<td>500</td>
</tr>
<tr>
<td>pSTAT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td></td>
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</tbody>
</table>

**Figure 5.8** LIF activation of STAT3 in strain 129 and CBA ES cells. ES cells were treated with control medium and medium containing LIF (500U/ml to 2000U/ml) in the absence of U0126. Cell lysates were separated by SDS-PAGE, blotted and probed with antibody specific to the phosphorylated form of STAT3 (pSTAT3). The increase in STAT3 phosphorylation in response to LIF stimulation is comparable in strain 129 ES cells and CBA ES cells. Re-probing with an antibody recognising total STAT3 levels shows that LIF stimulation has no effect on the total level of STAT3.
The blot was probed with an antibody specific to phosphorylated STAT3 which revealed that LIF-mediated STAT3 phosphorylation levels are comparable in both CBA and strain 129 ES cells. Re-probing the membrane with an antibody specific to total STAT3 revealed that total levels of STAT3 were not affected by LIF stimulation.

5.3 Discussion

5.3.1 Summary of results

In the previous chapter isolation of CBA ES cells was not achieved in the presence of the MEK/ERK inhibitor PD98059. In this chapter, LIF was used at an increased concentration and the MEK/ERK inhibitor U0126 was used instead of PD98059. The addition of these supplements established conditions where the MEK/ERK pathway was inhibited and STAT3 activation was increased. Under these conditions, ES cell lines were derived from CBA embryos at a frequency of 22.6%. ES cell isolation experiments using medium supplemented with U0126 or LIF alone, or U0126 and a low concentration of LIF did not facilitate CBA ES cell isolation. Therefore, both MEK/ERK inhibition with U0126 and an increased level of LIF activity were required to isolate CBA ES cells.

5.3.2 Factors affecting CBA ES cell isolation

It was hypothesised that CBA ES cell progenitors required greater gp130 stimulation for self-renewal than strain 129 ES cells. Greater gp130 stimulation would be expected to lead to greater levels of STAT3 activation and this was achieved by the addition of $2 \times 10^3 \text{U/ml LIF}$. STAT3 activation by 500U/ml LIF is sufficient to
isolate strain 129 ES cells, however ES cells cannot be isolated from CBA embryos under these conditions. CBA ES cell progenitors may require a higher concentration of LIF to attain the equivalent level of STAT3 activation achieved in strain 129 ES progenitors. Although this could be the result of a difference in ligand: gp130 receptor affinity between CBA and 129 inbred strains, differential STAT3 activation by the same amount of LIF was not observed in strain 129 and strain CBA ES cells (figure 5.8). However, this hypothesis is supported by strain-specific differences in STAT3 activation observed in response to viral infection. Ning et al. (2003) observed differential activation of STAT3 splice variants in resistant and sensitive mouse strains following infection with mouse hepatitis virus type III. Alternatively, CBA ES cell progenitors may require a greater overall level of STAT3 activation for self-renewal. This hypothesis is supported by the comparable STAT3 activation by LIF observed in strain 129 and strain CBA ES cells in figure 5.8. It would be further supported if strain specific differences in the expression levels downstream targets of STAT3 were identified, however these targets have not been characterised in murine ES cells (Burdon et al., 2002).

Supplementation with $2 \times 10^3$ U/ml LIF was sufficient to isolate CBA ES cells only when 2μM U0126 was also included. This suggests that MEK/ERK inhibition by U0126 has a role in preventing differentiation in ES cells, preventing proliferation in somatic cells or increasing active STAT3 levels.

MEK/ERK pathway inhibition by U0126 may have prevented ES cell precursor differentiation during CBA ES cell isolation. CBA ES cells were isolated in a high
concentration of LIF. This high concentration of LIF is likely to have increased activation of the MEK/ERK pathway and, therefore had a negative effect on ES cell self-renewal (Burdon et al., 1999). There is evidence that differentiation of epiblast cells in vivo is under the influence of MEK/ERK signalling. Mouse embryos in which SHP-2 activity is inhibited do not activate MEK/ERK and fail to gastrulate normally (Burdon et al., 1999; Saxton et al., 1997). Gastrulation is also inhibited by mutation of the serum response factor (srf) gene (Arsenian et al., 1998). This MEK/ERK-dependent transcription factor is dispensable for ES cell self-renewal.

ES cells are developmentally equivalent to epiblast and MEK/ERK activation may also regulate ES cell differentiation. ES cells grown in suspension in the absence of LIF differentiate to form embryoid bodies (EBs; see 1.3.2.2). Differentiation of ES cells in EBs is inhibited when they are grown in the presence of PD98059 (Burdon et al., 1999). Activation of the MEK/ERK pathway is therefore involved in differentiation of epiblast cells in vivo and ES cells in vitro. Inclusion of 2μM U0126 substantially downregulates the MEK/ERK pathway in CBA ES cells (fig. 5.7a) and may have protected ES cells from the harmful effects of increased MEK/ERK activity that are stimulated by high levels of LIF.

Inhibition of MEK/ERK by U0126 may inhibit proliferation of somatic cells. Since transition through the cell cycle requires MEK/ERK activation in many differentiated cell types (see 4.3.2; Pages et al., 1993), inhibition of MEK/ERK in somatic cells may prevent their proliferation. Signals from somatic cells are believed to induce differentiation in ES cell progenitors and removal or reduction of these signals could be important in establishing early ES cells (McWhir et al., 1996; Brook & Gardner,
By inhibiting proliferation of non-ES cells we may reduce their influence through cell-cell signalling.

A third consequence of MEK/ERK inhibition by U0126 could be to indirectly increase the level of STAT3 activation. Inhibition of MEK/ERK has been shown to enhance gp130-mediated STAT3 activity in a variety of cell types (Bonni et al., 1997; Ihara et al., 1997). There is also evidence that ERK2 directly inhibits STAT3 activity (Jain et al., 1998). Burdon et al., (1999) described murine ES cells containing a mutated gp130 receptor unable to activate the MEK/ERK pathway. Self-renewal of these ES cells was not impaired and they could be maintained in 100-fold lower concentrations of LIF. The inclusion of 2μM U0126 may therefore have indirectly contributed to enhanced STAT3 activation. Analysis of LIF-mediated STAT3 activation in strain 129 and CBA ES cells did not reveal a strain-specific difference in the absence of U0126 (figure 5.8). A comparison of STAT3 activation by LIF in the presence of U0126 would reveal if U0126 was capable of indirectly increasing STAT3 phosphorylation.

The failure to isolate CBA ES cells in medium supplemented with PD98059 in chapter 4 supports the hypothesis that increased STAT3 activation is also required. PD98059 inhibits phosphorylation of MEKI and, to a lesser extent, phosphorylation of MEKII (fig 5.1). It was hypothesised that residual ERK activation prevented CBA ES cell isolation in chapter 4, however this is opposed by two observations. Firstly, although PD98059 does not have the same affinity for MEKII as for MEKI (Alessi et al., 1995; Dudley et al., 1995), substantial inhibition of ERK phosphorylation was
observed in the presence of PD98059 (figure 4.2). Secondly, U0126 was used at 2µM concentration which did not completely inhibit ERK phosphorylation in ES cells of either strain 129 (figure 5.3) or strain CBA (figure 5.7). As MEK/ERK inhibition does not have to be complete it may be possible to isolate CBA ES cells in the presence of 25µM PD98059 and 2×10³ U/ml LIF. If CBA ES cells were isolated in PD98059 and 2×10³ U/ml LIF this would further support the hypothesis that increased STAT3 activation is the primary requirement for CBA ES cell isolation. Stronger evidence would be provided if CBA ES cells could be isolated by enhanced STAT3 activation independently of MEK/ERK activation by gp130 stimulation. By uncoupling STAT3 activation from MEK/ERK pathway activity the role of STAT3 in CBA ES cell isolation may be more clearly characterised. This could potentially be achieved by engineering cytokine receptors in CBA ES cells. Receptors with the capacity to activate STAT3, but not MEK/ERK, would allow the role of STAT3 to be investigated. Studies of this type have previously been used to characterise STAT3 and MEK/ERK activation in strain 129 ES cells (Niwa et al., 1998; Burdon et al., 1999).

Increased STAT3 signalling as a route to ES cell isolation is not inconsistent with other reports of CBA ES cell isolation (McWhir et al., 1996; Brook & Gardner, 1997). The removal of somatic cells from CBA ES cell isolation cultures had a positive effect on self-renewal in ES cell precursors. Under normal ES cell isolation conditions, somatic cells appear to provide cues which lead to differentiation of CBA ES cell precursors. ES cell differentiation is accompanied by a rapid downregulation of activated STAT3 (Nemetz et al., 1998). Eliminating somatic cells removes the
source of differentiation and allows STAT3 levels to be maintained. Isolation of CBA ES cells has also been reported using medium conditioned by rabbit fibroblasts transfected with a rabbit genomic LIF vector (Schoonjans et al., 2003). The CBA ES cells were maintained as undifferentiated cell lines and therefore must have sustained the level of STAT3 activation that is required for self-renewal (Niwa et al., 1998). In addition to rabbit LIF, the conditioned medium used in this experiment contained many uncharacterised factors that are likely to have influenced both STAT3 and MEK/ERK activity. Without a breakdown of the components of this medium it is difficult to speculate which aspects of signalling are being affected, although an increase in STAT3 activity may result from rabbit LIF. Other signals may attenuate the level of MEK/ERK activity which would be compatible with the results described in chapter 5.

5.3.3 Conclusion

Increased gp130 stimulation and MEK/ERK inhibition facilitated the isolation of CBA ES cells. This is the first time that manipulation of signalling pathways has been used as a method of CBA ES cell isolation. The MEK/ERK pathway is implicated in many types of cellular differentiation (Seger & Krebs, 1995), therefore isolation in the presence of U0126 may have altered the differentiation capacity of the ES cell lines. Additionally, U0126 was observed to have a toxic effect on feeder cells when used at high (10μM) concentration. If the approach used in chapter 5 is to have broader applicability it is necessary to comprehensively characterise the CBA ES cell lines and determine that they have not been compromised by isolation in the presence of U0126. This characterisation is performed in chapter 6.
Assuming cells isolated by manipulation of signalling pathways are shown to have the defining properties of murine ES cells, including germline competence, the method may be applied to ES cell isolation from other mouse strains and species, including chickens.

Most murine ES cell lines in common use are derived from strain 129 embryos. This can be problematic when studying gene function in mice following HR of ES cells. Inbred mice have strain-specific phenotypic characteristics that complicate observations of gene function (Linder, 2001). Strain 129 mice in particular have a range of abnormalities including poor reproductive performance (Festing, 1976), behavioural abnormalities (Lush, 1988) and brain abnormalities (Livy and Wahlsten, 1997). The ability to isolate murine ES cells from wider variety of inbred strains will allow more accurate study of gene function in inbred strains that do not show the phenotypic abnormalities associated with strain 129.

Manipulation of cell signalling pathways may also provide a new direction for ES cell isolation from other species. The method developed in this chapter was largely based on the work Niwa et al. (1998) and Burdon et al. (1999) who both used chimaeric receptors to elucidate the precise mechanisms of murine ES cell signalling. This information is not available in other species and, ironically, the inability to isolate and manipulate ES cells is one of the main barriers. Human ES cells do not depend on LIF (Thomson et al., 1998) but can be manipulated by HR (Zwaka and Thomson, 2003). It should therefore be possible to elucidate in detail the signalling
mechanisms that operate to maintain human ES cells through the use of chimaeric receptors. Undifferentiated chicken cells have been isolated and maintained in vitro (Pain et al., 1996). If chimaeric receptors could be expressed in these cells following manipulation by HR then signalling mechanisms responsible for self renewal could also be characterised in chickens. In the absence of undifferentiated cell lines that can be manipulated by gene targeting, these types of study are not possible. However there is evidence that the roles of STAT3 and MEK/ERK are conserved between chickens and mice (see 1.5.1 and 1.5.3). Therefore manipulation of these two pathways was explored as an approach to chicken ES cell isolation in chapter 7.
Chapter 6 Characterisation of CBA ES cell lines

6.1 Introduction

The CBA ES cell lines isolated in chapter 5 phenotypically resembled established lines of murine ES cells. Additional characterisation of these cell lines was required to confirm ES cell status. This was particularly important because a modified isolation method may have resulted in abnormalities. Cells were isolated and maintained in culture medium containing the MEK/ERK pathway inhibitor U0126. The MEK/ERK pathway is implicated in many aspects of differentiation (see 1.5.1.3.). Characterisation of CBA ES cells was carried out to establish any major phenotypic consequences of isolation and culture in the presence of U0126.

6.1.1 Characteristics of murine ES cells

ES cells from strain 129 mice were first isolated in 1981 (Evans & Kaufman, 1981, Martin, 1981) and have been extensively characterised (see 1.3.2.2.). Murine ES cells are characterised by large nuclei relative to cytoplasmic volume, express high levels of TNAP and a specific subset of cell surface markers including SSEA-1 (Abbondanzo et al., 1993; Wobus et al., 1984; Solter & Knowles, 1978). The transcription factor oct4 is expressed in ES cells and is tightly down-regulated following ES cell differentiation (see 1.5.2; Scholer et al., 1990b). ES cells are derived from epiblast cells of the early mouse embryo and they retain the capacity to differentiate into the three germ layers (Evans & Kaufman, 1981; Martin, 1981; Brook & Gardner 1997). Differentiation can be induced in vitro using embryoid body intermediates or by tumour formation in vivo following injection into either mice with a compromised immune system or syngeneic mice (see 1.3.2.1.). When
ES cells are introduced into early embryos they re-enter embryonic development (see 1.3.2.2.). Pluripotent ES cells contribute to all embryonic cell lineages in chimaeras including the germ line (Bradley et al., 1984). The capacity to contribute to the germ line is a key characteristic of ES cells which allows the production of ES cell-derived mice by breeding from chimaeras (see fig 1.5). It is prudent to analyse the karyotype of ES cells prior to analysis in chimaeras. Although cells with abnormal karyotypes can differentiate into the three germ layers they rarely proceed through meiosis to produce functional gametes (Stewart & Mintz, 1981). If the CBA ES cells isolated in chapter 5 possess all of these properties they can be considered pluripotent ES cells. The differentiation profile of these ES cells is of particular interest given the inclusion of U0126 in the culture medium.

6.1.2 Aims of chapter 6

1) to characterise cell lines isolated from CBA blastocysts
2) cells were analysed for phenotypic characteristics of established ES cells
3) *oct4* expression at the RNA and protein levels was analysed
4) differentiation, *in vitro* and *in vivo*, was examined
6.2 Results

6.2.1 Phenotype of CBA ES cell lines

The CBA ES cell lines were examined for typical phenotypic characteristics of murine ES cells. CBA ES cell lines were compared to a strain 129-derived ES cell line previously shown to have germline colonising potential (HM1, Magin et al., 1992). All twelve CBA ES cell lines grew in tightly packed colonies and showed a high ratio of nuclei to cytoplasm with prominent nucleoli (figure 6.1 a; CBA ES cell line C2 shown). Strain 129-derived ES cells have similar morphology (figure 6.1b). Five CBA ES cell lines (C2, L1, N2, J1 and P1) were examined for TNAP activity. High levels of TNAP activity were observed in all CBA cell lines tested and in strain 129-derived control ES cells (figure 6.1 c & d; CBA ES cell line J1 shown). Four of the twelve CBA ES cell lines (C2, L1, N2, and P1) were analysed for expression of SSEA-1 by immunocytochemistry using an SSEA-1 specific antibody. SSEA-1 was detected at high levels on all CBA ES cell lines tested and in strain 129-derived ES cells (figure 6.1e & f; CBA ES cell line L1 shown). The CBA ES cells derived in chapter 5 had typical ES cell morphology and expressed two markers indicative of murine ES cells: TNAP and SSEA-1.

6.2.2 Oct4 expression in CBA ES cell lines

Expression of the transcription factor \textit{oct4} is required to establish and maintain undifferentiated murine ES cells (Nichols et al., 98; Niwa et al., 2000). RT-PCR and immunocytochemistry were used to establish \textit{oct4} expression in CBA ES cell lines. Total RNA was isolated from four CBA ES cell lines (K1, C2, H1 and G4).
Figure 6.1 CBA ES cells have morphology and express markers indicative of ES cells. a & b) CBA ES cells and control 129-derived ES cells have large nuclei relative to cytoplasm and grow in tightly packed colonies. c & d) CBA ES cells and control strain 129-derived ES cells express TNAP (blue, nuclei counterstained red), e & f) CBA ES cells and control strain 129 ES cells express the cell surface epitope SSEA-1 (green, nuclei counterstained red). Scale bars: 100μm.
Total RNA was used to generate cDNA. PCR primers specific to \textit{oct4} were used to amplify a band of 520bp by PCR using cDNA as a template. The 520kb band was amplified from the four CBA ES cell lines and control ES cell cDNA (figure 6.2a). No PCR product was observed in control no-RT samples indicating PCR amplification from contaminating genomic DNA was not responsible for the 520kb band. \textit{Oct4} was transcribed in CBA ES cells.

CBA ES cell lines C2 and F4 and strain 129 ES cell line HM1 were probed with an OCT4-specific antibody to determine if OCT4 protein was present. OCT4 expression was visualised by FITC which co-localised with DAPI staining indicating the expected nucleus-specific expression. OCT4 protein expression was detected in >98\% of CBA ES cells and all strain 129 ES cells (figure 6.2b; cell line F4 shown). OCT4 is expressed at the protein level in CBA ES cells.

6.2.3 \textbf{In vitro differentiation of CBA ES cell lines}

To establish that CBA ES cells lines could differentiate \textit{in vitro}, cell lines were used to produce embryoid bodies and cultured in differentiation medium. Endoderm differentiation was detected by \textit{\alpha}-fetoprotein expression. Mesoderm and ectoderm differentiation were identified by cardiac troponin T and \textit{\beta}-tubulin III expression respectively.

CBA ES cell lines F4 and L1 were induced to form embryoid bodies by growth in suspension in the absence of LIF. CBA ES cells formed aggregates which expanded \textit{in vitro} over the next seven days. During the first 48 hours, cells of the outer surface
layer appeared to undergo endoderm-like differentiation (figure 6.3a). Embryoid bodies were grown in suspension for three to seven days. RNA was isolated from a proportion and the remainder were allowed to attach to slides and grown in differentiation medium.

**Figure 6.2 Oct4 is expressed and translated in CBA ES cells** a) *Oct4* expression detected by RT-PCR in four CBA ES cell lines (K1, C2, H1 & G4). A 520bp band specific to *Oct4* cDNA was amplified from strain 129 ES cell cDNA. A 520bp band was also amplified from CBA ES cell cDNA, b) *OCT4* expression is detected in CBA ES cell line F4 and strain 129 ES cell line HMI by immunochemistry. *OCT4* expression visualised with FITC (green) corresponds to staining with the nuclear marker DAPI (blue). >2% of CBA ES cells in the frame do not express OCT4 (e.g. white arrows). Scale bars: 100μm.
Figure 6.3 CBA ES cells differentiate via embryoid bodies into the three germ layers  

a) CBA ES cells form EBs when cultured in suspension in the absence of LIF, b) Expression of α-fetoprotein (endoderm) was detected by RT-PCR, c & d) Expression of the neural marker β-tubulin III (ectoderm) was detected in EB-derived cells, but not CBA ES cells, e & f) Expression of the cardiac marker troponin T (mesoderm) was detected in patches of cells derived from EBs but not CBA ES cells. Scale bars: 100μm.
Endoderm

Alpha-fetoprotein is widely expressed in endoderm-derived cells (Abelev, 1971). The time of onset of α-fetoprotein expression was unknown, therefore total RNA was isolated from embryoid bodies grown in suspension for three or seven days to increase the chance of detecting α-fetoprotein expression. RNA from day three and day seven embryoid bodies was used to generate cDNA and analysed by PCR. PCR primers were designed to amplify an α-fetoprotein-specific 200bp product from cDNA. Alpha-fetoprotein expression was detected in both day three and day seven embryoid bodies (figure 6.3b). Expression of α-fetoprotein was detected in embryoid bodies from both CBA ES cell lines tested (F4 and L1). Alpha-fetoprotein expression was not detected in undifferentiated CBA ES cells prior to differentiation indicating expression was induced during embryoid body formation and growth. Alpha-fetoprotein expression was not observed in control no-RT samples indicating PCR amplification from contaminating genomic DNA had not generated the 200bp band. Thus CBA ES cells differentiated in vitro into cells expressing α fetoprotein, an endoderm specific gene.

Mesoderm

Cardiac troponin T is expressed by a subset of mesoderm-derived cardiac cells (Malouf et al., 1992). After seven days growth in suspension, embryoid bodies were plated in cardiogenesis differentiation medium for 7-10 days. During this period colonies of synchronously beating cells were frequently observed, suggesting differentiation of cardiomyocytes. These cultures were examined for expression of a mesoderm-specific marker, cardiac troponin T, by immunochemistry. Embryoid
body-derived cells were probed with a primary antibody specific to cardiac troponin T and the presence of cardiac troponin T was detected using a FITC-conjugated secondary antibody. Expression was detected in small patches of cells that had previously observed to beat synchronously (figure 6.3e). Expression was detected in embryoid body-derived cells from both CBA ES cell lines tested (F4 and L1). Cardiac troponin T expression was not detected in undifferentiated CBA ES cells. Thus CBA ES cells differentiated in vitro into cells expressing a mesoderm-specific marker.

Ectoderm

Ectoderm differentiation can be detected by β-tubulin III which is expressed in neural cells (Lee et al., 1990). Embryoid bodies were plated in neurogenesis differentiation medium after seven days growth in suspension. Embryoid bodies attached and differentiated for a further 7-10 days. Ectodermal differentiation was examined by using fluorescent immunocytochemistry. Embryoid body-derived cells were probed with a β-tubulin III-specific primary antibody and visualised with a FITC-conjugated secondary antibody. Embryoid body derived cells from CBA ES cell lines F4 and L1 expressed β-tubulin III, indicating neural differentiation (figure 6.3c). Beta-tubulin III expression was not detected in the CBA ES cells before differentiation. Thus CBA ES cell lines have the capacity to differentiate in vitro into cells expressing an ectoderm-specific marker.
In conclusion, CBA ES cells differentiated \textit{in vitro}, via embryoid body intermediates, to express markers of endoderm, mesoderm and ectoderm. Expression of these markers was not detected prior to embryoid body formation.

6.2.4. \textbf{In vivo differentiation of CBA ES cell lines in SCID mice}

The capacity of CBA ES cell lines to differentiate \textit{in vivo} to produce tumours was examined. Strain 129 derived ES cells have previously been shown to differentiate into the three germ layers in teratomas (Martin, 1981; Wobus \textit{et al}., 1984). CBA ES cells (line H1) were injected into the hind leg thigh muscle of two SCID mice (approximately $1 \times 10^7$ cells per mouse). After 35 days, tumours had formed and were visible as bulges beneath the skin. Mice were sacrificed and tumours was dissected, weighed and photographed (figure 6.4a). Tumour 1 weighed 2.23g and tumour 2 weighed 1.38g. Tumours were fixed in paraformaldehyde, embedded in paraffin and cut into sections. Haematoxylin eosin (HE) staining of tumour 1 sections revealed a wide variety of differentiated cell types. Mucous secreting epithelial cells suggesting endoderm differentiation were identified by HE staining (fig. 6.4a). Cells with an epithelial phenotype were identified using an ezrin-specific antibody (figure 6.4c). Ezrin is expressed by intestinal cells and is indicative of endoderm (Bretscher, 1986). Patches of cartilage cells (fig 6.4d) and muscle cells (fig 6.4e), both derived from mesoderm were frequently observed. Immature neural cells, originating from the ectoderm, were also present (figure 6.4f). In conclusion, CBA ES cells differentiated \textit{in vivo} to form teratomas containing cells derived from the three germ layers.
Figure 6.4 *In vivo* differentiation of CBA ES cells in SCID mice

a) Tumours generated from CBA ES cells contained many differentiated cell types identifiable by haematoxylin eosin staining, including b) mucous-secreting epithelium, c) ezrin expressing cells were detected by immunohistochemistry (dark blue), d) cartilage, e) muscle cells, f) immature neural cells. Scale bars in b-f: 100μm.
6.2.5. Preparation of CBA ES cells for chimaera analysis

The most stringent test of an ES cell line is formation of functional germ cells in chimaeras. Prior to using the CBA ES cells for blastocyst injection, karyotype analysis was performed. Metaphase spreads of chromosomes were prepared from four cell lines: F4, G4, H1 and C2. The chromosome complement was counted in 50 metaphase spreads from each line and the percentage of normal (40) chromosome complements was calculated as a percentage. Normal chromosome complements were observed in the majority of chromosome spreads from all four CBA ES cell lines (figure 6.5a and b, table 6.1a & b). Only cell lines with normal karyotype frequency greater than 85% percent are considered for blastocyst injection. All four cell lines tested were above this threshold.

<table>
<thead>
<tr>
<th>CBA ES cell line</th>
<th>Percentage of spreads with 40 chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4p8</td>
<td>88%</td>
</tr>
<tr>
<td>G4p8</td>
<td>94%</td>
</tr>
<tr>
<td>H1p8</td>
<td>96%</td>
</tr>
<tr>
<td>C2p8</td>
<td>96%</td>
</tr>
</tbody>
</table>

Table 6.1 Percentage of normal chromosome spreads in four CBA ES cell lines  Metaphase spreads of CBA ES cell lines were prepared and chromosomes counted. Fifty metaphase spreads were counted for each cell line.

CBA ES cell lines were labelled by stable transfection with the PGKGF plasmid. Transfected cells were clonally expanded before blastocyst injection. ES cell-derived chimaeras were expected to be identified on the basis of agouti coat colour contribution and GFP expression would permit embryo analysis in the event of premature termination.
Two CBA ES cell lines with >85% normal karyotypes (C2 and F4) were transfected with a DNA construct containing a GFP gene under the control of the phosphoglycerate kinase (PGK) promoter. The construct also contained a selectable neomycin gene driven by the SV40 early promoter. The plasmid construct was introduced to the cells by lipofection and cells were cultured for 24 hours. Selection was applied (200μg/ml G418) and after seven days 48 surviving colonies from C2 and F4 (24 each) were picked and expanded. Expression of GFP was monitored and
six lines each from C2 and F4 were chosen for expansion (figure 6.1c & d). These lines were selected for strong expression of GFP and with the least variegation. Variegation occurs when GFP expression is downregulated in a proportion of stably transfected cells. GFP-positive CBA ES cell lines were expanded and a normal chromosome complement was re-confirmed. These cell lines are referred to as CBA(C2 or F4) GFP followed by the number used to identify the original transfected colony.

Contribution of CBA ES cell lines to chimaeras following blastocyst injection is currently under investigation. CBAF4GFP1 cells were injected into 13 C57BL/6 blastocysts and transferred to a pseudopregnant F1 (C57BL/6 x CBA) recipient. Subsequently two chimaeric pups, one of either sex, were born. The female chimaera exhibited strong agouti coat colour contribution to the host blastocyst-derived black colour (figure 6.6). The male chimaera was not identifiable by coat colour, however, under UV light, green fluorescence was observed on skin patches in the ears and feet. This fluorescence was too weak to be photographed but both animals are being tested for germline contribution by backcrossing to C57BL/6 mice.

6.3 Discussion

6.3.1 Summary of results

CBA ES cells were tested for the characteristics typical of established ES cell lines (table 6.2). CBA ES cells displayed phenotypic characteristics associated with established murine ES cell lines. CBA ES cells had relatively large nuclei compared to cytoplasm and grew in densely packed colonies; they expressed high levels of
endogenous TNAP and SSEA-1 (fig.6.1). Oct4 expression is a defining characteristic of murine ES cells (Pesce et al., 1998). CBA ES cells were shown to express Oct4 by RT-PCR (fig. 6.2a) and by immunocytochemistry (fig. 6.2b).

Figure 6.6 Chimaeras derived from CBAF4GFP1 ES cells a) I) age-matched C57BL/6 mouse, II) chimaeric female mouse derived from CBAF4GFP1 ES cells, III) male chimaera, with no obvious agouti contribution to coat colour, b) male (left, front) and female (right, front) chimaeras with agouti-coloured F1 (C57BL/6 x CBA) surrogate mother (centre, back).
In the absence of LIF and U0126, CBA ES cells formed embryoid bodies with endoderm-like cells on the outer surface (fig. 6.3a). CBA ES cells differentiated from embryoid bodies to express markers associated with the three germ layers. Endoderm, ectoderm and mesoderm were detected by expression of α-fetoprotein, β-tubulin III and cardiac troponin T (fig. 6.3). CBA ES cells also differentiated in teratomas containing a wide variety of cell types when injected into SCID mice (fig. 6.4a). Expression of Ezrin and the presence of mucous secreting epithelial cells indicated endoderm differentiation (6.4e and f). Cartilage and muscle (fig 6.4b and c) are derived from mesoderm and early neural cells (fig. 6.4d) derived from the ectoderm were identified by HE staining. CBAGFP ES cells have contributed to hair (fig. 6.6) and skin colour (not shown) in chimaeras which further demonstrates the capacity of these cells to differentiate into ectodermal lineages. The capacity of CBA ES cells for germline transmission following in vivo differentiation following blastocyst injection is currently under investigation.

<table>
<thead>
<tr>
<th>ES cell property</th>
<th>Cell lines tested</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>All CBA ES cell lines</td>
</tr>
<tr>
<td>TNAP activity</td>
<td>C2, J1, L1, N2, P1</td>
</tr>
<tr>
<td>SSEA-1 expression</td>
<td>C2, L1, N2, P1</td>
</tr>
<tr>
<td>Oct4 RT-PCR</td>
<td>C2, G4, H1, K1</td>
</tr>
<tr>
<td>OCT4 immunochemistry</td>
<td>C2, F4</td>
</tr>
<tr>
<td>In vitro differentiation</td>
<td>F4, L1</td>
</tr>
<tr>
<td>Tumour analysis</td>
<td>H1</td>
</tr>
<tr>
<td>Blastocyst injection</td>
<td>CBAF4GFP1</td>
</tr>
</tbody>
</table>

Table 6.2 Summary of CBA ES cell line characteristics The specific CBA ES cell lines tested for each characteristic are listed for each ES cell characteristic.
6.3.2 CBA ES cells and established ES cell lines

When cultured in the presence of $2 \times 10^3$ U/ml LIF and 2μM U0126, CBA ES cells maintained the same phenotypic characteristics as established strain 129 ES cells cultured in 500U/ml LIF. Murine ES cells exist in a developmentally static condition which is maintained by at least three transcription factors. A balance of STAT3, Oct4 and nanog expression are all required to maintain undifferentiated morphology (Niwa et al., 1998; Niwa et al., 2000; Mitsui et al., 2003; Chambers et al., 2003). In the absence of these signals, murine ES cells differentiate, which is accompanied by a change in morphology (see figure 4.4) and downregulation of TNAP and SSEA-1 (Zandstra et al., 2000). CBA ES cells were maintained in conditions which supported a typical ES morphology and expression of high levels of oct4, TNAP and SSEA-1 (figure 6.1 & 6.2). CBA ES cell culture conditions are hypothesised to achieve sufficient STAT3 activation to prevent differentiation. The stimuli required to maintain strain 129 and the strain CBA ES cells described here are different. Although downregulation of oct4, TNAP and SSEA-1 following withdrawal of LIF and U0126 was not examined, when LIF and U0126 were withdrawn, CBA ES cells displayed morphological changes associated with differentiation. These changes in morphology strongly suggest that both LIF and U0126 are required to prevent CBA ES cell differentiation. If expression of Oct4, TNAP and SSEA-1 were downregulated in response to reduced levels of LIF and U0126 this would provide further evidence that they are both required for CBA ES cell self-renewal. Continued expression of oct4, TNAP and SSEA-1 supports the hypothesis that $2 \times 10^3$ U/ml LIF and 2μM U0126 maintain undifferentiated CBA ES cells.
The hypothesised role of LIF in CBA ES cell isolation was to achieve elevated STAT3 activation. High levels of LIF are not known to induce differentiation in ES cells. Inhibition of the MEK/ERK pathway by U0126 would not be expected to prevent self-renewal in CBA ES cells because the MEK/ERK pathway appears to be largely redundant in ES cells (Burdon et al., 1999). MEK/ERK activity regulates transition through the cell cycle in differentiated cells and only appears to become important in the differentiated descendants of ES cells (Burdon et al., 2002). Although U0126 is hypothesised to have a role in preventing CBA ES cell differentiation, some differentiation was observed in the presence of $2 \times 10^3$ U/ml LIF and 2μM U0126. A small proportion of, mostly peripheral, CBA ES cells failed to show OCT4 expression by immunochemistry (fig. 6.2b, white arrows). This is possibly a result of Oct4 downregulation following spontaneous differentiation of CBA ES cells. It is not uncommon for ES cells to differentiate, particularly when grown at low density or when at the periphery of colonies. Although not observed in strain 129 ES cells, spontaneous differentiation of CBA ES cells may indicate normality, as a level of spontaneous ES cell differentiation has been suggested to indicate good differentiation potential (Smith, 2001). U0126 does not appear to completely inhibit spontaneous differentiation in CBA ES cells.

### 6.3.3 Differentiation of CBA ES cells and strain 129 ES cells

CBA ES cells described here have demonstrated the capacity to differentiate into the three germ layers *in vitro* or *in vivo* in teratomas. There was no difference between strain CBA and strain 129 in expression of markers of differentiation *in vitro*. A comparison of differentiation between strain CBA and strain 129 ES cells in
teratomas was not examined, however differentiation of CBA ES cells was consistent with published reports of strain 129 ES cell differentiation (Martin, 1981; Wobus et al., 1984). Differentiation of CBA ES cells in chimaeras has also been demonstrated, however germline transmission from CBA ES cells remains to be shown. The differentiation capacity of CBA ES cells is of particular importance for two reasons. Firstly, CBA ES cells were isolated in the presence of U0126, a drug hypothesised to prevent differentiation. It was therefore necessary to establish that MEK/ERK inhibition during ES cell isolation had not compromised the differentiation capacity of these cells. Secondly, manipulation of cell signalling pathways was intended for use as an approach to ES cell isolation from other species. ES cells from other species would have considerably more applications if they differentiate normally when re-introduced into an early embryo (see 1.3.2.2).

Endoderm

Activation of the MEK/ERK pathway is required for proliferation of endoderm cells including hepatocytes (Coutant et al., 2002) and pancreatic cells (Burns et al., 2000). Therefore the presence of a MEK/ERK inhibitor during isolation may have compromised differentiation into endoderm cells. CBA ES cells differentiated into cells expressing α-fetoprotein in embryoid bodies and ezrin in teratomas. Expression of these markers is indicative of cells of endoderm origin (Abelev, 1971; Bretscher, 1986), and demonstrates that endoderm differentiation is not compromised.
Mesoderm

Cardiac fibroblasts and cartilage cells are both derived from mesoderm and both require MEK/ERK activity for DNA synthesis (Hafizi et al., 1999; Yosimichi et al., 2001). Inhibition of the MEK/ERK pathway during isolation may therefore have compromised the capacity of CBA ES cells to differentiate into mesoderm cells. CBA ES cells differentiated \textit{in vitro} to express cardiac troponin T and \textit{in vivo} into cells with a cartilage and muscle phenotype. Differentiation of CBA ES cells into cardiac and cartilage cells demonstrates that MEK/ERK inhibition during isolation did not prevent subsequent mesoderm differentiation.

Ectoderm

MEK/ERK activity is required for DNA synthesis in neural progenitors (Li et al., 2001) and for proliferation in neuroretina cells (Peyssonaux et al., 2000). MEK/ERK inhibition during isolation may therefore have compromised the capacity of CBA ES cells to differentiate into ectoderm cells. CBA-ES-derived cells expressed β-tubulin III following \textit{in vitro} differentiation and gave rise to primitive neural cells in teratomas. Differentiation of CBA ES cells into β-tubulin III-expressing cells \textit{in vitro}, and cells with primitive neural morphology \textit{in vivo} demonstrates that ectoderm differentiation has not been compromised by MEK/ERK inhibition.

For MEK/ERK inhibition to be used as part of a strategy for isolating ES cells from other species, CBA ES cell differentiation in chimaeras is required. At the time of writing, CBA ES cells have been used to generate chimaeras which are being tested
for germline transmission. In addition to regulating differentiation in the three germ layers, MEK/ERK activity regulates many aspects of mammalian germ cell development (Sun et al., 1999). Although inhibition of the MEK/ERK pathway during ES cell isolation has not inhibited subsequent differentiation into the three germ layers, it may prevent the formation of functional germ cells. Germline transmission from CBA ES cells in chimaeras would eliminate this possibility and support application of the method to ES cell isolation from other species.

6.3.4 Conclusion

CBA ES cells described here have demonstrated the same differentiation potential as strain 129 ES cells, with the exception of germline competence. If germline competence is established in CBA ES cells then they may become an alternative inbred strain to study gene function. CBA ES cells have previously been shown to contribute to the germline in chimaeras (McWhir et al., 1996; Brook & Gardner, 1997; Schoonjans et al., 2003), however, gene modification by HR in this strain has not yet been demonstrated. ES cell isolation by the method described in chapter 5 will provide more cell lines and could potentially be applied to ES cell isolation from other inbred mouse strains. The availability of ES cells from a broader range of mouse strains will address some of the problems associated with gene targeting in strain 129 mice (see 5.3.3)

The study of gene function in animals through HR in ES cells has been limited to the mouse. This is because of the inability to isolate germline competent ES cells in other species. If the method described here could be translated and applied to ES cell
isolation from other species then a new range of animal models would become available. This would have importance in models of human disease where murine pathology is qualitatively different from human pathology, e.g. cystic fibrosis (Gray et al., 1994). ES cells from other species would also be of great interest to the biotechnology industry. For example, cells could be modified by HR and used to create animals capable of producing valuable pharmaceutical proteins (see 1.1).
Chapter 7 Stimulation of STAT3 and MEK/ERK inhibition in chicken blastodermal cell culture

7.1 Introduction

Multipotent CBA ES cell lines were derived using modified isolation medium. This medium contained supplements to manipulate signalling downstream of the gp130 receptor. Adaptation of this approach to chicken ES cell isolation requires manipulation of functionally equivalent pathways.

7.1.1 LIF and CNTF

Murine ES cells are isolated and maintained in medium supplemented with LIF. The isolation of CBA ES cells described in chapter 5 required a high concentration of LIF which resulted in increased STAT3 phosphorylation. It is hypothesised that increased STAT3 phosphorylation was necessary for the isolation of CBA ES cells.

STAT3 activation of by LIF is required for murine ES cell self-renewal (Niwa et al., 1998). A chick homologue of murine LIF has not been identified. CNTF and LIF are both members of the IL-6 family of cytokines and can both maintain murine ES cells (Smith et al., 1988, Conover et al., 1993; see 1.5.1). Chick CNTF has been isolated and characterised (Helfand et al., 1976; Barbin et al., 1984) and could potentially be substituted for LIF in chick ES cell isolation experiments. CNTF stimulates STAT3 phosphorylation in both chick neurons and chick myocytes (Wishingrad et al., 1997; Wang & Halvorsen, 1998) and may therefore stimulate phosphorylation of STAT3 in chick embryo-derived cells. CNTF may provide
equivalent signals to chick embryo-derived cells as LIF delivers to ES cell precursors during murine ES cell isolation.

7.1.2 The MEK/ERK pathway in chicken cells

Inhibition of the MEK/ERK pathway with 2µM U0126 was necessary for the isolation of CBA ES cells. The MEK/ERK pathway is activated by LIF in murine ES cell and their progenitors. CNTF operates through the same receptor subunit as LIF (gp130) and activates the MEK/ERK pathway in a wide variety of cell types (Boulton et al., 1994). Activation of the MEK/ERK pathway is implicated in neural differentiation in chicken cells (Wu & Howard, 2001). Differentiation of chick neurons was inhibited by the MEK/ERK inhibitor PD98059 which was used to inhibit MEK/ERK in murine ES cells in chapter 4. The CBA ES cell isolation technique described in chapter 5 required inhibition of the MEK/ERK pathway in embryo-derived cultures. Inhibition of the MEK/ERK pathway by U0126 may also facilitate chicken ES cell isolation.

7.1.4 Aims of chapter 7

1) to determine if MEK/ERK is activated by CNTF and inhibited by U0126 in CEFs

2) to determine if CNTF stimulates STAT3 phosphorylation in CBCs

3) to evaluate a modified disaggregation protocol

4) to apply conditions that increase STAT3 phosphorylation and inhibit MEK/ERK in chicken ES cell isolation experiments
7.2 Results

7.2.1 The MEK/ERK pathway is inhibited by U0126 in CEFs

To establish if the MEK/ERK pathway could be inhibited by U0126 in chicken cells, CEFs were stimulated with CNTF and examined by western analysis. Analysis of MEK/ERK activation in CEFs by SDS-PAGE allowed U0126-mediated inhibition to be titrated. CEFs were cultured in control medium and medium containing U0126 (1μM, 2μM, 4μM, 6μM, 8μM and 10μM) for four hours. Two hours prior to treatment CEFs were starved of serum to reduce background ERK phosphorylation. In both the continued presence of U0126 and absence of serum CEFs were stimulated with 20ng/ml CNTF for ten minutes. Cell lysates were prepared and analysed by SDS-PAGE and western blot using a phosphorylated ERK (pERK)-specific antibody (figure 7.1). In the absence of U0126 high levels of pERK were detected following stimulation with CNTF. The level of pERK decreased in higher concentrations of U0126 and was undetectable above 4μM. The membrane was stripped and re-probed with an antibody that recognises total ERK protein. The total level of ERK protein was the same in all samples. When CEFs were cultured for longer than 24 hours in medium containing U0126 at concentrations higher than 6μM increased cell death was observed. Thus the MEK/ERK pathway is inhibited by U0126 in CEFs. Therefore U0126 was used to inhibit the MEK/ERK pathway in subsequent chicken ES cell isolation experiments.
Figure 7.1  ERK phosphorylation is inhibited by U0126 in CEFs  CEFs were stimulated with 20ng/ml CNTF in control medium and medium containing U0126 (1μM, 2μM, 4μM, 6μM, 8μM & 10μM). Cell lysates were blotted and probed with an antibody specific to phosphorylated ERK (pERK). a) CNTF-mediated ERK phosphorylation is inhibited by the presence of U0126 in CEFs, b) The total level of ERK protein is unaffected by U0126 as revealed by stripping the membrane and re-probing with an antibody recognising total ERK.

7.2.2  CNTF stimulates STAT3 phosphorylation in CBCs

Disaggregated cells from 16 stage X chick blastoderms were seeded into four double chamber slides (two embryos per chamber) and grown in vitro for 24 hours. CBCs in two of the double chamber slides were stimulated with 20ng/ml CNTF for 10 minutes. Two control slides were not treated with CNTF. Undifferentiated CBCs were identified by morphology before analysis by immunochemistry. Phosphorylated STAT3 was detected at higher levels in undifferentiated CBCs following stimulation with 20ng/ml CNTF (figure 7.2b). Control CBCs did not show elevated levels of phosphorylated STAT3 (figure 7.2a). CNTF treated and control cells were also examined with an antibody recognising total STAT3. CNTF treatment did not affect the intensity of staining with this antibody (figure 7.2c and d). Therefore the overall level of STAT3 was unaffected by CNTF treatment.
Treatment with 20ng/ml CNTF resulted in increased STAT3 phosphorylation in undifferentiated CBCs.

Figure 7.2 CNTF stimulates STAT3 phosphorylation in CBCs CBCs were stimulated with 20ng/ml CNTF. a & b) an antibody specific to phosphorylated STAT3 (pSTAT3) revealed an increase in phosphorylation following CNTF treatment (green; nuclei counterstained with DAPI: blue), c & d) no change in the level of total STAT3 was detected following stimulation with CNTF (green; nuclei counterstained with DAPI: blue). Scale bars: 50μm.

7.2.3 Gentle disaggregation enhances CBC proliferation

The blastoderm disaggregation method used in chapter 3 was hypothesised to be stressful to CBCs, therefore it was compared to two alternative treatments. The three treatments evaluated were: no disaggregation, disaggregation into small clumps of
cells and disaggregation into single cell suspension (table 7.1). Fifteen embryos were dissected intact and seeded directly onto STO feeder cells. All fifteen intact embryos failed to attach to feeder cell layers. Following gentle disaggregation small clumps of cells from fifteen blastoderms were seeded onto STO feeder cells. Colonies from these cells in eleven (73%) of these cultures proliferated rapidly and required passage after 48-72 hours. Some of these colonies had relatively large nuclei compared to cytoplasmic area and expressed SSEA-1 (figure 7.3). Cells from these cultures failed to proliferate following mechanical passage. Cells from fifteen blastoderms were dispersed into a single cell suspension and seeded onto a feeder cell-coated surface. Cells in each of these cultures attached to the surface but failed to proliferate. Dispersal of blastoderms into small clumps improved proliferation of CBCs during early culture and was used in subsequent chicken ES cell isolation experiments.

<table>
<thead>
<tr>
<th>Disaggregation strategy</th>
<th>Embryos</th>
<th>In vitro cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No disaggregation</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Small clumps</td>
<td>15</td>
<td>11 (73%)</td>
</tr>
<tr>
<td>Single cells</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7.1 Gentle disaggregation promotes primary CBC proliferation. Embryos were seeded in culture whole (no disaggregation), in small clumps or as single cells. The number of cultures containing proliferating cells that required passage is listed (passage 1).
CBCs proliferate in the presence of 20ng/ml CNTF and accumulate lipid vesicles following passage

To establish if STAT3 upregulation and MEK/ERK pathway inhibition facilitated chicken ES cell isolation, an ES cell isolation experiment was performed using medium supplemented with CNTF and U0126. Stage X blastoderms were dissected from 409 eggs, gently disaggregated and seeded onto STO feeder cells. Blastoderms were cultured in control medium (103), control medium + 20ng/ml CNTF (102), control medium + 2μM U0126 (102) or control medium + 20ng/ml CNTF + 2μM U0126 (102). CBC cultures were closely monitored and colonies of undifferentiated cells were passaged onto freshly prepared feeder cell layers. Five methods of passage were evaluated. Cells were passaged in 1 mM EDTA solution, 1mM EGTA solution, 0.25% TEG solution, 0.025% pronase EDTA or by mechanical
disaggregation. CBC proliferation was frequently observed following mechanical disaggregation but not after passage using 1mM EDTA solution, 1mM EGTA solution, 0.25% TEG solution or 0.025% pronase EDTA. CBCs were therefore passaged by mechanical disaggregation.

In the absence of CNTF proliferating cells were not observed following passage. In the presence of CNTF some CBC cultures continued to proliferate requiring two (57 cultures in total; table 7.2) or three passages (8 cultures in total; table 7.2).

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Blastoderms/Passage 1</th>
<th>In vitro cell proliferation at passage 2</th>
<th>In vitro cell proliferation at passage 3</th>
<th>In vitro cell proliferation at passage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>103</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ 20ng/ml CNTF</td>
<td>102</td>
<td>26</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>+ 2μM U0126</td>
<td>102</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+20ng/ml CNTF + 2μM U0126</td>
<td>102</td>
<td>31</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7.2 CBCs proliferate in the presence of 20ng/ml CNTF  CBCs were gently disaggregated and cultured in control medium or medium supplemented with 20ng/ml CNTF, 2μM U0126 or 20ng/ml CNTF + 2μM U0126. In the absence of 20ng/ml CNTF cells failed to proliferate after passage 1. Proliferating cells gradually became fewer until undetectable following passage 3.

Proliferation occurred in the presence or absence of 2μM U0126. Following passage, CBCs with undifferentiated morphology were not observed. CBCs at passage 3 ceased to proliferate and appeared to accumulate lipid vesicles. Oil red O staining confirmed that these vesicles contained lipid (figure 7.4). Accumulation of lipid vesicles occurred in all CBC cultures that reached passage 2 or 3 regardless of medium used and method of disaggregation.
Figure 7.4 CBCs accumulate lipid vesicles following passage  CBCs accumulated lipid vesicles identified by staining with oil red O when grown in a) control medium, passage 1, b) control medium + 20ng/ml CNTF, passage 2, c) control medium + 2\mu M U0126, passage 1, d) control medium + 20ng/ml CNTF + 2\mu M U0126, passage 2. Scale bars: 100\mu m.

7.2.5 Medium supplementation with charcoal stripped serum does not prevent accumulation of lipid vesicles in CBCs

CBC culture medium is routinely supplemented with 10% foetal calf serum. The potential of charcoal stripped serum (CSS) to reduce accumulation of lipids was evaluated. Hormones and lipids are depleted from CSS and it was hypothesised that CSS may therefore prevent accumulation of lipid vesicles. Cell proliferation had consistently been enhanced in the presence of 20ng/ml CNTF which was therefore
used in all media in these experiments. Stage X blastoderms (481) were
disaggregated and cultured in control medium (121), control medium and 2μM
U0126 (120), medium supplemented with CSS (120) and medium containing CSS
and 2μM U0126 (120). Undifferentiated colonies of cells were passaged by
mechanical disaggregation. Colonies of TNAP-expressing CBCs were observed in
cultures following disaggregation of the blastoderm (figure 7.5a). CBCs
accumulated lipid vesicles following passage in all culture media (figure 7.5b). CBC
proliferation in general was reduced and fewer colonies in total were observed in
medium supplemented with CSS (table 7.3). During this experiment a line of cells
was isolated in control medium supplemented with FCS that maintained
undifferentiated characteristics at passage 4 and survived to passage 9 (see 7.2.6).

Figure 7.5 CBCs accumulate lipid in the presence of CSS CBCs were grown in culture medium
supplemented with CSS, a) colonies of TNAP positive (blue) CBCs were present in primary cultures, b)
morphology of CBCs changed following passage and lipid vesicles, identified by oil red O staining,
accumulated. Lipid accumulation was observed in all cultures regardless of the presence or absence of
U0126. Scale bars 100μm.
Table 7.3  Medium supplemented with CSS does not support CBC proliferation  Stage X blastoderms were dissociated and grown in medium containing either FCS or CSS with or without 2μM U0126. Cells from all embryos survived to passage 1. Cells consistently survived for longer in the presence of FCS.  *A single cell line derived in control medium was maintained until passage 9 (see 7.2.6).

7.2.6  A CBC line maintained for seven weeks

A line of cells was derived in control medium containing 20ng/ml CNTF and FCS. This cell line first appeared as colonies of phenotypically undifferentiated cells at passage 2 (figure 7.6a). These colonies were passaged by mechanical disaggregation and re-plated onto fresh feeder layers (figure 7.6b). Cells proliferated rapidly during early culture, gradually slowing down until passage 9 when they had ceased to proliferate. Cells grew for a total period of approximately seven weeks (passage 9). During this period cell morphology changed considerably (figure 7.6b & d). Accumulation of lipid vesicles was not observed in this cell line but, spontaneous neural differentiation, identified by morphology, was frequently observed. CBCs at passage 4 were shown to express the cell surface marker SSEA-1 by immunochemistry (figure 7.6c). By passage 9, CBCs ceased to proliferate and had a differentiated morphology (fig. 7.6d). Despite intensive attempts to isolate CBC lines under the same conditions, CBC lines were not isolated from a further 392 cultured blastoderms. A line of cells with some of the characteristics of undifferentiated chicken cells at three to four weeks was maintained in vitro for approximately seven weeks.
A CBC line isolated in the presence of 20ng/ml CNTF A line of CBCs was isolated in control medium and maintained in vitro for nine passages. a) colonies of undifferentiated cells proliferated following 2\textsuperscript{nd} passage of CBCs, b) Following 3\textsuperscript{rd} passage CBCs maintained a compact morphology, c) Cells at passage four expressed the SSEA1 epitope (green, nuclei counterstained with DAPI: blue), d) By passage eight, cell morphology had changed significantly and cells had ceased to proliferate. Scale bars 100\mu m.

7.3 Discussion

7.3.1 Summary of results

STAT3 phosphorylation increased in undifferentiated CBCs following treatment with 20ng/ml CNTF (figure 7.2). U0126 was shown to inhibit the MEK/ERK pathway in CEFs following stimulation with CNTF (figure 7.2). Therefore, prior to
chicken ES cell isolation experiments, STAT3 upregulation and inhibition of the MEK/ERK pathway were established in primary chicken cells.

A major modification was made to the method of blastoderm disaggregation. In earlier experiments (chapter 3), blastoderms were disaggregated into a single cell suspension using the method of Pain et al. (1996). It was hypothesised that a gentler disaggregation protocol may promote CBC proliferation. A comparison of disaggregation techniques showed that disaggregation of the blastoderm into clumps encouraged CBC growth and was used in subsequent experiments.

CBCs maintained in the presence of CNTF and U0126 consistently accumulated lipid vesicles and ceased proliferating by the third passage. Accumulation of lipid vesicles was not prevented by the substitution of CSS in place of untreated serum as a medium supplement. During these experiments, a single line of cells that maintained features of undifferentiated cells was maintained for seven weeks. This line eventually assumed morphology characteristic of differentiated cells and stopped proliferating.

7.3.2 STAT3 stimulation and MEK/ERK inhibition in CBC culture

The potential of STAT3 upregulation by CNTF and U0126-mediated MEK/ERK inhibition in chicken ES cell isolation was examined in 7.2.4. Colonies of CBCs proliferated for longer in vitro in the presence of 20ng/ml CNTF however, no effect was observed on CBC proliferation by 2μM U0126. CBC proliferation in the presence of CNTF was accompanied by a change in morphology. Cells derived from
CBCs accumulated lipid vesicles and ceased to proliferate following a maximum of three passages. Lipid accumulation occurs during adipogenesis and is not a feature associated with undifferentiated cells. The presence of CNTF facilitated culture of CBCs beyond passage 3 but it is unclear whether lipid accumulation was a direct consequence of CNTF signalling or resulted from an extended period of growth in vitro. STAT3 and the MEK/ERK pathway are both involved in adipogenesis, however the specific functions remain to be clearly resolved. STAT3 is activated by IL-6 cytokines in adipocytes (Stephens et al., 1998) but it is not known if STAT3 activation is required for adipogenesis. There is evidence that activation of the MEK/ERK pathway is required for differentiation into adipocytes (Sale et al., 1995; Yarwood et al., 1999) but another report suggests that MEK/ERK pathway inhibition with PD98059 does not inhibit adipogenesis (Font de Mora et al., 1997). MEK/ERK activation may only be required at early stages of adipocyte differentiation (Engelman et al., 1998). Inclusion of 2μM U0126 in CBC culture medium did not inhibit lipid accumulation in CBC-derived cells following passage (7.2.4), however, the MEK/ERK pathway was not completely inhibited as some ERK activation was observed at this concentration (figure 7.1). In the absence of complete inhibition it is not possible to judge if MEK/ERK activation stimulated adipogenesis in CBCs. If increased STAT3 phosphorylation has a role in adipogenesis, CBC culture in a lower concentration of CNTF may support CBC growth without stimulating the accumulation of lipid.

Culture medium is routinely supplemented with FCS which is known to contain hormones and lipids. The presence of hormones and lipids in serum may have
directly contributed to the accumulation of lipid vesicles by CBCs. By depleting serum of hormones and lipids by charcoal treatment it was hypothesised that lipid accumulation in CBCs might be prevented. However, CBCs accumulated lipid vesicles regardless of whether FCS or CSS was used. CBCs also grew more slowly and for less time in CSS supplemented medium than CBCs in control medium. It is likely that CSS was deficient in many factors required for in vitro growth of CBCs.

Chicken ES cells were not isolated in the presence of CNTF and U0126. Assumptions were made about conservation of STAT3 and MEK/ERK pathway function in the maintenance of mouse and chick ES cell precursors. STAT3 activity is required for murine ES cell proliferation (Niwa et al., 1998) which is enhanced by MEK/ERK inhibition (Burdon et al., 1999). In murine cells STAT3 activity is downregulated by MEK/ERK (Jain et al., 1998). Inhibition of the MEK/ERK pathway therefore directly prevents MEK/ERK-dependent downregulation of STAT3. In chicken cells STAT3 may be downregulated by a mechanism independent of MEK/ERK. Therefore, assuming STAT3 activity is required by chicken ES cells, MEK/ERK inhibition may not prevent STAT3 downregulation in chick cells.

Although STAT3 activity was upregulated in CBCs, it may have a different function in precursors of chicken ES cells than murine ES cells. Mechanisms governing self-renewal of undifferentiated cells have only been characterised in murine ES cells (Niwa et al., 1998). Although it was possible to exploit signalling pathways characterised in strain 129 ES cells to isolate strain CBA ES cells (chapter 5), these
pathways may not be relevant when designing strategies for chicken ES cell isolation. In the absence of detailed information regarding self renewal in chicken cells, it was hypothesised that STAT3 may maintain undifferentiated CBCs. CNTF stimulation resulted in an increase in STAT3 activity (fig. 7.2) but did not facilitate the isolation of undifferentiated cell lines. The hypothesis that increased STAT3 activity is required for chicken ES cells is only weakly supported by the results of chapter 7. Although STAT3 activation may be necessary for chicken ES cell isolation, it does not appear to be sufficient. The signalling pathways that operate in murine ES cells were elucidated using chimaeric receptors (Niwa et al., 1998; Burdon et al., 1999). Undifferentiated CBCs can be maintained in culture for long periods of time (Pain et al., 1996) and could potentially be used in a similar approach to characterise the signals responsible for self-renewal. Identification of appropriate signals for self-renewal would allow an ES cell isolation strategy involving stimulation of specific receptors to be used.

MEK/ERK activation by CNTF and MEK/ERK inhibition by U0126 was confirmed by western blot. 2μM U0126 was used in subsequent experiments despite detectable levels of ERK phosphorylation. Low levels of MEK/ERK activity in the presence of 2μM U0126 had not prevented CBA ES cell isolation in chapter 5. An additional benefit of using 2μM U0126 was the lack of toxicity that was previously observed on STO feeder cells (see 5.2.2) or CEFs (see 7.2.2). MEK/ERK inhibition was hypothesised to prevent differentiation in undifferentiated chicken cells on the basis that murine ES cell self-renewal is enhanced in conditions that reduce MEK/ERK activity (Burdon et al., 1999). This hypothesis makes the assumption that the
MEK/ERK pathway controls the same cellular functions in mouse and chicken ES cells. The role of the MEK/ERK pathway in undifferentiated chicken cells has not been characterised and it may not be dispensable. In this case, inhibition of the MEK/ERK pathway would prevent chicken ES cell isolation. As with STAT3, analysis of MEK/ERK function in undifferentiated chicken cells is desirable prior to designing an ES cell isolation strategy based on manipulated cell signalling.

A line of blastoderm-derived cells was isolated and maintained in vitro for seven weeks. This cell line expressed SSEA-1 at passage 4. By passage 9 these cells had ceased to proliferate and cell morphology had changed significantly. As this cell line was unique it is difficult to draw conclusions about what features of the culture method had facilitated its isolation. This cell line was isolated and cultured in medium supplemented with 20ng/ml CNTF. Lipid accumulation was not observed at any stage. This suggests that neither CNTF nor increased MEK/ERK activity was principally responsible for lipid accumulation observed in other cultures. CBCs were frequently observed to spontaneously differentiate into cells with a neural morphology. This spontaneous differentiation is potentially a result of growth in 20ng/ml CNTF, a cytokine known to have a major role neural differentiation (Heller et al., 1996). Attempts to isolate further lines of blastoderm-derived cells, using the same conditions, were unsuccessful.

7.3.3 Conclusion

Undifferentiated chicken cells have previously been isolated and maintained in culture (Pain et al., 1996). There are important differences between the culture
medium used by Pain et al. and the medium used in chapter 7 that could explain the failure to isolate undifferentiated chicken cell lines. The medium used by Pain et al. contained IL-11, IGF-1 and an anti retinoic acid monoclonal antibody (ARMA). The roles of IGF-1 has not been characterised in CBCs, however, IL-11 belongs to the same family of cytokines as CNTF (see 1.5.1) and may be expected to have overlapping effects. Retinoic acid can stimulate adipogenesis (Bost et al., 2002) and the depletion of retinoic acid may have prevented the accumulation of cellular lipid (Pain et al., 1996). However, lipid accumulation was observed in the presence of CSS suggesting other factors may also regulate lipid accumulation. If lipid accumulation could be prevented, prolonged culture of undifferentiated chicken cells in the presence of CNTF and U0126 may be possible.

Despite the failure to isolate undifferentiated ES cell lines from chickens, manipulation of cell signalling pathways may still have application in ES cell isolation. The method could potentially be used to isolate ES cells from other species, especially when there is more information about the maintenance of pluripotency. Extensive efforts are being made to identify the signals that maintain human ES cells (e.g. Lim & Bodnar, 2002). Once these pathways are elucidated, the method could be adapted to the requirements of human ES cells and used to simplify current human ES cell isolation protocols (Thomson et al., 1998; Trounson, 2001).
Chapter 8 Discussion

8.1 Summary of results

The objective of the experiments described in this thesis was to isolate pluripotent chicken cell lines. The techniques used in murine ES cell isolation were applied to chicken ES cell isolation. New strategies were explored using strain CBA mice and applied in chicken ES cell isolation experiments.

In chapter 3, CBCs and genital ridges were explanted and cultured using techniques, feeder cells and growth factor supplements previously applied in murine and chicken ES cell isolation. Undifferentiated chicken cell lines were not established in murine ES cell isolation conditions suggesting that chicken ES progenitor cells have different requirements from murine ES cell progenitors. Chicken ES cell lines were not isolated using conditions similar to those used by Pain et al. (1996). A major difference between the experiments described in chapter 3 and those of Pain et al. (1996) was the depletion of retinoic acid from culture medium, which was not performed in chapter 3. Retinoic acid is a potent activator of differentiation and the depletion may prevent differentiation in early chick cultures. Retinoic acid can also be stripped from medium by pre-treating serum with dextran charcoal (Gellersen et al., 1992). The use of charcoal-stripped serum was examined in chapter 7.

Attempts to prevent differentiation in early murine ES cell cultures focussed on the MEK/ERK inhibitor PD98059 in chapter 4. Although medium supplemented with this drug lead to an increase in frequency of ES cell isolation of from strain 129 mice that was close to but outwith statistical significance, it did not support isolation of
strain CBA ES cells. It was hypothesised that MEK/ERK inhibition could be used to isolate CBA ES cells as part of an expanded strategy in parallel with increased gp130 stimulation.

Increased gp130 stimulation and MEK/ERK inhibition using an alternative inhibitor, U0126, was successfully applied to isolate strain CBA ES cells. It was hypothesised that increased STAT3 activation as a consequence of gp130 stimulation was primarily responsible for CBA isolation and MEK/ERK inhibition suppressed the undesired consequences of this signalling pathway.

CBA ES cells isolated in chapter 5 were characterised in chapter 6 and found to have comparable properties to existing strain 129 ES cell lines. Isolation and maintenance in the presence of U0126 had not impaired the differentiation capacity of CBA ES cells. Contribution to the germline in chimaeras is currently being evaluated.

The method used to isolate strain CBA ES cells was adapted and applied to culture CBCs in chapter 7. This was done in the absence of detailed knowledge of the signals responsible for maintenance of undifferentiated chicken cells. Undifferentiated chicken cell lines were not isolated in medium containing CNTF and U0126 that stimulated STAT3 and inhibited the MEK/ERK pathway respectively. It was concluded that the signals responsible for CBA ES cell isolation may not be the same for chicken ES cell isolation or that other unknown factors preclude chicken ES cell isolation.
8.2 Potential applications of the CBA ES cell isolation method

Many important applications of murine ES cells depend on the capacity to form functional germ cells when introduced into chimaeras (see 1.3.2.2). Therefore, germline transmission from CBA ES cells cultured in the presence of U0126 must be demonstrated before the method can be broadly applied to murine ES cell isolation. Any persistent inhibition of MEK/ERK activity is likely to seriously compromise the differentiation potential of ES cells, including germline potential as MEK/ERK regulates aspects of both male and female germ cell differentiation (DiAgostino et al., 2002; Kalab et al., 1996). The germline potential of these CBA ES cells is under investigation.

ES cells have been isolated from a wide variety of inbred mouse strains at high frequencies using feeder cells transfected with a rabbit genomic LIF vector (Schoonjans et al., 2003). Although expression of rabbit LIF from these feeder cells was not formally demonstrated, rabbit LIF may achieve CBA ES cell isolation by a similar mechanism to that hypothesised to facilitate CBA ES cell isolation. Rabbit LIF may activate STAT3 with greater efficiency than the MEK/ERK pathway in murine ES cell precursors. Western analysis of STAT3 and MEK/ERK phosphorylation following stimulation with rabbit and murine LIF could potentially resolve this possibility. If IL-6 cytokines of rabbit origin were shown to preferentially activate STAT3 over MEK/ERK this would be consistent with the CBA ES cell isolation described in chapter 5.
If CBA ES cells described here demonstrate germline potential then this method may have broad application in ES cell isolation from many inbred mouse strains. ES cells from a range of inbred strains would reduce the current overdependence on strain 129 ES cells (Hooper, 1992). Gene targeting in inbred mice allows the phenotypic consequences of a precise genetic modification to be compared between animals with a controlled genetic background. However, certain inbred mouse strains, particularly strain 129, have associated phenotypic abnormalities (Festing, 1976; Lush, 1988; Livy and Wahlsten, 1997; see 5.3.3). ES cells from a range of inbred strains would provide a choice of background murine phenotypes in which to compare the effects of single gene modifications.

Germline transmission from CBA ES cells described here would support the use of this method in ES cell isolation from other animals. Germline competent ES cells from laboratory animals e.g. rat and zebrafish and commercially important livestock animals e.g. cow and pig have proved elusive for many years (see 1.3.2.2). Isolation of CBA ES cells was only possible because of the detailed information of signalling in murine ES cells (Niwa et al., 1998; Burdon et al., 1999). Therefore, non-murine ES cell isolation strategies are likely to involve a substantial amount of guesswork. However, murine signalling mechanisms may exhibit a greater degree of conservation with other mammalian signalling mechanisms than between the murine and chicken signalling mechanisms examined in chapter 7.

If the CBA ES cells described here do not demonstrate germline competence, the method may still be used to isolate undifferentiated cell lines from livestock species.
ES-like cells from livestock species may be useful as nuclear donor cells in nuclear transfer experiments, as germline competency is not required in nuclear donors. The degree of efficiency in nuclear transfer appears to be inversely related to the degree of differentiation of the nuclear donor cell (Hochedlinger & Jaenisch, 2002b). As the CBA ES cells described here can be maintained in the undifferentiated state for at least thirty passages in culture, they could be potentially be modified by HR in vitro prior to use as nuclear donors.

Isolation of human ES cell lines is another application of this method that does not require germline competence. The medical applications of human ES cells have been the subject of much speculation since their isolation (Thomson et al., 1998). Applications in regenerative medicine include neural regeneration following damage to the central nervous system (Cao et al., 2002) and differentiation into insulin producing cells to treat diabetes type 1 (Kaczorowski et al., 2002). Other suggested uses are drug discovery and toxicity testing in human ES cell-derived hepatocytes (Rambhatla et al., 2003). For all of these applications it would be advantageous to have available human ES cell lines from a range of genotypes. Human ES cell isolation is a skilled procedure which, for legislative reasons, often uses "spare" embryos, donated from couples undergoing fertility treatment. These embryos have already been rejected during fertility treatment and are likely to be poor quality starting material in ES cell isolation experiments. ES cell isolation frequency from these embryos is often low and may be increased by using the method developed in chapter 5. An increased number of human ES cell lines would benefit the medical applications of human ES cell technology.
8.3 Cell signalling and chicken ES cell isolation

The signalling pathways stimulated by LIF in murine ES cells have been characterised in detail (Niwa et al., 1998; Burdon et al., 1999). These studies have made use of gene targeting technology to precisely identify the key signalling intermediates that are required for murine ES cell self renewal. HR has not been demonstrated in undifferentiated CBC lines and regulators of self-renewal in embryonic chicken cells are largely unknown. Without this information it is necessary to draw parallels between mouse and chicken signalling pathways. This approach makes many assumptions of conserved function of growth factors and signalling intermediates.

The maintenance of pluripotency through gp130 has been extensively characterised in the mouse. However, the biological function of gp130 raises doubts whether the mouse is an appropriate model for comparison to the chicken. In vivo, gp130 signalling maintains undifferentiated epiblast cells during embryonic diapause (see 1.5.1.1). Embryonic diapause is the temporary developmental arrest that occurs when females are still lactating following a previous litter. In gp130 null mice, arrested embryos do not resume normal development and do not give rise to ES cells (Nichols et al., 2001). Therefore signals that maintain undifferentiated epiblast cells in vivo function through the same receptor to maintain undifferentiated cells in vitro. Embryonic diapause is not observed in chickens; therefore gp130 signalling may play a different role in the avian embryonic cells. Embryonic diapause is also not observed in humans and gp130 signalling does not maintain human ES cells.
(Thomson et al., 1998). Therefore the signals that maintain human ES cells may be more appropriate for comparison when designing chicken ES cell isolation strategies. These signals have not yet been identified but are the subject of much research effort (Lim & Bodnar, 2002).

Although chick cells do not undergo diapause they can be maintained at low temperatures without loss of developmental potential. Identification of the signals that maintain undifferentiated chicken cells at low temperatures in vivo may inform future strategies to isolate and maintain chicken ES cells in vitro.

Pain et al. (1996) isolated and maintained undifferentiated CBC lines. These cell lines share many of the properties of murine ES cells with the exception that they fail to contribute to the germline in chimaeras after prolonged culture. The depletion of retinoic acid appears to have prevented differentiation in these cell lines. Signalling pathways other than MEK/ERK are involved in cellular differentiation and retinoic acid depletion appears to inhibit differentiation more potently than U0126. Cellular lipid accumulation was observed in many cultures of differentiating chicken embryo-derived cells in chapter 7. This high frequency suggests that the default differentiation pathway in chick embryo cells involves lipid accumulation. Retinoic acid stimulates adipogenesis in murine ES cells by activating the MEK/ERK pathway (Bost et al., 2002). Retinoic acid depletion may inhibit adipogenesis more effectively than MEK/ERK inhibition and thereby facilitate prolonged culture of undifferentiated chicken cells.
Inhibition of the MEK/ERK pathway appeared to reduce the influence of differentiating cells in the CBA ES cell isolation experiments described in chapter 5. Inhibition of other signalling pathways may be more appropriate to inhibit differentiation in chicken embryo cell cultures. For example, activation of p38 MAPK is associated with adipogenesis (Hata et al., 2003) and accumulation of lipid vesicles was a feature of chicken ES cell isolation experiments. Inhibition of p38 MAPK by SB203580 has been shown to inhibit adipogenesis in 3T3 L1 fibroblasts (Engelman et al., 1998). Activation of peroxisome proliferator-activated receptor gamma (ppary) by stress-activated protein kinases (SAPK) is also implicated in adipogenesis (Camp et al., 1999). Commercially available inhibitors of ppary or p38 MAPK may therefore be used to inhibit adipogenesis in chicken ES cell isolation experiments.

Undifferentiated chicken cells (Pain et al., 1996) could potentially be used to elucidate the signalling pathways responsible for maintenance. It may be possible to manipulate these cells by HR to examine signalling mechanisms using chimaeric receptors. This type of study was used to elucidate the signalling pathways that operate to maintain murine ES cells (Niwa et al., 1998; Burdon et al., 1999). HR has been demonstrated in the transformed DT40 transformed chicken cell line (Buerstedde & Takeda, 1991) but not in CBCs. Therefore an attractive alternative strategy may be to employ RNA interference (RNAi). Gene function can be effectively ablated by introducing short RNA oligonucleotides into cells and this method has been used to "knockdown" gene function in murine ES cells (Kunath et al., 2003). RNAi may be used to study gene function where gene targeting is not
practical and could potentially be used to elucidate signalling pathways in embryonic chicken cell lines. Gene function has been studied in whole chick embryos using RNAi (Hernandez-Hernandez et al., 2001; Pekarik et al., 2003). Identification of the key signals that maintain undifferentiated chicken cells would lead to improved design of strategies using biochemical inhibitors.

8.4 Alternative methods for chicken ES cell isolation

ES cells have been isolated from the CBA strain by selective ablation (McWhir et al., 1996) and microsurgery (Brook and Gardner, 1997). These techniques both removed differentiating cells from early cultures to facilitate expansion of ES cell precursors. The MEK/ERK inhibitor U0126 appears to reduce the influence of differentiating cells, however complete removal of differentiated cells may be necessary to facilitate chicken ES cell isolation.

The selective ablation approach used the oct4 promoter to drive neo in undifferentiated transgenic CBA ES cell precursors (see 1.3.2.2; McWhir et al., 1996). It has not been possible to apply this approach to chicken ES cell isolation because a chicken homologue of oct4 has not been identified (Soodeen-karamath & Gibbins, 2001). The selective ablation method requires a promoter that is only expressed in undifferentiated cells. Nanog is a transcription factor with a stem cell specific expression pattern (Mitsui et al., 2003; Chambers et al., 2003). The nanog promoter could potentially be used in a selective ablation approach. If a chicken homologue of nanog was identified, its promoter could potentially be used in a selective ablation approach. Nanog could be employed to drive antibiotic resistance
in the undifferentiated cell population of primary CBC cultures. Other cell types would be removed by the antibiotic and this may facilitate expansion of undifferentiated cells. However, this approach would also require an efficient method of transgene delivery (see 1.3.1.2 and 1.4.1).

Physical methods to remove non-ES precursors have been successfully used in murine ES cell isolation (Martin, 1981; Brook & Gardner, 1997). There are several approaches that could be taken to enrich embryo cell cultures for precursors of chicken ES cells. SSEA-1 positive CBCs could potentially be enriched by magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS). The drawback of these procedures is that they require a single cell suspension and cause considerable stress to cell membranes (Seidl et al., 1999). As CBCs did not proliferate following single cell suspension (see chapters 3 and 7) these techniques may not yield viable cells. An antibody-based method of purification may enrich germline competent CBCs. Stage X chicken blastodermal cells are undifferentiated and contribute to chimaeras when transferred to recipient embryos (Petitte et al., 1990, Carscience et al., 1993, Thoraval, 1994). Although CBCs contribute to the germ lineage in chimaeras, PGC fate may be specified by the inheritance of maternally-derived cytoplasmic factors (Tsunekawa et al., 2000; see 1.4.1). Only Cvh-positive blastodermal cells may contribute to the germ lineage. This contrasts with mouse germ cells, which have not yet been determined in the epiblast (Tam & Zhou, 1996; see 1.2.1). Germline competent CBCs express the SSEA-1 epitope in the stage X blastoderm which could potentially be used to purify CBCs by MACS or
Germline competence may be maintained for longer periods *in vitro* when a pure SSEA-1 positive population of CBCs is used as starting material.

### 8.5 Conclusion

A major assumption of this thesis has been that the reasons underlying the inability to isolate CBA ES cells have similarities to those preventing chicken ES cell isolation. A recent report provides evidence that ES cell isolation from CBA and non-murine species may have important differences. Buehr *et al.* (2003) employed a selective ablation strategy to remove differentiating cells from rat embryo cultures. ES cells could not be isolated from rat blastocysts expressing a selectable marker under the control of the mouse *oct4* promoter. This strategy has previously been used to isolate ES cells with a CBA inbred background (McWhir *et al*., 1996; Gallagher *et al*., 2003). ES cell precursors in rat and mouse blastocysts appear to behave differently during the selective ablation approach. CBA strain mouse blastocysts are more “permissive” for ES cell isolation than rat blastocysts under selective ablation. Although chickens do not appear to have an *oct4* gene (Soodeen-karamath & Gibbins, 2001), this highlights the dangers of making cross-species comparisons.

The isolation of strain CBA ES cells by a new method has been described in this thesis. Although this method was not applied successfully to chicken ES cell isolation, it represents a new direction in ES cell isolation with potential applications in many species.
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Appendix: publications
Isolation of murine embryonic stem and embryonic germ cells by selective ablation

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Abstract
The isolation of murine embryonic stem (ES) cells has been almost exclusively from the 129 mouse strain. Other mouse strains, such as CBA, have proven refractory to ES cell isolation by conventional means. We previously reported the isolation of 87.5% CBA ES lines by selective ablation of differentiating cells (McWhir et al., 1996). Here, we report the isolation of ES and EG cells from 94% CBA embryos hemizygous and homozygous for a neomycin-resistance transgene under the transcriptional control of the Oct3/4 promoter (Oct/neo). Since expression of the Oct/neo transgene only confers drug resistance to undifferentiated cells of the inner cell mass, selection results in the ablation of differentiating cells from the culture. The efficiency of ES isolation by selective ablation in homozygotes is twice that in heterozygotes. ES isolation frequency in permissive strain 129 embryos is enhanced by treatment with an inhibitor of the extracellular-signal-regulated kinase (ERK) pathway but this effect is not sufficient to permit ES isolation from the CBA strain.

Introduction
Embryonic stem (ES) cells allow the generation of precise genetic modifications to provide animal models of human genetic disease or to elucidate gene function. Under altered conditions of culture ES cells generate a wide variety of embryonic lineages in vitro and provide models of both mammalian development and ES-derived tissue transplantation therapy (reviewed in Thomson & Odorico, 2000). The recent isolation of human ES cells (Thomson et al., 1998; Pera et al., 2000; Reubinoff et al., 2000) and EG cells (Shamblott et al., 1998) was motivated by the therapeutic potential of ES-derived somatic cells in a broad range of diseases. The efficiency of ES isolation may become a limiting factor in the application of therapeutic cloning to provide syngeneic stem cells for individual patients (Lanza et al., 1999). There is also great interest in the potential of ES cells in livestock species. Where the interest is focused on germ line modification, either for production of therapeutic proteins in milk or to facilitate xenotransplantation. However, proven ES cells from domestic species have not yet been reported; even among mouse strains there is an apparent genetic variation in the ease with which ES lines are isolated. We sought to investigate the mechanisms by which some strains and species are refractory to ES isolation.

Although ES-like cells have been reported in rabbit (Graves & Moreadith, 1993), pig (Wheeler, 1994), cattle (Stice et al., 1996), rhesus monkey (Thomson et al., 1995) and human (Thomson et al., 1998; Pera et al., 2000; Reubinoff et al., 2000), as yet only a few strains of mice (predominantly C57BL/6 and strain 129) have successfully produced totipotent ES cells capable of colonising the germ line (Ledermann & Burki, 1991). The most compelling evidence for ES cells in a non-murine species is in man, in which derivatives of all three primary germ layers can be obtained from differentiating ES cells in vitro. We have previously shown in the mouse, that predominantly CBA embryos could give rise to ES lines only when these embryos had been genetically modified.
to allow selection against non-ES cell lineages within the explant (McWhir et al., 1996). Gardner and colleagues have also shown that several strains of mice refractory to traditional ES isolation methods can yield ES lines following microdissection of the early embryo prior to culture (Brook & Gardner, 1997). This highly skilled technique requires the physical removal of the ES progenitors from the influence of other embryonic lineages such as the primitive endoderm. This approach demonstrates that for non-permissive strains of mice it is necessary to remove the influence of the inhibitory cell types that are probably causing the ES precursor cells to differentiate. While it is apparent that there is a strain barrier to ES derivation, it is unclear if the biological mechanism underpinning this is also that which operates across non-human non-murine species (Kawase et al., 1994). However, the dependence of ES isolation in some genotypes on physical or selection-mediated removal of other lineages is consistent with the view that the strain barrier arises from differences in the extent to which the embryo/explant exerts control over the proliferation of ES precursor cells.

Germ line competent ES cells were isolated by selective ablation from the otherwise non-permissive mouse line (87.5% CBA, 12.5% C57BL/6 embryos, McWhir et al., 1996). Our approach required the generation of transgenic mice that expressed the selectable neomycin phosphotransferase (neo) gene under the transcriptional control of a 1.9kb proximal Oct3/4 promoter that was active only in the ES and EG cells and their precursors (Yeom et al., 1996; Pesce et al., 1998). Differentiating cells in an explanted embryo down regulated expression of neo rendering them susceptible to the neomycin analogue G418. Drug selection ablates the embryonic cells that hinder ES isolation whereas the Gardner approach requires the skilled microsurgical removal of such cells prior to derivation. Using this selective ablation approach 87.5% CBA ES lines could be generated with similar frequency to ES lines from the permissive 129 strain. We concluded that removal of differentiating lineages relaxes inhibition of the stem cell population, permitting the isolation of ES cells from an otherwise non-permissive genotype (Figure 1 for a schematic overview).

A similar approach has been taken by others to study the loss of pluripotency as ES cells are induced to form aggregates in suspension culture (Mountford et al., 1998). Aggregation of ES cells induces differentiating structures known as embryoid bodies, which lose their pluripotent stem cell population. However, when selection is applied to aggregates carrying a selectable marker under the transcriptional control of the Oct3/4 promoter, the controlling influence of differentiating cells is removed, allowing survival and proliferation of the stem cell population. In common with the microsurgical based isolation approach (Brook & Gardner, 1997) and selective ablation, the necessity of sequestering stem cells away from other embryonic lineages appears to be key to the success of the procedures.

Here we show that selective ablation also facilitates the isolation of embryonic germ cells and describe the effects of G418 concentration and transgene copy number on the efficiencies of ES isolation. We also show that down regulation of the MEK/ERK pathway with a chemical inhibitor (PD98059) leads to an increased frequency of ES isolation in strain 129 but is insufficient alone, to permit ES isolation from strain CBA.
Materials and methods

ES cell isolation – selective ablation

Transgenic mice were generated by pronuclear micro-injection of pOctneo1 DNA into F1 (CBA × C57BL/6) embryos (McWhir et al., 1996). The transgenic lines 72–49, were back-crossed to the CBA mouse strain to produce homozygous 87.5% CBA, 6% C57BL/6 mice (transgenic strain Octneo87). Hemizygous Octneo embryos were obtained by mating Octneo87 males to wild type CBA females to produce 94% CBA blastocysts. Homozygous 87.5% CBA blastocysts were obtained by mating Octneo87 males with Octneo87 females.

ES maintenance medium was: Glasgow's minimum essential medium (GMEM; Sigma) supplemented with 5% newborn calf serum and 5% fetal calf serum (Globepharm), 0.1 mM non-essential amino acids (Life Technologies), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies) and 500 U/ml recombinant murine leukaemia inhibitory factor (LIF; Life Technologies). When required, medium was supplemented with 25 μM PD98059 (NEB) or 50–200 μg/ml G418 (geneticin). ES isolation media consisted of ES maintenance media supplemented with fetal calf serum to a final concentration of 10%.

Isolation and maintenance of ES cells was as previously described (McWhir et al., 1996). Uterine horns were removed and flushed with approximately 0.5 ml of isolation medium. Embryos were washed three times in isolation medium and explanted individually into wells of a 24-well plate, pre-seeded with mitotically inactivated STO feeder cells (Robertson, 1987). In the absence of G418 ES isolation was as described (Robertson, 1987). Primary explants in unselected groups were picked at day 6 or 7 of culture depending upon the size of the 1CM outgrowth and the extent of differentiation. The strategy was to maximise the former and minimise the latter. First-passage wells were then monitored daily for the appearance of ES-like colonies which were picked and replated to remove them from the influence of other cell types. In selected groups the appearance of ICM outgrowths was delayed and explants remained unpicked for periods ranging from 14 to 21 days. At first passage primary ES explants were picked and disaggregated by gentle trypsinisation into clumps of cells, then transferred onto feeder layers in single wells of a 24-well plate. These wells were trypsinised approximately 1 week later and the ES cells plated into 1 well of 6-well plate. Primary ES cell lines were further expanded until passage 5 or 6, frozen in ES medium plus 10% DMSO and stored in liquid nitrogen. Newly derived ES lines were moved to ES maintenance medium after passage 4.

EG cell isolation

The procedure for isolation of EG cells was based on that of Cooke et al. (1993). Wild type and 94% CBA foetuses were collected at days 8.5, 9.5 and 12.5 post-coitus. For 8.5 and 9.5 day embryos, the posterior 1/3 including the allantois was removed and dissociated into single cells by incubation at 37°C solution of 0.0025% trypsin and 0.02% EDTA in phosphate buffered saline (trypsin) for about 10 min. The single cell suspension was then seeded into single wells of a 24-well plate with an inactivated St4-m220 cell feeder layer (Koshimizu et al., 1996). Genital ridges were removed from day 12.5 embryos by microdissection and disaggregated in trypsin. The single cell suspension from one embryo was plated onto 4 wells of a 24-well plate on inactivated feeder cells. Cells were cultured in ES cell isolation medium supplemented with 20 ng/ml bFGF. When G418 selection was used, the drug was included in the isolation media at the required concentration.

Primary EG colonies were apparent after 7–10 days of culture. They were picked, trypsinised, and passaged onto fresh St4-m220 feeders. As for ES cells, lines were expanded, frozen and stored in liquid nitrogen.

Immunoblotting

ES cells were plated in 6-well plates (1 × 10⁶ cells per well) in standard ES cell medium. Twenty-four hours later medium was replaced with ES medium containing 0.5% serum and without LIF. After a further 24 h cells were re-fed with medium lacking both serum and LIF. Four hours later cells were stimulated with LIF for 10 min, washed once with ice cold PBS and scraped off in pre-warmed lysis buffer (100 mM Tris–HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol). Samples were boiled for 5 min and stored in aliquots at −80°C. Ten microliters aliquots were loaded directly onto a 12% Tris–glycine gel (Novex, Invitrogen), fractionated and electroblotted onto PVDF membrane (Hybond-P, Amersham). Membranes were then probed with
Table I. Selective ablation 94% CBA oct/neo murine embryos for the isolation of ES cells (CBA embryos heterozygous and homozygous for the Oct/neo transgene were placed under varying levels of G418 selection)

<table>
<thead>
<tr>
<th>G418 concentration (µg/ml)</th>
<th>0</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morulae (day 2.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octneo CBA hemizygotes</td>
<td>0/18</td>
<td>0/40</td>
<td>0/24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocysts (day 3.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octneo CBA hemizygotes</td>
<td>0/46</td>
<td>9/132</td>
<td>5/66</td>
<td>2/78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homozygotes</td>
<td>(0%)</td>
<td>(7%)</td>
<td>(7.5%)</td>
<td>(2.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myf/PGKneo hemizygotes</td>
<td>0/12</td>
<td>9/64</td>
<td>15/103</td>
<td>11/88</td>
<td>4/104</td>
<td></td>
</tr>
</tbody>
</table>

a Forty of these blastocysts were cultured in vitro from I-cell embryos prior to G418 selection (3/40, 7%).

b Only 50% of the embryos carry the Myf/PGKneo transgene.

anti-ERK antibodies according to the manufacturers directions (Cell Signalling Technology).

Results

Isolation of ES cells from 94% CBA mice by selective ablation

We previously isolated ES cells from non-permissive 87.5% CBA 12.5% C57BL/6 Octneo mice by selective ablation (McWhir et al., 1996). As it was formally possible that isolation of ES cells from these mice arose as a consequence of residual C57BL/6 alleles we performed a further round of backcrossing to CBA mice to generate Octneo embryos with a 94% CBA genotype. Blastocysts hemizygous for the Oct/neo transgene, were cultured under G418 selection at a concentration of 50 µg/ml (Table I) and gave rise to ES lines at a comparable frequency to that of the 87.5% CBA mice in the previous study (McWhir et al., 1996). This rate was also comparable to that observed for 129 blastocysts in the absence of selection (7% v.s. 10%, respectively; Table I). Increasing the G418 concentration to 75 µg/ml had no effect on the overall efficiency of isolation from hemizygotes. However, increasing selection to 100 µg/ml G418 reduced the frequency of hemizygote ES isolation to 2.5% (two lines from 78 embryos, Table I). These data demonstrate that it is possible to isolate ES cells from the non-permissive 94% CBA strain at a comparable frequency to that observed for the isolation of ES cells from the 87.5% CBA mouse strain.

To investigate the effect of applying G418 selection at an earlier stage of development we cultured hemizygous Octneo morulae in 50 and 100 µg G418/ml. Unselected morulae developed in vitro to the blastocyst stage and terminally differentiated, giving rise to no ES lines. Although the Oct3/4 promoter is known to be active at the morula stage (Yeom et al., 1996), G418 selection of Octneo morulae resulted in arrested embryo development and cell death; no ES lines were isolated (Table I). Since ES lines can be isolated from dissociated strain 129 morulae, the transition from morula to blastocyst is unnecessary for ES isolation and the failure of the selective ablation approach at this stage may be associated with either a non-specific effect of G418 or insufficient levels of Oct/neo expression due to site of incorporation effects.

There are important differences in the way in which ES lines arise under selective ablation. Octneo explants under G418 selection are reduced to a very small number of cells by 12 days of selection (Figure 2(A)). There are few or no differentiating cells associated with such explants, implying that all potential inhibitory cells have been removed from the culture. This is consistent with the view that their removal allows the expansion of ES precursors and eventually of ES cells themselves. By 20 days of culture primary colonies have a typical ES morphology and, unlike conventional primary ES colonies, continued their undifferentiated growth upon passage in all cases. In conventional ES isolation first passage is frequently followed by terminal differentiation (often to trophectoderm), and hence, failure to isolate ES lines.
Figure 2. The derivation of a primary ES cell colony from an explanted 94% CBA embryo expressing the Oct/neom transgene. Panel A depicts the morphological changes of a single embryo explant under selective ablation conditions. There was initial growth particularly from the ICM up to day 6, after which the explants shrunk in size as non-resistant cells were ablated by G418 action. At day 12 there appeared to be only a small group of ES precursor cells that were resistant to G418, compared to the many differentiating cell types observed without selection. Thereafter the primary ES colony expanded until day 22, when it was picked and further expanded. It must be noted that fresh feeder cells were added between days 17 and 18. Panel B depicts two control embryos of the same genotype that have been cultured in the absence of G418. At day 12 onwards it was evident that many terminally differentiated cells such as giant trophoblast cells (top row) or endoderm like cells (bottom row) were the major cell types produced as opposed to a few ES precursor cells.

Transgene copy number

The two transgenic lines that repeatedly gave rise to ES lines under selective ablation were both low copy number transgenics (one or two copies, McWhir et al., 1996). We speculated that high levels of neo expression in multi-copy integrants may have led to continued drug resistance after transcriptional down regulation and hence to loss of ES precursors. This may occur as an indirect consequence of the survival
Table 2. Isolation of EG cells from sv129 mice and from 94% Octneo CBA foetuses in the absence and presence respectively of G418 selection (embryos homozygous and heterozygous for the Oct/neo transgene were dissected and the disaggregated PGC containing tissue subjected to EG isolation).

<table>
<thead>
<tr>
<th>Genotype and day of development</th>
<th>G418 concentration (ug/ml)</th>
<th>Total number of lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>sv129 dl2.5</td>
<td>5/30</td>
<td></td>
</tr>
<tr>
<td>sv129 d8.5</td>
<td>2/30</td>
<td></td>
</tr>
<tr>
<td>CBAOctneo dl2.5</td>
<td>0/30$^a$</td>
<td>4/30$^a$</td>
</tr>
<tr>
<td>hemizygotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBAOctneo d9.5</td>
<td>0/10</td>
<td>2/10</td>
</tr>
<tr>
<td>homozygotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBAOctneo d8.5</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>homozygotes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$The cells from each of the 30 embryos were split over the four treatments, hence the absolute number of lines is an underestimate and had all the material been plated under the same conditions would have yielded on average, 16 lines.

Table 3. Isolation of ES cells in the presence of the MEK 1 inhibitor (blastocyst embryos from strain 129 or CBA mice were subjected to ES cell isolation in the presence and absence of the inhibitor PD98059).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PD98059 Control</th>
<th>PD98059 25 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sv129</td>
<td>74</td>
<td>27</td>
</tr>
<tr>
<td>CBA</td>
<td>84</td>
<td>28</td>
</tr>
</tbody>
</table>

The effect of G418 is lineage-specific

We next investigated the possibility that selective advantage accrued to ES progenitor cells by non-specific action of G418 and not as a result of epiblast-specific expression of the Oct/neo transgene. Since ES lines could be generated most readily at a very low level of G418 selection (50 µg/ml), we were concerned that this level of selection may not have been sufficient to account for the observed cell death. Established ES lines for example are routinely selected in 300 µg/ml and will survive for variable periods in 50 µg/ml. Although 50 µg/ml G418 was sufficient to cause the death of explanted wild type embryos (data not shown), selective ablation may have arisen in transgenic embryos as a result of differential sensitivity to a non-specific effect rather than restricted transgene expression. This question was addressed by applying 50 µg/ml G418 selection to transgenic blastocysts that expressed neo from the constitutive PGK (phosphoglycerate kinase) promoter. Although embryos survived and grew vigorously, none of these embryos produced colonies resembling ES cells (Table 1). This result suggests that ES lines arise under selective ablation at 50 µg G418/ml as a consequence of the restricted expression of the Oct/neo transgene in the epiblast.

94% CBA EG cells by selective ablation

EG cells can be isolated from strain 129 mice by cultivation of a crude dissection of the allantoic region at day 8.5 or from germinal ridges taken up to day...
12.5 (Matsui et al., 1992). Although it is unclear from the literature if the same strain restrictions for ES isolation also apply for EG cells, in our hands, 94% CBA germinal ridges are totally refractory for EG isolation using conventional methods (Table 2). When G418 selection was imposed, it became possible to isolate EG cell lines from 94% CBA Octneo foetuses at days 12.5 and 9.5 but not from day 8.5 (Table 2). The frequency of EG isolation from day 12.5 94% CBA embryos by selective ablation was comparable to that obtained at day 12.5 from strain 129 mice in the absence of selective ablation.

Direct comparisons of the frequencies of EG isolation across the three developmental stages are less meaningful because the number of PGCs and hence, potential precursor cells rises from approximately 1000 at day 9.5 to 26,000 at day 12.5 (Tam & Snow, 1981). In addition, pairs of disaggregated ridges at day 12.5 were each split across the four treatments while at days 9.5 and 8.5, individual embryo dissections were plated into a single well/treatment. However, each line isolated at day 12.5 arose from a single colony. Hence, the number of lines isolated in each treatment at day 12.5 reflects on average 1/4 of the full potential of the primary explant to give rise to EG lines in those conditions. An approximate comparison across stage can therefore be calculated by adjustment for the relative number of potential precursors and the number of treatments over which explants were split. Standardising to efficiency per 1000 PGCs gives an adjusted frequency at day 12.5 of 2% versus 20% at day 9.5 at 50 µg G418/ml. These results are consistent with previous work on EG cell isolation from C57BL/6 or 129/sv mouse strains (Labosky et al., 1994; Matsui et al., 1992).

We were unable to obtain EG lines with any treatment from dissections of Octneo foetuses at day 8.5, although lines could be obtained from strain 129 mice by conventional means. We were also unable to obtain EG lines from day 12.5 foetuses at G418 levels above 100 µg/ml.

Down-regulation of MEK I lead to enhanced ES isolation frequency in strain 129

ES cell self-renewal is enhanced in the presence of the MEK 1 inhibitor PD98059 (Burdon et al., 1999). PD98059 prevents ERK activation that appears to have an adverse affect on ES cell self-renewal. We hypothesised that MEK 1 inhibition may be an alternative method with which to enhance the isolation of ES cells by preventing the appearance of differentiated cells.

Figure 3. A primary ES colony emerging some distance from the original embryo explant. Occasionally we observed the apparent migration of ES precursor cells away from the original site of explant attachment.
in embryo explants. Blastocysts from both strain 129 and CBA mice were cultured with and without 25 μM PD98059 (Table 3). Among strain 129 blastocysts we observed a relatively significant ($p < 0.059$) increase in the frequency of ES isolation from 10/84 (12%) to 20/74 (27%). From CBA embryos we were unable to derive lines in either treatment or control groups, suggesting that down-regulation of the MEK 1 pathway is not sufficient for ES isolation in this genotype. We confirmed the effect of PD98059 action on 129 and CBA ES cells by Western analysis of the ERKI/2 proteins. MEK 1 was unable to phosphorylate ERKI/2 in the presence of the inhibitor for both cell types (Figure 4).

**ES progenitors migrate away from the explant**

Although Figure 2 is representative of most explants under selective ablation, not all primary ES colonies appeared in the same manner. A subset of primary colonies appeared at the periphery of the initial outgrowth (Figure 3). These colonies typically grew as a crescent shaped mass of cells and were apparently inhibited from making contact with a culture surface that had previously been in contact with the primary explant.

<table>
<thead>
<tr>
<th></th>
<th>HM1</th>
<th>HM1</th>
<th>HM1</th>
<th>N13</th>
<th>N13</th>
<th>N13</th>
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<tbody>
<tr>
<td>LIF</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PD</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 4.** PD98059 inhibits ERK phosphorylation in CBA and strain 129 ES cells. ES cells with a 129 background (HM1) or a 94% CBA (N13) background were cultured in the presence of PD98059 before stimulation with LIF for 10 min. Western analysis of extracts revealed that LIF dependent phosphorylation of ERK1/2 is blocked by PD98059 in both cell types. Treatment with PD98059 does not affect the levels of non-phosphorylated ERK1/2.

**Discussion**

Further development of the selective ablation strategy is constrained by the necessity to generate transgenic animals for the production of embryos. One way around this dilemma would be the use of episomal vectors. Our observation that ES isolation can be enhanced by higher levels of expression of the Oct/neo transgene provides some comfort that, provided tissue-specificity is retained, episomal, viral or transient expression strategies may prove to be consistent with selective ablation. We have also extended the utility of the approach to include the isolation of EG cells from the otherwise refractory 94% CBA embryos. Furthermore, we have demonstrated an alternative approach to selective ablation that involves inhibition of the MEK/ERK pathway, shown to enhance ES cell self-renewal (Burdon et al., 1999). Although this approach gives rise to heightened frequency of ES isolation in strain 129 mice, it does not overcome the barrier to ES isolation in the CBA strain.

Here we do not present data confirming the pluripotency of the new cell lines or the effects that G418 drug selection may have on pluripotency. However, we have previously demonstrated that ES cells generated under G418 drug selection are pluripotent and have proven to be germ line competent (McWhir et al., 1996). Based on the work of Burdon et al. (1999), similar concerns over the toxic impact that PD98059 has on newly derived ES lines can be set aside. They also isolated ES lines in the presence of PD98059, of which two were reported to be capable of germ line transmission.

It is interesting to note in Figure 1, Panel A, that cells of the explant remaining after 12 days of selection are reduced in number to form what seems like a core of ES precursor cells. There are very few or no differentiating cells associated with such explants, implying that all embryo cells of an inhibitory nature have been removed from the culture, allowing expansion and production of primary explants with a classic ES cell morphology. Interestingly others have noted that removal of the differentiating cells from ES aggregates, in suspension culture, was essential to prevent the loss of the totipotent cell population (Mountford et al., 1998). Our observations also indicate that the removal of the differentiating cells and presumably their inductive signals was crucial to maintaining the pluripotent cells and their proliferation.
A subset of primary colonies appeared at a distance from the initial explant outgrowth (Figure 3), and typically grew as a crescent shaped mass of cells on the periphery of the dying explant. ES progenitor cells appeared to have migrated away from the other cell lineages of the explanted embryo. Parallels may be drawn with the physical separation of germ cells in the embryonic yolk sac throughout a critical period of development in which embryonic patterning is being laid down. There is a deliberate physical separation of the germ cells throughout this period of development to protect them from differentiating influences of somatic cell lineages. The enclave hypothesis states that 'germ cells arise from regions and/or cells that have not been specified in a positional sense, that is, embryonic enclaves'. In other words germ cells are segregated at a time and place that allows them to avoid the influence and process of specialisation (Dixon, 1994). This hypothesis is consistent with our observation that the separation of the ES progenitor cells from the suppressive influence of the differentiating embryo cell types by selective means is a necessary requirement for the preservation and procurement of these naïve stem cells from non-permissive mouse strains. This physical separation can occur by microsurgical removal or by selective ablation and occasionally by migration away from inhibitory cell types of the embryo.

We observed a 2-fold higher ES isolation frequency for embryos expressing two copies of the transgene, under selective ablation conditions. These explants also had fewer differentiating cells an observation that is also apparent when isolating EG cells from PGCs of 94% CBA mice under G418 selection. This result indicates, as with ES cell isolation, that selective ablation could facilitate the efficiency of ES isolation from the 129 mouse strain by selective inhibition of the MEK/ERK pathway. Assuming the biological mechanism underlying the control of ES cell proliferation is similar in other species, then the immediate challenge is to apply this technique to non-murine species using an episomal approach.

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