Experimental Approaches to Establish Rat Embryonic Stem Cells

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I declare that the work described in this Thesis is my own, except where otherwise stated.

Stephen Earl Meek

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Abstract

The rat has been an established experimental animal model within many areas of biological investigation for over one hundred years due to its size, breeding characteristics, and knowledge of its physiology and behaviour. In recent years its status as a leading biomedical model has been somewhat surpassed by the mouse. This is largely the result of the isolation and application of mouse embryonic stem (ES) cells. Mouse ES cells have the capacity for unlimited self-renew *in vitro* whilst maintaining pluripotency and germline competence, and most importantly are amenable to sophisticated reverse genetics strategies such as gene targeting, which have provided a route to germ line modification. Thus far, the derivation of rat ES cells has proved elusive. The generation of rat ES cells would therefore facilitate equivalent applications to rat genetics and significantly strengthen the rat as an experimental model system.

Previous attempts to derive rat ES cells led to the isolation of rat ES-like cells. However, whilst these cells exhibit extensive self-renew *in vitro*, it was known that they fail to maintain significant levels of the key functional ES cell marker Oct4 and do not contribute to chimeras. Rather, these cells express the trophectoderm markers Cdx2 and CyclinD3, and have been termed ExS cells due to their probable extra-embryonic nature. In the work described in this thesis, further investigation of ExS cells revealed the absence of expression of the key pluripotency gene Nanog, although the expression pattern of Nanog in the rat embryo was shown to be similar to that of mouse.

It was hypothesised that expression of exogenous Oct4 and Nanog or Sox2 genes could facilitate reprogramming of ExS cells into a 'true' ES cell state. Initial

work described in this thesis demonstrated that it was possible to introduce transgenes into rat ExS cells and obtain stable transformants with long term transgene expression. On this basis Oct 4, Nanog and Sox2 transgene expression vectors were constructed and stably integrated into ExS cells, and transgene expression verified. However, no reactivation of an endogenous gene expression profile, characteristic of a true ES cell-like state, was observed in any of the transgenic lines produced.

Concurrent with work on ExS cells, investigations by others using chemically defined, serum-free medium containing small molecule inhibitors of MEK and GSK3 (called 3i/2i medium) had demonstrated that it was possible to readily isolate mouse ES cells, even from strains known to be refractory to ES cell isolation. Therefore, the ability of this culture system to facilitate rat ES cell derivation was investigated. Rat 3i/2i cell lines were established from ICM outgrowths of Fischer, DA and Sprague Dawley E4.5 rat embryos. These cells maintained expression of Oct4 and Nanog and could generate complex teratomas consisting of all three germ layers. They were distinct from epiblast stem cells (EpiSC) in that they expressed Klf4, Rex1 and Stella and most importantly, they could contribute to the formation of adult chimaeras and demonstrated germline competency.

Isolation of these authentic rat ES cells paves the way for gene targeting in the rat, a development that should greatly facilitate new biomedical discoveries.

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Abbreviations

bp	base pairs
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
d-CTP	deoxycytidine triphosphate
DIA-M	differentiation-inhibitory activity, matrix-bound form
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
d.p.c.	days postcoitum
DTT	dithiotreitol
EDTA	diaminetetraacetic acid disodium salt
ES	embryonic stem
FACS	fluorescent activated cell sorter
FGF	fibroblast growth factor
FCS	foetal calf serum
g	gravitational force
GFP	green fluorescent protein
GMEM	Glasgow's modified Eagle's medium
GSK3	glycogen synthase kinase 3
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP	horseradish peroxidase
ICM	inner cell mass
KOH	potassium hydroxide
KOAc	potassium acetate
KSOM	potassium simplex optimisation medium
LB	Luria-Bertani
LiCl	lithium chloride
LIF	leukaemia inhibitory factor
MEF	mouse embryonic fibroblasts
MgOAc	magnesium acetate
MOPS	3-(N-morpholino) propane sulfonic acid
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
nt	nucleotides
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PFA	paraformaldehyde
PGC	primordial germ cells
PVDF	polyvinylidene fluoride
RCGM	rat cell growth medium
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficiency

SDS	sodium dodecyl sulphate
SR	serum replacement
TU	transfection units

Chapter 1

Introduction

1.1 The rat model

Over the last century the rat has become established as the leading biomedical model within a wide variety of disciplines, and as such, a large number of relevant inbred disease models exist that make it the researcher's preferred choice of model for the study of human disease (Jacob, 1999). The rat has several advantages over other species. Its size lends itself to practical considerations of animal husbandry such as space and cost, yet it is large enough to allow sophisticated surgical techniques to facilitate phenotypic analysis and the taking of multiple samples, unlike the mouse.

However, despite these advantages, in recent years the mouse has succeeded the rat as the preferred mammalian experimental organism. Whilst the mouse, due to its smaller size and shorter reproductive cycle, may be better suited with regard to animal husbandry, it is not readily suited to some investigations requiring microsurgical techniques. Rather, the increased popularity of mouse is largely due to advances in genetic manipulation made possible by the derivation and maintenance of mouse embryonic stem (ES) cells.

1.2 Mouse ES cells

1.2.1 Blastocyst formation

Mouse ES cells are derived from the inner cell mass (ICM) of the pre-implantation embryo (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). The ICM is formed at the first differentiation step during early mouse embryogenesis, when the sixteen-cell embryo undergoes compaction to form two distinct cell populations (Gardner, 1983; Johnson and Ziomek, 1981). The inner cells, which constitute the ICM, are smaller and more tightly packed than the outer cells, which become polarised and flatten to form the trophectoderm (TE). The TE is responsible for the subsequent formation of the blastocyst cavity, a fluid-filled cavity (Biggers et al., 1977; Borland et al., 1977). As the cavity grows it forces the ICM to one end of the embryo. At this stage the embryo is referred to as a blastocyst and the second differentiation step occurs in which the ICM differentiates into the epiblast and primitive endoderm. The primitive endoderm forms on the side of the epiblast facing the blastocyst cavity and further differentiates into visceral and parietal endoderm (Gardner, 1982). Whilst the trophectoderm and the visceral and parietal endoderms go on to contribute to the placental epithelium and yolk sac respectively, epiblast cells are responsible for contributing to all the tissues of the adult organism. For this reason, the cells of the ICM or epiblast are termed pluripotent.

1.2.2 ES cell characteristics

Whilst *in vivo* the cells of the ICM commit to differentiate, *in vitro*, ES cells are capable of prolonged self-renewal, whilst retaining their pluripotential state and the ability to differentiate and colonise all the tissues, including the germ line, when

transferred back into a host blastocyst (Beddington and Robertson, 1989; Bradley et al., 1984).

1.2.3 ES cells as a research tool

The characteristics of mouse ES cells have allowed for the generation of mice with designed genetic modifications. Using techniques such as gene targeting, specific and precise genetic alterations can be made to the genome of ES cells cultured *in vitro* that can then be introduced into the germ line for the study of phenotypic changes *in vivo.* Thus, genetic modification of ES cells has become the primary strategy for investigating mammalian gene function *in vivo*, and for modelling diseases. In addition, ES cells have been useful for unravelling the molecular mechanisms that control self-renewal and pluripotency. The ability to control the differentiation of ES cells *in vitro* into specific cell types has facilitated investigations into mechanisms of lineage-specific differentiation and in the future may provide the basis for regenerative therapies. Collectively, these capabilities have made ES cells an invaluable and essential tool to the researcher.

1.2.4 ES cell derivation

When ICMs are cultured *in vitro* under permissive conditions, the disaggregated outgrowths give rise to colonies of undifferentiated, pluripotent ES cells. Originally ES cells were derived and maintained in the undifferentiated state on a layer of mitotically inactivated fibroblasts (Evans and Kaufman, 1981; Martin, 1981). Typically primary embryonic fibroblasts or immortalised cells such as STO cells are required to provide support for the growing ES cells. The nature of this

support may be multifactorial, such as improving attachment, by direct cell signalling effects, or by conditioning the medium with necessary factors. Whilst feeder cell-conditioned medium demonstrated only limited ability to maintain ES cells and prevent differentiation, it was discovered that ES cells could be maintained independent of feeder cells in the presence of media conditioned by the Buffalo rat liver cell line (Smith and Hooper, 1987). It was later discovered that the key constituent responsible for ES cell maintenance in both these culture conditions was the polypeptide factor, leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). Germ-line competent ES cells can be propagated and maintained independent of feeders whilst in the presence of LIF (Nichols et al., 1990). However, in the absence of LIF, ES cells spontaneously differentiate.

1.3 LIF signalling

1.3.1 LIF/gp130/STAT3

LIF is a member of the IL (interleukin)-6-type family of cytokines that signal via the transmembrane receptor glycoprotein 130 (gp130). LIF binds to its low-affinity receptor LIFR, which complexes with gp130 to form a high-affinity receptor (Davis et al., 1993; Gearing et al., 1992; Gearing et al., 1991). However, LIF receptor homozygous null (*Lifr*$^{-/-}$) ES cells can be maintained in the presence of IL-6 together with its soluble receptor, sIL-6R. The IL-6/sIL-6R complex signals via a gp130 homodimer, demonstrating that, gp130 and not LIFR, is the key signal transducer of LIF (Murakami et al., 1993; Yoshida et al., 1994).

The intracellular response of gp130 is initiated by Janus kinase (JAK). JAKs bind to the intracellular domain of gp130. Upon cytokine binding the receptor

undergoes a conformational change that brings the JAK molecules into close proximity to each other, resulting in their subsequent activation by auto- or trans-phosphorylation. The activated JAKs then phosphorylate tyrosine residues of gp130 that serve as binding sites for proteins containing Src-homology-2 (SH2) domains, which have a high affinity for phosphorylated tyrosine. STAT3 (signal transducer and activator of transcription 3) is one such protein (Stahl et al., 1995).

STATs are latent cytoplasmic transcription factors. They are recruited to activated receptors that have become activated by tyrosine phosphorylation in response to a variety of extracellular signals. Receptor-bound STAT is subsequently activated via a tyrosine kinase associated with the receptor, such as JAK (Ihle, 1996). This creates a further STAT3 docking site and thus mediates dimerisation of the molecule. Upon dimerising, the STAT3 molecules undergo a conformational change that releases them from the receptor, allowing their translocation to the nucleus whereby they can interact with the DNA to induce transcription of target genes.

It has been demonstrated that STAT3 activation by LIF is required for mouse ES cell self-renewal. Expression of a dominant negative STAT3 or mutation of the STAT3 docking sites on the gp130 receptor causes ES cells to differentiate even in the presence of LIF (Niwa et al., 1998), whilst an activatable STAT3-ER fusion supports ES cells self-renewal in the absence of LIF (Matsuda et al., 1999). Interestingly, LIF also activates the MAPK/Erk1/2 and PI3K signalling pathways, which are involved in differentiation and propagation, respectively.

1.3.2 Role of MAPK/Erk

Mitogen-activated protein kinases (MAPK), or Extracellular signal-regulated kinases (Erk) as they were originally called, are involved in a wide range of cellular processes, such as cell motility, proliferation and differentiation, and are expressed, to varying extents, in all tissues (Binetruy et al., 2007). They are intracellular serine/threonine kinases that become activated in response to a host of different extracellular signals such as growth factors (e.g. FGFs) and cytokines (e.g. LIF) that initiate a series of phosphorylation events (Cowley et al., 1994). This phosphorylation cascade is initiated when the extracellular signalling molecule binds to its receptor resulting in phosphorylation of the receptor's tyrosine residues. This occurs either directly, due to intrinsic receptor tyrosine kinase activity, as in the case of the FGF receptor (Heldin, 1996) or indirectly, via their association with non-receptor protein kinases such as JAK, in the case of gp130.

As mentioned earlier, these active tyrosine residues act as binding sites for proteins containing SH2-domains. In the case of Erk1/2 activation, the protein tyrosine phosphatase, SHP-2, is recruited to the receptor. SHP-2 can activate Erk by complexing with adaptor molecules such as Growth factor receptor-bound protein 2 (Grb2). Grb2 consists of an SH2 domain and an SH3 domain (Lowenstein et al., 1992). It binds to SHP-2 via its SH2 domain and couples this to Erk activation via the binding of the Ras GTP-GDP exchange protein, Son of Sevenless (SOS) to its SH3 domain (Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993)**.** SOS increases the proportion of active, GTP-bound Ras leading to the downstream phosphorylation and activation of Erk. The targets of activated Erk are located throughout the cell, including the cytoplasm (e.g. p90/rsk) (Gavin and

Nebreda, 1999) as well as nuclear transcription factors (e.g. c-Myc) (Gupta, Seth et al. 1993).

It has been demonstrated that Erk activation by LIF is dispensable for ES cell self-renewal. A mutant gp130 receptor that prevents SHP2 binding, fails to activate Erk1/2, yet continues to support ES cell self-renewal. This was supported by the maintenance of ES cells in the presence of the mitogen-activated protein kinase kinase (MEK) inhibitor PD098059 (Burdon et al., 1999). Not only did MEK inhibition enhance ES cell self-renewal it also inhibited embryoid body differentiation, suggesting that Erk signalling is necessary for lineage commitment of ES cells.

In vivo, *Erk2*$^{-/-}$ embryos fail to form mesoderm and die post-implantation due to trophoblast defects (Saba-El-Leil et al., 2003). Although *Erk2*$^{-/-}$ ES cells can be propagated *in vitro*, they are deficient in neural and mesoderm commitment (Kunath et al., 2007). The importance of Erk signalling for differentiation was further confirmed when it was demonstrated that only a short period of Erk activation is required to initiate neural specification (Stavridis et al., 2007). These findings demonstrate the importance of Erk signalling for the commitment of ES cells to differentiate. Therefore, the dual, yet conflicting activation of STAT3 and Erk by LIF, suggests the balance between these contributes toward ES cell maintenance.

1.3.3 Role of PI3K

LIF is also known to activate the class I_A family of Phosphoinositide 3-kinase (PI3K). PI3Ks are involved in a wide range of cellular processes such as proliferation, cell migration, cell survival and trafficking. SHP-2 forms a complex

with PI3K via the adaptor molecule Grb2-associated binding protein 1 (Gab1) thereby translocating PI3K to the membrane, where it can act upon its substrate.

PI3K is a lipid kinase that phosphorylates the membrane phospholipid, phosphatidylinositol 3,4-bisphosphate (PIP_2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 is capable of binding proteins containing a pleckstrin homology (PH) domain and in so doing is responsible for recruiting proteins to the membrane. One such protein is protein kinase B (PKB/AKT). Membrane-localised PKB/Akt activation relies on phosphorylation of both Thr308 by Phosphoinositide-dependent kinase I (PDK1) (Alessi et al., 1997; Stokoe et al., 1997), which is also localised to the membrane via its PH domain, and Ser473 by MTORC2, a complex containing mammalian target of rapamycin (mTOR) (Sarbassov et al., 2005).

The effect of the PI3K signalling pathway on mouse ES cells was first demonstrated through the discovery of the tumour suppressor Phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN is a lipid phosphatase which dephosphorylates PIP_3 to PIP_2 (Maehama and Dixon, 1998), essentially counteracting PI3K activity and preventing membrane localisation and activation of PKB/Akt and its subsequent downstream signalling. PTEN null ($PTEN^{-/-}$) mice fail to survive beyond day E9.5, but demonstrate regions of enhanced growth. Similarly, $PTEN^{-/-}$ mouse ES cells exhibit an increased growth rate and enhanced ability to form teratomas (Di Cristofano et al., 1998; Stambolic et al., 1998). Increased levels of PKB/Akt phosphorylation and activation accompany this proliferative advantage, suggesting that PKB/Akt promotes ES cell proliferation and that it is negatively

regulated by PTEN. Furthermore, when the ubiquitously expressed PKB/Akt isoform, Akt-1, was also mutated in PTEN null cells, the observed growth advantage was reversed (Stiles et al., 2002). This effect on mouse ES cell proliferation was supported by the demonstration that the ES cell-expressed Ras (Eras) binds to and activates PI3K and that $Eras^{-/-}$ ES cells exhibited decreased cell growth and PKB/Akt phosphorylation. However, $Eras^{-/-}$ cells could generate viable animals demonstrating that it is not essential for ES cell pluripotency (Takahashi et al., 2003).

One downstream target of PI3K, which may mediate this proliferative effect, is the mammalian target of rapamycin (mTOR). mTOR appears to be ubiquitously expressed in most tissues, including mouse ES cells. Activation of P70-S6 kinase-1 by mTOR, leads to an increase in protein synthesis and cell proliferation (Kawasome et al., 1998). mTOR deficient embryos die around E6.5-7.5 due to impaired proliferation and although null blastocysts appear normal, they fail to produce ES cells (Murakami et al., 2004).

These findings suggest the importance of PI3K in ES cell proliferation. However, there are also indications of an effect of PI3K on ES cell self-renewal. Differentiation was enhanced in the presence of the PI3K inhibitor, LY294002, or upon expression of a more specific dominant-negative PI3K subunit (Paling et al., 2004). This appeared to be a direct result of enhanced Erk activation, since self-renewal was restored upon addition of the MEK inhibitors PD98059 or U0126.

A constitutively active form of PKB/Akt could maintain mouse ES cells in the undifferentiated state independent of LIF (Watanabe et al., 2006). However, the downstream effector of PKB/Akt that is implicated in ES cell self-renewal remained unidentified. Storm *et al* demonstrated that PI3K inhibition resulted in a down-

regulation of Nanog expression, a reduction in Nanog protein levels, and a loss of self-renewal (Storm et al., 2007). The loss of self-renewal by PI3K inhibition could be prevented by activation of an inducible Nanog. Furthermore, the group demonstrated that loss of Nanog and self-renewal was mediated by Glycogen synthase kinase 3 (GSK-3). Inhibition of GSK3 has been implicated in ES cell self-renewal (Sato et al., 2004) and PKB/Akt-mediated phosphorylation inactivates GSK3 (Cross et al., 1995). Indeed, Storm *et al* demonstrated that inhibition of GSK-3 reversed the effect of PI3K inhibition and could maintain Nanog levels and self-renewal (Storm et al., 2007).

1.3.4 GSK3

GSK3 is a constitutively active serine/threonine kinase that was initially demonstrated to phosphorylate and inactivate glycogen synthase, an enzyme responsible for polymerizing glucose molecules into glycogen for storage (Embi et al., 1980). GSK3 regulates its substrates by phosphorylating serine and/or threonine residues that typically results in their inhibition. Phosphorylation by GSK3 is significantly enhanced in proteins that are pre-phosphorylated, or 'primed' by another kinase (Doble and Woodgett, 2003). The primed phospho-residue binds to a positively charged pocket within GSK3. This correctly orients both the kinase domain and the substrate within the catalytic region for efficient phosphorylation. In the case of glycogen synthase inactivation, the enzyme is pre-phosphorylated, or primed, by casein kinase II (CK2) prior to undergoing GSK3-mediated multisite phosphorylation (Fiol et al., 1990).

As it is constitutively active, regulation of GSK3 takes the form of inhibition. This occurs as a result of phosphorylation at serines 9 or 21 by an inactivating kinase. The mechanism of GSK3 inhibition takes advantage of its positively charged pocket's preference for phospho-residues. As such the phosphorylated S9/21 residues act as pseudo-substrates, binding to the positively charged pocket and occupying the catalytic domain, thereby competing with genuine substrates. For example, in response to insulin, GSK3 is inactivated in a PI3K-dependent manner via phosphorylation by PKB (Cross et al., 1995). Although there are several regulators of GSK3, including PI3K/PKB and MAPK, probably the best studied is Wnt.

GSK3 plays a central and inhibitory role in the Wnt/β-catenin signalling pathway. In the absence of Wnt stimulation, GSK3 is in its active state and forms part of what is known as the 'destruction complex'. The destruction complex consists of GSK3, Axin and adenomatous polyposis coli (APC) protein. Both Axin and APC are phosphorylated by GSK3, which increases and stabilises their binding to β-catenin. Within this complex GSK3 phosphorylates β-catenin which initiates its ubiquitylation and subsequent proteosome degradation, thereby limiting the cytoplasmic pool of β-catenin (Doble et al., 2007). Stimulation by Wnt activates the cell-surface receptor Frizzled that phosphorylates Dishevelled and subsequently prevents GSK3-mediated phosphorylation of β-catenin. As a result β-catenin accumulates within the cytoplasm and translocates to the nucleus where it can interact with T-cell factor (TCF)/ Lymphoid enhancer factor (LEF) transcription factors and regulate transcription of their downstream targets.

GSK3 exists as two homologues, GSK3α and GSK3β encoded by separate genes. Alleles of each homologue were sequentially knocked-out in mouse ES cells

to generate five lines (Doble et al., 2007). In both single homozygous knock-out lines, the level of β-catenin not altered. A gene dosage effect was demonstrated when either both alleles of one homologue and one of the other, or both alleles of each homologue were deleted. Therefore, with respect to the Wnt/β-catenin pathway, redundancy exists between GSK3α and GSK3β function. Although both ES cell morphology and expression of Oct4 and Nanog were maintained, *GSK3α*$^{-/-}$ and *GSK3β*$^{-/-}$ double knockout (DKO) cells also expressed the early mesoderm marker Brachyury, a TCF-responsive gene upregulated in response to increased levels of β-catenin. In this regard, these cells are similar to early mesoderm–specified (EM) progenitors (Suzuki et al., 2006). Furthermore, the ability of GSK3α/β DKO cells to differentiate was severely restricted as demonstrated by their failure to generate cardiomyocytes in embryoid body (EB) assays and the absence of neural tissue in teratomas. The influence of GSK3 on ES cell differentiation supports other studies which suggest a role for GSK3 inhibition in maintaining ES cell self-renewal, whether directly (Sato et al., 2004) or indirectly via upstream regulation by PI3K/PKB (Paling et al., 2004; Storm et al., 2007) or stabilisation of downstream targets such as c-myc (Cartwright et al., 2005).

1.3.5 c-Myc

A molecule that may reconcile the apparently conflicting downstream activities of LIF toward a common goal of ES cell maintenance is the transcription factor c-Myc. c-Myc is transcriptionally regulated by STAT3 and has been demonstrated to promote ES cell self-renewal by blocking differentiation (Cartwright

et al., 2005). Whilst STAT3 is involved in the transcriptional regulation of c-Myc, both Erk and PI3K are involved in its post-transcriptional regulation.

Degradation of c-Myc protein occurs following GSK3-mediated phosphorylation of Threonine 58 (T58). As a result, the protein is poly-ubiquinated and subsequently degraded by the proteosome. A stable form of c-Myc, in which the T58 residue is mutated, facilitates ES cell self-renewal independent of LIF (Cartwright et al., 2005). In the presence of LIF, Erk is activated and stabilises c-Myc by phosphorylating Serine 62 (S62), a step that is also required to prime c-myc for GSK-mediated phosphorylation of T58. However, this activity of GSK3 is prevented by PI3K.

In short, LIF stimulation up-regulates c-Myc via STAT3 and stabilises c-Myc protein via Erk activation and subsequent S62 phosphorylation as well as PI3K blocking of GSK-mediated degradation.

1.4 Intrinsic determinants

1.4.1 Oct4 and Cdx2

Oct4 is an octamer binding protein and member of the POU family of homeodomain transcription factors. Oct4 is maternally expressed, observed in the unfertilised oocyte, but not in sperm or testis. During development, expression progresses through the cleavage stage embryo becoming restricted to the pluripotent compartments of the developing embryo, namely the ICM of the blastocyst and epiblast of the egg cylinder. Expression of Oct4 is gradually downregulated after gastrulation (as cells are differentiated into somatic lineages) and becomes limited to primordial germ cells (PGC) (Scholer et al., 1990; Yeom et al., 1996). Intercrossing

mice heterozygous for an Oct4 deletion failed to produce any term or midgestation homozygous Oct4-deficient pups (Nichols et al., 1998). At E5.5, embryonic material was absent in 18 of 61 implantation sites analysed. Whilst E3.5 blastocysts appeared normal, with normal cell numbers, they failed to cavitate fully. Further expansion was not observed upon delayed implantation, rather, the embryos collapsed. Attempts to derive mouse ES cells from intact blastocysts or isolated ICMs of homozygous mutants yielded only trophoblast giant cells. Therefore, although Oct4-deficient cells are viable, they fail to develop beyond an immature blastocyst *in vivo* or to generate ES cells *in vitro*, demonstrating that Oct4 is essential for establishment of a pluripotent ICM. Knockdown of Oct4 in mouse ES cells using siRNA (Hay et al., 2004) or a tetracycline-regulated transgenic system (Niwa et al., 2000) demonstrated differentiation into trophectoderm, confirming the importance of Oct4 in maintaining ES cell self renewal. Interestingly, the precise level of Oct4 is critical. Not only does repression of Oct4 induce differentiation, but less than a two-fold over-expression induces differentiation into mesoderm and primitive endoderm (Niwa et al., 2000). Confocal imaging of immunohistochemical-stained embryos revealed that at the first differentiation stage during development, Oct4 protein becomes segregated to the ICM cells and is lost in surrounding trophectoderm (Palmieri et al., 1994). The primitive endoderm cells which evolve at the second differentiation step demonstrate higher levels of Oct4 than ICM cells. This data confirms that regulation of Oct4 protein levels determines three distinct cell populations leading to the formation of a mature blastocyst.

Whilst Oct4 is first downregulated in the trophectoderm, conversely, the Caudal-type homeobox transcription factor, Cdx2, is specifically expressed in the

trophectoderm of the blastocyst (Beck et al., 1995). *Cdx2*$^{-/-}$ embryos do not form a mature blastocoelic cavity or distinct epithelial outer layer and fail to implant (Strumpf et al., 2005). Interestingly, in wild type embryos, although Oct4 and Cdx2 expression are segregated in the blastocyst and specify distinct cell populations, they are initially co-expressed in cells of the early embryo (Niwa et al., 2005; Strumpf et al., 2005). Immunohistochemical staining of *Cdx2*$^{-/-}$ blastocysts demonstrated ectopic Oct4 expression in cells of the trophectoderm, suggesting that Cdx2 is necessary to restrict Oct4 expression to the ICM (Strumpf et al., 2005). Niwa *et al* demonstrated that over-expression of Cdx2 induced trophectoderm differentiation even in the continued presence of transgenic Oct4 (Niwa et al., 2005). This suggests that Oct4 cannot block Cdx2-induced trophectoderm differentiation, and that loss of Oct4 is not a pre-requisite for differentiation to proceed.

The fact that blastocysts develop in *Oct4*$^{-/-}$ and *Cdx2*$^{-/-}$ embryos, suggests that segregation of inner and outer layers is not dependent on either factor, but rather both are important for the specification of lineage identity, such that subsequent differentiation ensues correctly (Smith, 2005).

1.4.2 Nanog

Nanog is a homeoprotein which, when overexpressed, is capable of mediating LIF-independent ES cell self-renewal. It was originally identified in two ways. The first employed a functional expression cloning strategy, using *Lifr*$^{-/-}$ ES cells in the absence of cytokine, to identify cDNAs that could maintain mouse ES cells independent of LIF (Chambers et al., 2003). The second utilised *in silico* differential display to compare expressed sequence tag (EST) libraries from mouse ES cells and

various somatic tissues to identify genes expressed in ES cells as specifically as Oct4. One of the nine candidates identified was then shown to facilitate LIF-independent self-renewal upon forced expression (Mitsui et al., 2003). This protein was Nanog.

Nanog is endogenously expressed in ES cells and is downregulated upon differentiation. In fact, expression is confined to pluripotent cells, such as ES, embryonic germ (EG) and embryonic carcinoma (EC) cells, but is absent from adult tissues (Chambers et al., 2003; Mitsui et al., 2003). *In vivo*, expression first appears in the compacted morula where it is localised to the central compartment that will later become the ICM. Indeed, expression is confined to the ICM and epiblast of early and late blastocysts, but absent from the trophectoderm and primitive endoderm. By the implantation stage, expression is downregulated, but reappears at E11.5 in the genital ridges where staining is localised to the primordial germ cells (PGC) (Chambers et al., 2003).

Whilst endogenous levels of Nanog are required for maintaining self-renewal, ES cells are still reliant upon LIF signalling. LIF-independent self-renewal and resistance to differentiation are only mediated upon over-expression of Nanog. Interestingly, the self-renewing capacity of Nanog over-expressing ES cells is enhanced in the presence of LIF. However, Nanog does not appear to be a downstream target of LIF/gp130/STAT signalling, nor regulate STAT3 itself, suggesting that this combinatorial effect on self-renewal is a result of parallel action (Chambers et al., 2003). Likewise, Erk activation does not repress, nor is repressed by, Nanog. However, in the absence of Oct4, Nanog over-expressing cells differentiate, indicating that Oct4 expression is required for Nanog action.

Sequential targeted disruption of the homeodomain in both alleles of the Nanog gene was performed to generate Nanog-deficient ES cells. These cells adopted a parietal endoderm-like morphology in the absence of feeder cells and expressed endoderm transcription factors GATA4 and GATA6, as well as parietal and visceral endoderm markers (Mitsui et al., 2003). Chambers *et al* also generated *Nanog*$^{-/-}$ ES cells by genetic deletion. By comparing *Nanog*$^{+/+}$, *Nanog*$^{+/-}$ and *Nanog*$^{-/-}$ cells in a clonal assay, they demonstrated a stepwise reduction in the number of undifferentiated, alkaline positive colonies. Interestingly however, although prone toward differentiation, *Nanog*$^{-/-}$ cells were still capable of forming undifferentiated colonies (Chambers et al., 2007). In fact, in routine wild type ES cell cultures they observed that Nanog was undetectable in a fraction of Oct4-expressing cells. Using a Nanog-eGFP reporter cell line they demonstrated that levels of Nanog fluctuated in ES cell cultures. Although cells with no Nanog were predisposed toward differentiation, they did not commit, but rather remained undifferentiated and continued to express Oct4 and had the ability to re-express Nanog (Chambers et al., 2007)**.** Surprisingly, aggregation experiments demonstrated that *Nanog*$^{-/-}$ ES cells retain their pluripotent potential and are capable of contributing to adult chimaeras. However, whilst Nanog-null cells populate the genital ridges, PGC development appears to be prevented beyond E11.5 (Chambers et al., 2007).

The precise requirement for Nanog in the developing early embryo was further highlighted by breeding of Nanog heterozygous mice to generate Nanog-null embryos. Whilst Nanog heterozygous mice appeared normal and were viable, homozygous embryos at E5.5 were highly disorganised with no distinct epiblast. Interestingly E3.5 null blastocysts appeared normal, although ICMs could not be

maintained *in vitro*, but differentiated entirely into parietal endoderm-like cells (Mitsui et al., 2003). Overall, these data suggest that Nanog is neither required for the maintenance of pluripotency nor the integration of ES cells into the embryo, but rather for the establishment of pluripotency and germ cell formation.

The mechanisms through which Nanog functions are still incompletely understood. However, recent studies have indicated that Nanog exists in both monomeric and dimeric forms. Homodimers form via a tryptophan repeat region that acts as a dimerisation domain. Importantly, it was demonstrated that specific deletion or mutation of this region eliminates Nanog-Nanog interaction and the ability to mediate LIF-independent self-renewal (Mullin et al., 2008). It is unclear how dimerisation is regulated. Nonetheless, dimerisation is also likely to be important for the binding of Nanog to its protein partners and targets, and this will be affected by the differing binding specificities of monomeric and dimeric forms as well as their relative levels *in vivo*.

1.5 ES cell derivation from other species

1.5.1 Human ES cells

Since the isolation of mouse ES cells nearly 30 years ago, derivation of comparable cell lines from other species has been largely unsuccessful, with the notable exception of human ES cells. Human ES (hES) cells were originally derived from *in vitro* fertilised embryos in conditions very similar to those used for isolation of mouse ES cells (Thomson et al., 1998). In common with their mouse counterparts, hES cells are capable of self-renewing *in vitro* whilst retaining expression of the core transcription factors Oct4, Nanog and Sox2, and the capacity to differentiate into

cells of all three germ layers when injected into immuno-compromised mice. However, for ethical reasons it is unknown if these cells will fulfil the critical ESC cell requirement and contribute to germline-transmitting chimaeras. More importantly, there are some key differences between mouse and human ES cells with regard to the markers they express, the conditions in which they grow and the signalling pathways involved in their maintenance.

Whilst mouse ES cells express the cell surface marker SSEA1, human cells do not, but rather, express SSEA3 and SSEA4 as well as other cell surface markers such as TRA-1-60 and TRA-1-81. In this respect these cells more closely resemble human embryonic carcinoma cells (Andrews et al., 1996; Kannagi et al., 1983). Furthermore, unlike mouse ES cells, hES cells are sensitive to single-cell passaging, which hinders clonal propagation and assays. Intriguingly, LIF, which is important for mouse ES cell self renewal, does not support hES cell growth, nor prevent differentiation in the absence of feeder cells or conditioned medium. Differentiation occurs despite the fact that hES cells express both LIFR and gp130, and demonstrate LIF-induced STAT3 phosphorylation (Daheron et al., 2004; Humphrey et al., 2004). The absence of STAT3 phosphorylation in undifferentiated hES cell cultures suggests that this pathway is not important for hES cell self-renewal. However, culture in the complete absence of STAT3 signalling is necessary to determine that the role of STAT3 function is indeed redundant in hES cell maintenance. Similarly, BMP4, which assists in maintaining mouse ES cells in feeder/serum-free conditions by blocking neuroectoderm differentiation, (Ying et al., 2003) has been demonstrated to induce trophectoderm differentiation in hES cells (Xu et al., 2002).

The key pathways involved in hES cell maintenance appear to be bFGF (FGF2) and TGFβ/Activin/Nodal. FGF2 at a concentration of 4ng/ml was demonstrated to enhance clonal expansion and long-term culture of hES cells in the presence of serum replacement medium (SR) (Amit et al., 2000). This medium could also support feeder-free growth of hES on Matrigel^{TM} or laminin when previously conditioned by MEFs (CM) (Xu et al., 2001). In unconditioned medium, serum replacement medium induces BMP activity and rapid differentiation. BMP antagonists, such as Noggin, are detected in CM that protect against SR-induced differentiation. Interestingly higher levels of FGF2 (100ng/ml) reduce BMP signalling and maintain hES cells in unconditioned medium (UM) (Xu et al., 2005). It has been demonstrated that FGF2 degrades rapidly in UM compared to CM, such that only suboptimal levels of FGF2 exist, which are incapable of blocking differentiation. At higher concentrations, the critical threshold is achieved that facilitates self-renewal (Levenstein et al., 2006). Whilst these results have helped to define the hES cell culture conditions and demonstrate a specific role for FGF2, the culture still includes the addition of cell matrix and unrefined SR of animal origin, and does not provide evidence of any positive or negative effects these may have on hES cell self-renewal.

Recently, the TGF/Nodal/Activin pathway has also been demonstrated to play a role in hES cell maintenance. TGFβ1, Nodal and Activin are members of the transforming growth factor beta (TGF-β) superfamily that modulate their intracellular signal via Smad2/3 phosphorylation (Shi and Massague, 2003). Amit *et al* demonstrated feeder- and serum-free culture of hES cells in medium composed of SR, FGF2, LIF and TGFβ1. Although individually, TGFβ1 (0.12ng/ml) and FGF2

(4ng/ml) could not efficiently maintain hES cells at the concentrations used, their combinatorial effect was comparable to feeder conditions (Amit et al., 2004). Whilst investigating the role of Nodal on modulating hES cell differentiation, Vallier *et al* demonstrated that forced transgenic expression of Nodal restricted differentiation during EB formation. Phenotypically distinct EBs formed which consisted of an outer layer of anterior visceral endoderm-like cells surrounding an inner core of cells that continued to express markers of undifferentiated hES cells (Vallier et al., 2004). When Nodal-expressing hES cells were grown on MEFs in a chemically defined medium (CDM) (Johansson and Wiles, 1995), without FGF or serum, they continued to express undifferentiated markers after six passages, whilst wild type cells differentiated. Indeed it was demonstrated that Nodal is endogenously expressed by hES cells and downregulated upon differentiation. However, inhibition of Nodal by Lefty (Meno et al., 1999) or Cerberus-Short (Piccolo et al., 1999) did not induce differentiation of hES cells on MEFs in SR medium (Vallier et al., 2005). This suggests that an alternative factor, also capable of activating Smad2/3, may be compensating. Further evidence that supported the importance of this pathway was demonstrated by addition of the TGF-β, Alk4/5/7 receptor inhibitor SB431542 to hES cell cultures, resulting in reduction of Smad2/3 phosphorylation and loss of Oct4 protein (James et al., 2005). Indeed, Activin, which is produced by MEFs, was demonstrated to maintain hES cells in feeder and SR-free CDM in short-term cultures (Vallier et al., 2005)**.** Long-term propagation however, required the addition of FGF2. Interestingly, the influence of FGF2 was blocked by SB431542, suggesting that it is dependent on Activin/Nodal signalling. Recent evidence has demonstrated that Smad2/3 can bind to the Nanog proximal promoter, the activity of which is

enhanced in response to Activin (Xu et al., 2008). A negative feedback may exist in which Activin-controlled expression of Nanog limits FGF-induced neuroectoderm differentiation and Smad2/3-induced endoderm differentiation, thereby maintaining pluripotency (Vallier et al., 2009).

In conclusion, Activin/Nodal and FGF2 are required, at least in part, for the maintenance of hES cells, in defined, feeder and serum-free, conditions, although components of animal origin were still present in the attachment matrix and CDM - which includes BSA. Whilst it was not demonstrated that these conditions could facilitate the *de novo* derivation of hES cells, they were recently demonstrated to be capable of deriving pluripotent stem cell lines from the post-implantation embryo of mouse and rat (Brons et al., 2007; Tesar et al., 2007). This further strengthens the belief that hES cells are distinct from their ICM-derived, mES cell counterparts and represent a developmental state equivalent to the late epiblast. Since the comparative developmental state of the cells of the ICM in mouse and human is unknown, the effect of similar culture conditions on the intrinsic state of these cells may differ.

1.5.2 Rat ES-like cells

Whilst ES cell derivation is routine for mouse strains such as 129/Ola, the degree of success varies greatly for other mouse strains, with some strains being refractory to derivation.

Although ES-like cell lines have been established from a vast number of different species (Li et al., 2003; Saito et al., 2002; Sukoyan et al., 1992; Sun et al., 1995; Tian et al., 2006), including the rat (Buehr et al., 2003; Iannaccone et al., 1994;

Ruhnke et al., 2003; Vassilieva et al., 2000), none as yet fulfil the necessary criteria to be deemed authentic ES cells.

These rat ES-like cells self-renew *in vitro*, are morphologically similar to mouse ES cells and express markers commonly, but not specifically, found in ES cells, such as Alkaline Phosphatase (AP) and SSEA1. However, they fail to maintain significant levels of the key ES cell transcription factor, Oct4, demonstrate limited or unconvincing differentiation capacity, and fail to contribute to the germline. Since the level of Oct4 is critical for the maintenance of pluripotency (Niwa et al., 2000), it is likely that appropriate expression of Oct4 throughout the derivation process is required. To investigate this, Buehr *et al* tracked Oct4 expression during the derivation process by generating transgenic rats in which the reporter construct lacZ/*neomycin* was under the control of the Oct4 promoter and enhancers (Buehr et al., 2003). Using typical mouse ES cell-derivation protocols, they demonstrated that, whilst Oct4 is expressed within the rat ICM, it is downregulated within the first few days during the derivation process. Importantly, this downregulation occurs with no obvious cell death, differentiation or morphological change, such that Oct4-negative and Oct4-positive cells are indistinguishable. This pattern of Oct4 downregulation was also observed in cultures of mouse ICMs for the strains CBA, MF1, C57BL/6 and 129. After 5-6 days, Oct4 expression was diminished or absent in both rat and mouse outgrowths, with the exception of 129, which gave rise to Oct4-positive colonies.

After 6 days, morphologically undifferentiated, Oct4-negative rat outgrowths could be propagated and the resultant lines maintained *in vitro*. Although the cells are resistant to enzymatic digestion, they could be routinely mechanically passaged

by trituration. Propagation of these cells is reliant, not only upon the presence of a feeder layer and LIF, but also on the addition of Matrigel^{TM}. Matrigel is a commercially available preparation that consists of a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that resembles tissue extracellular matrix and whose major components are laminin and collagen IV.

Similar to most ES cell-like cells, they express AP and SSEA1, but fail to maintain Oct4. They also failed to generate chimaeras or contribute to either adult or fetal tissues as determined by microsatellite analysis. Interestingly, they strongly express the trophectoderm markers Cdx2 and CyclinD3, and when cultured in conditions for trophoblast stem (TS) cell derivation, are capable of forming colonies resembling mouse TS cells. They can also spontaneously give rise to parietal endoderm and are thus believed to be extra-embryonic in nature and as such, have been termed Extra-embryonic Stem (ExS) cells to distinguish them from true ES cells.

This data demonstrates that upon exposure to typical *in vitro* ES cell culture conditions, Oct4 is readily extinguished from the explanted embryonic pluripotent cell population. Only the permissive mouse strain 129 can maintain Oct4 expression and generate ES cell lines. Although these Oct4-negative cells continue to proliferate without any obvious morphological change, the resultant cell lines have restricted developmental potential. This suggests that maintaining expression of Oct4, or protecting it from downregulation during the early stages of ES cell derivation, is critical for establishing true pluripotent ES cells

Recently one group claimed to have established Oct4-expressing rat ES cell lines capable of forming chimaeras (Ueda et al., 2008). The data in support of this

finding are limited. Characterisation of the cells demonstrated that they did not express AP, and although they expressed SSEA-1, they also expressed markers common to hES cells, but not mouse ES cells, such as SSEA-3, SSEA-4 and TRA-1-81. Whilst real-time PCR detected Oct4 and Nanog expression, the levels varied widely between lines and from those of mES cells. No other markers were examined to confirm the uncommitted state of the cells, such as Rex1 or FGF5.

The differentiation potential of the cells was tested by embryoid body differentiation as well as teratoma formation by injecting cells into Severe Combined Immunodeficient (SCID) mice. Immunohistochemical staining of *in vitro* differentiated cells was either sparse (as for α-SMA) or failed to demonstrate the morphological features typical of the differentiated cell type, such as neuronal processes (as for β-III-tubulin). Whilst teratomas formed *in vivo*, it was reported that no structures of ectodermal origin were found and only intestinal epithelium-like and cartilage-like structures were identified.

Contribution of these cells to chimaeric animals was reported, but surprisingly the strain combination of cells and host embryo used did not facilitate identification by either coat-colour transmission or microsatellite analysis. Rather confirmation relied upon PCR detection of a GFP transgene. Whether or not these cells are indeed capable of chimaeric contribution, the evidence provided is not thorough enough to convince the reader of their authenticity. Furthermore, the authors fail to demonstrate germline transmission or efficient *in vitro* differentiation protocols that are necessary for such cells to be fully utilised.

1.5.3 Epiblast Stem Cells (EpiSC)

Recently, pluripotent stem cells have been derived from the epiblast of post-implantation stage embryos of both mouse (E5.5-6.5) and rat (E7.5) using chemically defined medium containing activin (Brons et al., 2007; Tesar et al., 2007). These have been termed Epiblast Stem Cells (EpiSC) based on their tissue of origin and to distinguish them from ICM-derived ES cells. Interestingly, lines could also be efficiently derived from strains which are typically non-permissive for mouse ES cell derivation, such as NOD and CBA x C57BL/6. EpiSC can be continually passaged whilst retaining expression of Oct4, Nanog and Sox2 and the ability to differentiate into the three germ layers, as determined *in vitro* and *in vivo*, by embryoid body and teratoma formation respectively.

However, EpiSC differ in several ways from ES cells. The colonies are flatter in appearance and, unlike mouse ES cells, are sensitive to single cell dissociation. Examination of the expression profile revealed that, unlike ES cells, EpiSC failed to express the specific ICM marker Rex1, as well as having lower levels of other ICM-associated genes, such as *Gbx2*, *Pecam1* and *Tbx3*. Rather, they expressed higher levels of genes associated with the late epiblast and commitment, such as *Fgf5*, *Gata6*, and *Sox17*. However, following blastocyst injection, only two mouse chimaeras were born from 385 injected blastocysts. Although this may be a consequence of the requirement for cells to be single cell, morula aggregation experiments with clumps of cells failed to demonstrate any integration.

Furthermore, EpiSC cannot be propagated using ES culture conditions containing LIF and/or BMP4. Using the activin receptor inhibitor, SB431542, it was demonstrated that the presence of Activin is critical for the maintenance of EpiSC.

However, cultures were further improved by the addition of FGF2 - conditions which had previously been demonstrated to facilitate the long-term maintenance of human ES cells (Vallier et al., 2005). Conversely ICM cells differentiate when cultured in EpiSC conditions.

These data suggest that ES cells and EpiSC are distinct cell types representative of the tissue from which they were derived, namely the ICM and epiblast respectively. The inefficiency of EpiSC to contribute to chimaeras restricts their utility for generation of genetically engineered models. However, this inefficiency may be due to an incompatibility between the post-implantation origin of the cells with the pre-implantation environment of the host ICM cells, rather than an inherent deficiency of the cells themselves.

Intriguingly, EpiSC share several similarities with hES cells that are distinct from those of mouse ES cells, such as a dependence on Activin signalling, their sensitivity to single cell dissociation and BMP4-induced differentiation into trophectoderm. Comparison of the Oct4 targets within mouse ES cells, hES cells and EpiSC demonstrated a greater overlap of hES cells with EpiSC than with mES cells (Tesar et al., 2007). Therefore, hES cells may represent a later developmental stage than their mouse counterparts indicating either a difference in the starting cell population and/or a differing response to the culture conditions to which they are exposed. Recent findings have demonstrated the reversion of mouse EpiSC to ES cells by either a combination of ectopic Klf4 expression and LIF culture conditions or indeed LIF signalling alone (Bao et al., 2009; Guo et al., 2009). In addition conventional hES cells can be converted to a ‘naïve’ mouse ES cell state as a result of ectopic expression of Oct4, Klf2 and Klf4 in defined medium containing LIF

(Hanna et al., 2010). These results suggest that distinct states of pluripotency exist that are dependent on culture conditions and/or ectopic expression of key transcription factors, and that these conditions are dictated by species or strain specific requirements.

1.6 Defined culture conditions

An understanding of the precise cues and mechanisms that direct self-renewal and initiate differentiation into specific lineages is complicated and hindered by the complexity of the culture conditions and the conflicting negative and positive influences. There are at least two contributing sources of unknown variables in ES cell cultures; feeder cells and serum. Serum is an undefined complex mixture derived from blood, consisting of growth factors and a host of other components. Whilst feeder cell metabolism may affect ES cells indirectly by competing for nutrients in the medium and altering the metabolite, pH and O_2/CO_2 levels, they can also exert an influence directly via cell-to-cell signalling or paracrine signalling, by releasing factors into the medium. Dissecting the effect any of these may have in ones culture system is daunting. Defining the medium, by reducing the composition to a minimal core of identified essentials, will highlight key requirements for ES self-renewal and facilitate our understanding of pluripotency, differentiation and development, as well as aiding regenerative medicine and pharmaceutical research by eliminating animal-sourced factors. Even if a common mechanism exists which will establish ES cells from different species, failure to derive ES cells, even from species closely related to the mouse such as rat, suggests that subtle developmental or intrinsic differences exist which, when exposed to such undefined conditions, may prevent the necessary

status being established. Understanding the minimal essential pathway(s) may facilitate this or at least aid in delineating the key differences.

1.6.1 LIF+BMP4

Whilst it has been demonstrated that LIF/STAT3 can facilitate derivation and propagation of mouse ES cells independent of feeders (Matsuda et al., 1999; Nichols et al., 1990; Niwa et al., 1998; Smith et al., 1988; Williams et al., 1988), culture in serum-free N2B27 induces efficient neuroectoderm differentiation. Although neural differentiation is reduced by the addition of LIF, ultimately it cannot be prevented (Ying et al., 2003).

Bone morphogenetic protein 4 (BMP4) is a member of the TGFβ superfamily known to suppress neural differentiation (Tropepe et al., 2001; Wilson and Hemmati-Brivanlou, 1995). In feeder-free, serum-free culture and in combination with LIF, BMP4 blocks neural differentiation, facilitating ES cell self-renewal. The role of BMP4 in ES cell maintenance is somewhat surprising as it is involved in differentiation and patterning in the developing embryo (Jones et al., 1991). Indeed, in the absence of LIF, BMP4 induces ES cells to differentiate into non-neural, flattened epithelial-like cells. Therefore, BMP4 can substitute for serum by blocking neural differentiation, but is dependant upon LIF-mediated blockade of BMP4-induced non-neural differentiation (Ying et al., 2003). These observations extended to BMP2 as well as the BMP relative Growth and Differentiation Factor 6 (GDF-6), but not to other members of the TGFβ superfamily, TGFβ1 or Activin.

BMP4 activates Inhibitor of Differentiation (Id) genes via the Smad signalling pathway (Ying et al., 2003). Indeed, overexpression of Id1, 2 or 3 negates

requirement for BMP4, whereas, overexpression of inhibitory Smads 6/7 impairs self-renewal and increases neural differentiation. This occurs even in the presence of serum and LIF, suggesting that induction of Id genes via Smad signalling is activated in ES cells by serum. Interestingly, ES cells express BMP4 as well as BMP receptors (BMPR) indicating that they have the potential to mediate autocrine signalling. If autocrine BMP4 signalling occurs, it appears to have only limited capacity to mediate self-renewal in serum-free conditions. This suggests that the presence of BMP4 or other BMPR ligand in serum is a key contributory factor such that serum-free cultures require the addition of exogenous BMP4.

BMP4/Smad/Id does not appear to act by regulating gp130 signalling as STAT3 phosphorylation is not enhanced nor is Erk phosphorylation inhibited by BMP4 (Ying et al., 2003). Rather, the antagonistic activity of BMP4 on neurogenic basic helix-loop-helix (bHLH) transcription factors, such as Mash1, which are expressed in ES cells, may play a role in blocking neural differentiation.

1.7 Aim

The derivation of mouse ES cells, combined with the ability to manipulate their genome *in vitro*, has facilitated the generation of disease models in mice. Although mouse ES cells have become a valued tool, there are occasions where other species would serve as more appropriate models. However, despite significant progress towards understanding the signalling pathways and intrinsic determinants involved in the formation of pluripotency *in* vivo and its maintenance *in vitro*, there has been limited success in deriving ES cells from other species.

As discussed in this Chapter, the rat is a well established scientific model for which a large number of inbred disease models exist. The size of the rat favours microsurgery, sampling and phenotypic analysis, all of which are restricted in mouse. Furthermore, the rat is more intelligent than the mouse and is commonly used in studies of behaviour, cognition and memory. The failure to derive genuine rat ES cells capable of germline transmission is a major obstacle toward implementing genome modification in this species and suggests that unrefined culture conditions are unsuitable for capturing pluripotent rat ES cells.

The aim of this project was to generate and characterise authentic rat ES cells. A strategy was employed to reprogramme rat ES-like (ExS) cells by stable integration and expression of Oct4 and Nanog expression vectors. Also, a recently developed defined culture condition established for mouse ES cells that employed small molecule inhibitors of MEK and GSK3, had been applied to rat ES cell derivation protocols and this project concludes with the characterisation of the lines generated under these conditions.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General solutions

Annealing buffer (x2)	200mM KOAc
	60mM HEPES-KOH, pH 7.4
	4mM MgOAc
AP Buffer	100mM NaCl
(Alkaline Phosphatase)	50mM $MgCl_2$
	0.1% Tween-20
	100mM Tris pH 9.5
BCIP	50mg/ml w/v in 100% DMF
(5-bromo-4-chloro-	(N,N-dimethyl formamide)
3-indoyl-phosphate)	
Denaturing solution	1.5M NaCl
	0.5M NaOH

GTE	50mM Glucose
	10mM EDTA, pH 8.0
	25mM Tris-HCl, pH 8.0
HB	50% v/v Formamide
	5 x SSC pH 4.5, with citric acid
	50µg/ml Heparin
	0.1% v/v Tween 20
Laemmli buffer (x2)	125mM Tris, pH 6.8
(stock solution)	25% v/v Glycerol
	4% v/v SDS
Laemmli buffer (x1)	2x Laemmli buffer
(working solution)	100mM DTT
	Diluted with protease inhibitor-treated water
	(1 tablet/3.5ml water) (Roche, 11 836 153 001)
LB agar	1% w/v Bacto-tryptone (Difco, 211705)
	0.5% w/v Bacto-yeast extract (Difco, 212750)
	1% w/v NaCl
	1.5% w/v Bacto-agar (Difco, 214010)
	to pH 7.0 then autoclave.

LB medium	1% w/v Bacto-tryptone (Difco, 211705)
	0.5% w/v Bacto-yeast extract (Difco, 212750)
	1% w/v NaCl
	to pH 7.0 then autoclave.
Lysis buffer (Tissue)	100mM Tris-HCl, pH 8.5
	5mM EDTA, pH 8.0
	0.2% w/v SDS
	200mM NaCl
NBT	75mg/ml in 70% w/v DMF (N,N-dimethyl formamide)
(4-nitroblue tetrazolium)	
Neutralising solution, pH8	3M NaCl
	0.5M Tris
PBS	137mM NaCL
	2.7mM KCL
	4.3mM Na_2HPO_4
	1.47mM KH_2PO_4
	pH to 7.4

PBST	PBS
	0.1% v/v Tween-20
PFA (4%)	4% w/v in PBS, dissolved at 70^{o}C for 2 hour
PG-PBT	4% w/v PFA
	0.2% v/v Glutaraldehyde in PBT
RIPA	150mM NaCl
	1% v/v Nonidet-P-40
	0.5% w/v Sodium deoxycholate
	0.1% w/v SDS
	1mM EDTA
	50mM Tris, pH 8.0
SSC (20x)	3M NaCl
	0.3M Sodium Citrate
	to pH 7.0 with HCl
SSC-FT	2x SSC, pH 4.5
	50% v/v Formamide
	0.1% v/vTween-20

TAE (50x)	2M Tris
	50mM EDTA
	to pH 7.7 with acetic acid
TBST (10x stock)	8g NaCl
	0.2g Kcl
	25ml 1M Tris pH7.5
	10ml 10% v/v Tween-20
TE buffer	10mM Tris-HCl, pH 8.0
	1mM EDTA, pH 8.0

2.1.2 Cell culture solutions

3i medium	N2B27 medium
	3µM CHIR99021 (Dundee University)
	0.8µM PD184352 (Dundee University)
	2µM SU5402 (Calbiochem, 572630)
	Penicillin (100U/ml) } (Gibco,
	Streptomycin (100µg/ml) } 15140-122)
2i Medium	N2B27 medium
	3µM CHIR99021 (Dundee University)
	1µM PD032 (Dundee University)
	Penicillin (100U/ml) } (Gibco,
	Streptomycin (100µg/ml) } 15140-122)
DMEM	1x DMEM (high glucose) (Gibco, 11965-092)
	10% v/v FCS (Invitrogen, 10270-106)
	2mM L-glutamine (Invitrogen, 25030-024)
	1x Non-Essential Amino Acids (Gibco, 11140-035)
	Penicillin (100U/ml) } (Gibco,
	Streptomycin (100µg/ml) } 15140-122)
Freezing mix (2x)	N2B27/DMEM or GMEM + 10% v/v FCS
	20% v/v DMSO

GMEM	1x GMEM (Sigma, G5154) 0.1mM Non-essential amino acids (Invitrogen, 11140-035) 2mM L-Glutamine (Invitrogen, 25030-024) 1mM Sodium pyruvate (Invitrogen, 11360-039) β-mercaptoethanol (BDH, 44143-3A) 100U/ml LIF
KSOM^{AA}	KSOM dissolved in diluent (Chemicon, MR-020P-5D) 0.8x MEM non-essential amino acids (Sigma, M-7145) 1x BME amino acids (Sigma, B-6766) 5.56mM Glucose
LIF	Recombinant LIF was produced by Cos7 cells that had been transfected with the pC10-6R human DIA/LIF expression vector (Smith, Heath et al. 1988). The Cos7 conditioned medium was harvested and the concentration assayed using the mouse ES cell line, CP1, as indicator cells (Rathjen, Toth et al. 1990) then diluted to give a stock concentration of 100,000 units/ml.

Lysis buffer (ES cell)	100mM Tris-HCl, pH8.5
	5mM EDTA, pH8.0
	2% w/v SDS
	200mM NaCl
N2 supplement	DMEM/F12 (Gibco, 21331-020)
	50µg/ml BSA
	25µg/ml Insulin
	6ng/ml Progesterone
	16µg/ml Putrescine
	30nM Sodium Selenite
	100µg/ml Transferrin
Neurobasal/B27	Neurobasal medium (Gibco, 21103-049)
	2% v/v B27 (Gibco, 17504-044)
	L-glutamine (Invitrogen, 25030-024)
N2B27 medium	1:1 mix of N2 supplement : Neurobasal/B27

PB1 (Phosphate-buffered medium)	136.98mM Sodium chloride 2.73mM Potassium chloride 0.3mM Sodium pyruvate 1.42mM Potassium dihydrogen phosphate 0.48mM Magnesium chloride 8.05mM Sodium hydrogen phosphate 0.94mM Calcium chloride 0.01mg/ml Phenol Red 1mg/ml Penicillin G 0.05mg/ml Streptomycin 1mg/ml Glucose 3mg/ml BSA
RCGM	GMEM 15% v/v FCS (Invitrogen, 10270-106) Penicillin (100U/ml) } (Gibco, Streptomycin (100µg/ml) } 15140-122) 200U/ml LIF
Trypsin	0.025% w/v Trypsin (Invitrogen, 15090-046) 1mM EDTA 1% v/v Chicken serum (Sigma, C5405)

2.2 Cloning

Cloning of DNA fragments was achieved by ligating purified, restriction enzyme-digested DNA. DNA was digested with the appropriate restriction enzyme according to the manufacturer's instructions. Digested DNA was resolved on a TAE agarose gel to confirm digestion, and the desired fragment excised then purified using Geneclean II gel purification kit (Qbiogene, 1001-400) according to the manufacturer's instructions. Purified DNA concentrations were determined by TAE agarose gel electrophoresis alongside known standards. Typically a 3:1 insert:vector molarity ratio was ligated with 400U T4 DNA Ligase (NEB, M0202L) in a 20µl reaction overnight at 16^{o}C.

2.2.1 TOPO® Cloning

4µl of PCR products were cloned into the pCRII®-TOPO® vector using the TOPO® TA Cloning kit as manufacturer's instructions (Invitrogen, K4600-01).

2.2.2 Transformation of bacterial cells

Typically 50ng vector DNA or 20µl of ligation reaction were transformed into 100µl DH5α competent cells. The DNA/cell mixture was incubated on ice for 30 minutes prior to heat-shocking for 1 minute at 42^{o}C. The cells were allowed to recover on ice for 2 minutes before adding 800µl LB medium and incubating for 1 hour at 37^{o}C with shaking. Various amounts of culture were plated on to 9cm LB agar dishes, allowed to absorb at room temperature and then incubated overnight at 37^{o}C. Dishes were stored at 4^{o}C and colonies picked into LB medium containing the appropriate selective agent (typically 50µg/ml Ampicillin (Roche, 10835269001)).

2.3 Sample preparation

2.3.1 Plasmid vector DNA isolation (miniprep)

A single bacterial colony was picked and used to inoculate a culture of 3ml LB medium containing appropriate antibiotic selection (typically 50µg/ml Ampicillin (Roche, 10835269001)). The culture was incubated for 16 hours at 37^{o}C with shaking. 1ml of cell culture was harvested by centrifugation at 16,000*g* on a bench-top centrifuge (Hereaus Biofuge Pico) for 2 minutes. The supernatant was discarded and the pellet resuspended in 100µl cold GTE by vortexing. The cell suspension was lysed by addition of 200µl 0.2M NaOH / 1% SDS (w/v), mixed by inversion and incubated on ice for 5 minutes. The lysate was then precipitated by addition of 150µl 3M NaOAc, pH4.8, mixed by inversion and centrifuged at 16,000*g* for 10 minutes. The supernatant was transferred to 1ml of ice-cold 100% ethanol, mixed by inversion and spun at 16,000*g* for a further 10 minutes. The pellet was resuspended in 300µl 0.1M NaOAc / 50mM Tris, pH 8.0 and incubated at 70^{o}C for 10minutes. After cooling to room temperature, 5µl of 10mg/ml RNaseA (Roche, 10109169001) was added, mixed by inversion and incubated at room temperature for 1 hour. 300µl Phenol was added, vortexed and spun at 16,000*g* for 2 minutes. The aqueous phase was transferred to a fresh tube to which 300µl Chloroform was added, vortexed and spun for a further 2 minutes. DNA was then precipitated from the aqueous phase by addition of 750µl 100% ice-cold ethanol and centrifugation at 16,000*g* for 10 minutes. The DNA pellet was washed twice with 70% ethanol, then air-dried and resuspended in 50µl TE buffer.

2.3.2 Plasmid vector DNA isolation (maxiprep)

A single bacterial colony was picked and used to inoculate a starter culture of 3ml LB medium containing the appropriate antibiotic selection (typically 50µg/ml Ampicillin (Roche, 10835269001)). The culture was incubated for approximately 7 hours at 37°C with shaking then pelleted by centrifugation at 1500*g* for 3 minutes (MSE Mistral 2000). The resultant pellet was resuspended in LB medium then used to seed a 250ml LB culture containing the appropriate antibiotic selection. The cell culture was incubated for 16 hours at 37°C with shaking then harvested by centrifugation at 6000*g*, 4°C for 10 minutes (Sorvall SLA-1500 rotor). The supernatant was discarded and the pellet resuspended in 10ml cold GTE by pipetting. The cell suspension was lysed by addition of 20ml 0.2M NaOH / 1%SDS, mixed by inversion then incubated on ice for 5 minutes. The lysate was then precipitated by addition of 15ml 3M NaOAc, pH4.8, mixed by inversion and spun at 2000*g* for 10 minutes (MSE Centaur 2). The supernatant was divided equally into two 50ml Corning tubes and 25ml ice-cold Isopropanol added to each, mixed by inversion then centrifuged at 2000*g* for 10 minutes. Each pellet was resuspended in 1ml TE buffer, pooled, then 2.5ml of 5M LiCl added and mixed by inversion before centrifugation at 2000*g* for 10 minutes. The supernatant was transferred to 12.5ml of ice-cold 100% ethanol, mixed by inversion then centrifuged again at 2000*g* for 10 minutes. The pellet was resuspended in 500µl TE buffer then transferred to a 1.5ml eppendorf tube. 20µl of 10mg/ml RNaseA was added, mixed by inversion and incubated at room temperature for 1 hour. 500µl of 1.6M NaCl / 13% PEG 6000 was added, mixed by inversion, incubated on ice for 5 minutes then centrifuged at 16,000*g* for 5 minutes (Hereaus Biofuge Pico). The pellet was resuspended in 500µl TE buffer by

vortexing. 500µl Phenol was added, vortexed, then spun for 2 minutes at 16,000*g*. The aqueous phase was transferred to a fresh tube to which 500µl Chloroform was added, vortexed, then spun for a further 2 minutes. The aqueous phase was then precipitated in 1ml ice-cold 100% ethanol containing 50µl 3M NaOAc, pH5.5 by centrifugation at 16,000*g* for 10 minutes. The pellet was washed twice with 70% ethanol, then air-dried and resuspended in 500µl TE buffer.

2.3.3 ES cell genomic DNA isolation

Cells were lysed in 500µl ES cell lysis buffer containing 50µg Proteinase K (Sigma, P2308) for 4-24 hours at 37^{o}C. 500µl of room temperature Isopropanol was added and mixed by inversion for 10 minutes then centrifuged at 16,000*g* (Hereaus Biofuge Pico centrifuge). The pellet was washed twice with 70% ethanol then resuspended in 50µl TE buffer.

2.3.4 Tail and ear genomic DNA isolation

Tissue samples were lysed in 500µl tissue lysis buffer containing 500µg Proteinase K (Sigma, P2308) overnight at 55^{o}C. The lysates were cleared of debris by centrifugation at 9500*g* for 5 minutes, then transferring the supernatant to a fresh tube. The supernatant was then extracted, firstly with an equal volume of Phenol:Chloroform:Isoamylalchohol (25:24:1) and then Chloroform, by vortexing and then centrifuging at 9500*g* for 2 minutes. The final aqueous phase was precipitated in 2 volumes of ice-cold, 100% ethanol and 1/10th volume 3M NaOAc. The pellet was washed twice with 70% ethanol, air-dried and resuspended in TE buffer.

2.3.5 Blood cell genomic DNA isolation

Blood samples were centrifuged at 380*g* for 3 minutes (Hereaus Biofuge Pico centrifuge). The pellet was reusupended in 1ml Pharmlyse (BD Biosciences, #555899) and incubated in the dark for 15 minutes at room temperature. This was then centrifuged as before and the pellet resuspended in 500µl tissue lysis buffer containing 500µg Proteinase K (Sigma, P2308) overnight at 55^{o}C. The lysates were then processed as for tail and ear samples.

2.3.6 Protein isolation.

Cells were pelleted at 300*g* for 3 minutes (Eppendorf 5702 centrifuge) then washed with 5ml cold PBS. The final pellet was resuspended in 50µl 1x Laemmli buffer, sonicated (Branson sonifier 150, remote setting 2) until the lysate was no longer viscous (typically 2-5 second) then boiled for 10 minutes before storing at -20^{o}C.

2.3.7 RNA isolation

Cells were pelleted at 300*g* for 3 minutes (Eppendorf 5702 centrifuge) then washed with 5ml cold PBS. The resultant pellet was stored at -80^{o}C. RNA samples were isolated from cells using RNeasy^{TM} Mini Kit (Qiagen, #74104) as detailed in the manufacturer's instructions and included the recommended DNase treatment.

2.3.8 cDNA synthesis

cDNA was synthesized from RNA samples by Oligo-dT priming using the Superscript™ First Strand Synthesis System for RT-PCR (Invitrogen Corporation, #11904-018) as detailed in the manufacturer's instructions.

2.4 Detection methods

2.4.1 Western Blotting

Samples were boiled for 5 minutes prior to running on a 10% Bis-Tris polyacrylamide gel (Invitrogen Corporation, #NP0301BOX). The gel was electrophoresed for 50 minutes at 200V in MOPS-SDS running buffer (Invitrogen Corporation, #NP0001) then transferred on to Immobilon-P™ PVDF membrane (Millipore, IPVH08130) at 395mA for 70 minutes at 4°C. The membrane was washed twice for 5 minutes with TBST prior to blocking overnight with 10% milk solution at 4°C. After washing twice with TBST, the membrane was incubated for one hour at room temperature in the appropriate dilution of primary antibody (see Table 2.1) in 5% milk solution. Primary antibody was removed by washing the membrane four times for 15 minutes each in TBST before incubating for one hour at room temperature in the appropriate dilution of secondary antibody (see Table 2.1). Secondary antibody was removed by washing the membrane four times for 15 minutes in TBST. The bands were visualised using Supersignal™ West Pico (Pierce, #34080) as described in manufacturer's instruction and detected by exposing (typically 10s – 1 minute) on to Hyperfilm (Amersham, RPN2103) and developed using a Konica-Minolta SRX-101A developer.

Table 2.1 Antibodies and dilutions used for Western blotting.

Antibody	Dilution	Supplier, Catalogue number	Secondary Antibody
Cdx2	1:2000	Abcam, ab22586	α rabbit-HRP
Nanog (α75x36)	1:3000	Kind gift from Dr. I Chambers	α rabbit-HRP
αOct-4 (C10)	1:2000	Santa Cruz, Sc5279	α mouse-HRP
α mouse-HRP	1:2500	Amersham, NA931	N/A
α rabbit-HRP	1:2500	Amersham, NA934	N/A

2.4.2 Immunohistochemical staining.

Embryos were recovered from timed-matings by flushing the uteri/oviduct with PB1 + 10%FCS before transferring to PBS.

Embryos/cells were washed, twice with PBS, then fixed at room temperature for 10 minutes in 4% PFA. After washing twice for 5 minutes in PBST, the cells were then blocked and permeabilised at room temperature for 20 minutes in PBST containing 3% serum (derived from the same species as the secondary antibody was raised in) and 1% BSA (blocking solution). The cells were then incubated with the appropriate dilution of primary antibody (see Table 2.2) at 4^{o}C overnight in blocking solution. Primary antibody was removed with four washes in PBST for five minutes each. The cells were then incubated with secondary antibody (see Table 2.2) at room temperature, in the dark for 1 hour in blocking solution. Secondary antibody was removed by washing four times for 5 minutes with PBST before staining the cells with DAPI and observing under a fluorescence microscope for staining.

Table 2.2 Antibodies and dilutions used for immunohistochemical staining.

Antibody	Dilution	Supplier, Catalogue number	Secondary Antibody
Cdx2	1:80	Abcam, ab22586	α rabbit-HRP
Nanog (α75x36)	1:30,000	Kind gift from Dr. I Chambers	Alexa 488 goat α-rabbit IgG (H+L)
α-Oct-4 (C10)	1:200	Santa Cruz, Sc5279	Alexa 594 goat α-mouse IgG_{2b}
Alexa 488 goat α-rabbit IgG	1:1000	Invitrogen, A11008	N/A
Alexa 594 goat α-mouse IgG_{2b}	1:1000	Invitrogen, A21145	N/A

2.4.3 *In-situ* Hybridisation of embryos and cells

Early stage embryos were isolated as described below, placed on ice and washed twice with cold PBS before fixing overnight at 4^{o}C in 4% paraformaldehyde in PBS. Embryos were then processed as follows:

2.4.3.1 Pretreatment

- Washed twice with cold PBT before dehydrating by successive 5 minute washes of 25%, 50% and 75% methanol-PBS followed by two washes in 100% methanol.
- Rehydrated by successive 5 minute washes with 75%, 50% and 25% methanol in PBS.
- Washed three times with PBT at room temperature.

- Washed three times in RIPA (1% Triton X100 in PBS for cells), 30 minutes per wash.
- Post-fixed for 20 minutes in PG-PBT at room temperature with mixing.
- Washed five times with PBT at room temperature, for 5 minutes each time.

2.4.3.2 Hybridisation

- Washed for 15 minutes with 1:1 mix of Hybridisation Buffer (HB) and PBT at room temperature.
- Washed once with HB at room temperature.
- Prehybridised for 5 hours (overnight for cells) at 70^{o}C in HB containing 100µg/ml tRNA and 100µg/ml sheared denatured herring sperm DNA.
- Digoxigenin-labelled riboprobe (see below for preparation) denatured by heating at 95^{o}C for 10 minutes then placing on ice. NB. Anti-sense riboprobe used as a control.
- Hybridised in HB containing 100µg/ml tRNA and denatured DNA overnight at 70^{o}C in a chamber humidified with 50% formamide.
- Washed for 10 minutes in HB at 70^{o}C.
- Washed twice for 5 minutes with SSC-FT at room temperature.
- Washed three times for 30 minutes with SSC-FT at 65^{o}C.
- Cooled to room temperature and then washed three times with TBST.

2.4.3.3 Antibody binding

- Blocked in 10% heat-inactivated sheep serum in TBST for 1 hour.
- Block replaced with 1:2000 dilution of freshly preadsorbed anti-digoxigenin Fab-alkaline phosphatase conjugate (Boehringer Mannheim) in TBST with 1% heat-inactivated sheep serum and incubated overnight at 4^{o}C.

2.4.3.4 Washing and colour development

- Antibody conjugate removed and washed with TBST at room temperature three times for 5 minutes followed by four times for 30 minutes.
- Washed three times for 10 minutes with APB.
- Stained in a solution of 4.5µl/ml NBT and 3.5µl/ml BCIP in APB in dark until colour develops (NB. embryos transferred to glass dish prior to staining).
- Reaction stopped by washing three times in 1mM EDTA in PBS.

2.4.3.5 Riboprobe synthesis

The following were combined in an eppendorf tube –

1-3ug linearised template DNA
10µl 5x dNTP mix, containing 11-digoxigenenin-UTP
5µl 10x transcription buffer
50 units RNase inhibitor
50 units appropriate phage RNA polymerase
to 50µl final volume with DEPC-treated water.

The reaction was incubated at 37^{o}C for two hours then added 20 units of RNase-free DNase and incubated at 37^{o}C for 15 minutes. The reaction was stopped

by the addition of EDTA to a final concentration of 25mM. The DNA was precipitated with 1/10th volume 4M LiCl and three volumes of absolute ethanol and cooling overnight at -20^{o}C. The DNA was pelleted by centrifugation at 16,000*g* for 15 minutes. The pellet was washed twice, carefully, with 70% ethanol, then air-dried and resuspended in 100µl DEPC-treated water. Riboprobe size and quality was confirmed by agarose gel electrophoresis.

Table 2.3 Riboprobes for *in situ* hybridisation.

Riboprobe	**Vector**	**Polymerase**
Nanog sense	pPyCAG-rat Nanog-IP	T7
Nanog anti-sense	pPyCAG-rat Nanog-IP	SP6
Oct4 sense	pPyCAG-rat Oct4-IP	SP6
Oc4 anti-sense	pPyCAG-rat Oct4-IP	T7

2.4.4 RT-PCR

Typically one tenth of cDNA sample was amplified in a 50µl reaction on a PTC-200 thermocycler (MJ Research) using 2.5U GoTaq Flexi DNA Polymerase (Promega Corporation, #M8305) under the following conditions; 94^{o}C 2 minutes, followed by 30 cycles of 94^{o}C 20 seconds, 50^{o}C 20 seconds and 72^{o}C 1 minute, with a final extension at 72^{o}C for 5 minutes. The primer sequences used and the product sizes are listed in Table 2.3.

Table 2.4 Oligoncleotides used for RT-PCR analysis.

Rat mRNA	Sequence (5' to 3')	Product size
β-actin	For - CACTGGCATTGTGATGGACT	427
	Rev - ACGGATGTCAACGTCACACT	
Brachyury	For - AACTGCGAGTGGGTCTGGAAG	451
	Rev - TGGGTCTCGGGAAAGCAGTG	
Cdx2	For - CCGAATACCACGCACACCATC	394
	Rev - CTTTCCTTGGCTCTGCGGTTC	
c-Myc	For - CCTCAGTGGTCTTCCCCTACC	284
	Rev - GCTGTGTGGAGGTTTGCTGTG	
Eras	For - CGAGCGGTGTGGGTAAAAGTG	501
	Rev - GGTGTCGGGTCTTCTTGCTTG	
Errβ	For - TGTGCGGGGACATTGCTTCTG	436
	Rev - TCCCGATCTGCCAAGTCACAG	
FGF4	For - CGGGGTGTGGTGAGCATCTTC	202
	Rev - CCTTCTTGGTCCGCCCGTTC	
GATA6	For - TCATCACGACGGCTTGGACTG	467
	Rev - GCCAGAGCACACCAAGAATCC	
Kdr	For - ATACACCTGCACAGCGTACAG	271
	Rev - TCCCGCATCTCTTTCACTCAC	
Klf4	For - CAGTCGCAAGTCCCCTCTCTC	321
	Rev - CCTGTCGCACTTCTGGCACTG	
Nanog	For - GCCCTGAGAAGAAAGAAGAG	356
	Rev - CTGACTGCCCCATACTGGAA	
Nestin	For - AGAGAAGCGCTGGAACAGAG	234
	Rev - AGGTGTCTGCAACCGAGAGT	
Oct-4	For - GGGATGGCATACTGTGGAC	412
	Rev - CTTCCTCCACCCACTTCTC	
Pax6	For - GAGACTGGCTCCATCAGACC	212
	Rev – CTAGCCAGGTTGCGAAGAAC	

Rex1	For - TTCTTGCCAGGTTCTGGAAGC	277
	Rev - TTTCCCACACTCTGCACACAC	
Sox2	For - GGCGGCAACCAGAAGAACAG	414
	Rev – GTTGCTCCAGCCGTTCATGTG	
Sox17	For - AGGAGAGGTGGTGGCGAGTAG	268
	Rev - GTTGGGATGGTCCTGCATGTG	
Stella	For – TCCTACAACCAGAAACACTAG	304
	Rev – GTGCAGAGACATCTGAATGG	

Mouse mRNA	**Sequence (5' to 3')**	**Product size**
Nanog	For ATGAAGTGCAAGCGGTGGCAGAAA	464
	Rev CCTGGTGGAGTCACAGAGTAGTTC	

2.4.5 Southern Blotting

5µg of genomic DNA were digested with 40U of the appropriate restriction enzyme for 3-4 hours at the correct temperature. A further 40U of enzyme was then added and incubated overnight. The digested DNA was run overnight at 20V on a 0.7% agarose gel. The gel was stained with Ethidium bromide and photographed under short wave UV fluorescence to nick the DNA. The gel was then washed twice in denaturing solution for 20 minutes each with gentle agitation. A brief rinse in distilled water was carried out prior to two washes in neutralising solution for 20 minutes each. The gel was then blotted overnight onto charged nylon membrane (Millipore, INYC00010) in 20xSSC. Following transfer, the membrane was washed in 2xSSC and the DNA fixed by baking at 120^{o}C for 2 hours. The membrane was washed briefly in 2xSSC prior to hybridization with radiolabelled probes.

2.4.5.1 Radiolabelling of DNA probe

25ng of DNA in TE buffer was labeled with Rediprime™ II Random Prime Labelling System (GE Healthcare, #1633) following the manufacturer's protocol, using 50µCi Redivue™ [^{32}P] d-CTP (GE Healthcare, #AA0005). Unincorporated nucleotides were removed by passing the reaction through a ProbeQuant™ G-50 micro column (GE Healthcare, #27-5335-01) according to manufacturer's instructions. The probe was then denatured by boiling for 5 minutes and cooled quickly on ice for 5 minutes before adding to the hybridization mixture.

2.4.5.2 Hybridisation of membranes with radioactive probes

The membrane was washed briefly in 2xSSC, then pre-hybridised for 3 hours at 65^{o}C in 25ml of hybridization mix containing 1.25ml of pre-boiled 0.2M Tris pH8.0 and 10mg/ml Salmon sperm DNA in 0.4M NaOH (1:1). The denatured, radiolabelled probe was diluted in 10ml of hybridization mix containing Tris and Salmon sperm as before, then added to the membrane and incubated for 24 hours at 65^{o}C. The following day, the hybridization mix was removed and the membrane washed briefly with 2xSSC followed by two, 10 minute washes with 2xSSC/0.1%SDS and one, 5 minute wash with 0.1xSSC/0.1%SDS (wash solutions were pre-heated to 65^{o}C). The blot was then exposed for 3 days on Biomax XAR film (Kodak, #165-1512) and developed using a Konica-Minolta SRX-101A developer.

2.4.6 Microsatellite genotyping

Fluorescent-tagged oligonucleotides (Applied Biosystems) were used to amplify the rat microsatellite regions D1Rat122, D3Rat17 and D2Rat250. Genomic DNA was extracted from tail, ear and blood biopsies. PCR reactions were amplified in a PTC-200 thermocycler (MJ Research) using Taq DNA Polymerase (Invitrogen) under the following conditions; 95^{o}C 2 minutes, followed by 35 cycles of 94^{o}C for 30 seconds, 58^{o}C or 66^{o}C for 45 seconds and 72^{o}C for 45 seconds, with a final extension at 72^{o}C for 10 minutes. Annealing temperatures were 62^{o}C, 58^{o}C and 66^{o}C for D1Rat122, D3Rat17 and D2Rat250 respectively. PCR products were diluted 1 in 100 in sterile, distilled water. 2µl of this was diluted in 18µl Hi-Di Formamide (Applied Biosystems) containing LIZ-500 internal size standard (Applied Biosystems). The resulting products were detected on an ABI capillary 3730 DNA analyzer and visualised on a 4% agarose gel. D1Rat122 alleles are 230bp and 255bp for DA and Fischer rat strains respectively, D3Rat17 alleles are 140bp and 154bp for DA and SD strains respectively, and D2Rat250 alleles are 156bp, 168bp and 190bp for SD, DA and Fischer rat strains respectively.

Table 2.5 Oligoncleotide sequences for microsatellite analysis.

Microsatellite region	Direction	Oligonucleotide sequence	Fluorescent tag
D1Rat122	**Forward**	CTGCTCCACCTGCCTGTATT	6-FAM (blue)
	Reverse	TCCCTTTGCAATAGACAATGG	
D3Rat17	**Forward**	TCATTTTCCTTCCTCTCTCTCA	VIC (green)
	Reverse	AAGACAAAATGCTGGAGGGA	
D2Rat250	**Forward**	GTCCCTCTCCTGTCCCTCTC	PET (red)
	Reverse	GAAGTCTGAACGCTCATGCA	

2.4.7 Genomic PCR

100-200ng of genomic DNA was amplified using oligonucleotides designed against the rat Sry gene or GFP transgene. *Sry* primers give no product from mouse genomic DNA. IL2 oligonucleotides were included in each reaction as an internal control. The PCR was performed in a PTC-200 thermocycler (MJ Research). For *Sry* PCR, GoTaq Flexi DNA Polymerase (Promega) was used along with the following conditions; 95^{o}C for 2 minutes, followed by 35 cycles of 94^{o}C for 30 seconds, 50^{o}C for 1 minute and 72^{o}C for 1 minute, with a final extension at 72^{o}C for 10 minutes. For GFP PCR, Taq DNA Polymerase (Invitrogen) was used along with the following conditions; 95^{o}C for 2 minutes, followed by 35 cycles of 94^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30 seconds, with a final extension at 72^{o}C for 10 minutes. PCR products were visualised with ethidium bromide on a 2% agarose gel. The IL2 PCR product is 324bp, the *Sry* product is 104bp and the GFP product is 173bp.

Table 2.6 Oligoncleotide sequences for genomic PCR.

Oligonucleotides	Direction	Oligonucleotide sequence
Sry	**Forward**	CATCGAAGGGTTAAAGTGCCA
	Reverse	ATAGTGTGTAGGTTGTTGTCC
GFP	**Forward**	AAGTTCATCTGCACCACC
	Reverse	TCCTTGAAGAAGATGGTGCG
IL-2	**Forward**	CTAGGCCACAGAATTGAAAGATCT
	Reverse	GTAGGTGGAAATTCTAGCATCATCC

2.4.8 DNA sequencing

1µg of miniprepped vector DNA was mixed with 3.2pmoles of sequencing primer in a 6µl volume. Sequencing was carried out by University of Edinburgh, School of Biological Sciences Sequencing Service using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, #4337445) and analysed on a 3730 DNA Analyser (Applied Biosystems).

2.5 Vibratome sectioning

Fluorescent embryos were embedded in a 1:1 mixture containing 20% gelatin and 20% albumin in PBS then fixed overnight at 4^{o}C in 4% Paraformaldehyde. The gelatin blocks were then embedded in a 'setting solution' with 10% Glutaraldehyde overnight at 4^{o}C. Transverse sections were cut in a Series 1000 Vibratome at 100µm and then treated with PBST for 10 minutes prior to mounting under coverslips in Vectashield (Vector Laboratories) containing DAPI nuclear fluorescent stain. Confocal microscopy was performed on the sections within 24 hours of sectioning.

The 'setting solution' is prepared by combining 450ml PBS with 2.2g gelatin, 70g BSA and 90g sucrose. The gelatin is added to the PBS, heated to dissolve then allowed to cool. The BSA is then added, allowing a thin layer of albumin to form on the surface that dissolves very slowly. Once dissolved, the sucrose is added and the final solution aliquoted and stored at -20^{o}C.

2.6 Animals

Rats were housed and bred within the University of Edinburgh according to the Animals (Scientific Procedures) Act (UK) 1986. They were maintained on a

cycle of 12 hours dark, 12 hours light, with the midpoint of the dark cycle at 0100. Litters from natural matings were left with parents until 4 weeks of age, at which point they are weaned by separating the offspring from their parents. At weaning the animals were sexed and tail tips, ear punches and blood were taken for genotyping. Breedings, tissue biopsies, bleeding and maintenance of animal stocks were performed by the Biomedical Facility staff at the ISCR.

2.7 Cell culture

2.7.1 Cell attachment substrates

2.7.1.1 DIA-M feeder layer

The DIA-M feeder cells are C3H/10T1/2 mouse fibroblasts that have been stably transfected with an expression vector for the matrix-associated form of LIF (Rathjen et al., 1990). Cells were maintained in GMEM + 10% FCS, without LIF and grown to confluency in a 75cm^2 flask then gamma-irradiated and plated into gelatin-coated (0.1%) 4-well dishes (2cm^2/well) at a density of 1.5 x 10^4 cells/well.

2.7.1.2 Gelatin Coating

Wells were coated in 0.1% gelatin for five minutes at room temperature. The gelatin was aspirated and wells allowed to dry in tissue culture hood for 5 minutes.

2.7.1.3 Laminin Coating

Wells were coated in 10µg/ml Laminin (Sigma, L1010) overnight at 37^{o}C. Laminin was aspirated and wells washed three times with PBS before plating cells.

2.7.2 Immunosurgery

After removing the zona pellucida with acid Tyrode's solution, blastocysts were incubated for 1 hour at 37^{o}C (no CO_2) in PB1 (filter sterilised) containing rabbit anti-rat serum antibody (Sigma, R5256) at a dilution of 4:1. The blastocysts were then rinsed three times in successive drops of PB1 (filter sterilised) containing 10% FCS before transferring to PB1 (filter sterilised) containing rat complement serum (Sigma, S3394) at a dilution of 4:1. They were monitored frequently until the trophectoderm had lysed, then transferred to GMEM + 10% FCS for 30-60 minutes.

2.7.3 Derivation and culture of rat ExS cells

Rat blastocysts were collected at 4.5d.p.c and the zona pellucida removed with acid Tyrode's solution (Sigma, T1788). Immunosurgery was then carried out to remove the trophectoderm and isolate the ICM. The resulting ICMs were plated and cultured in Rat Cell Growth Medium (RCGM) on a layer of gamma-irradiated DIA-M cells. 80µg Matrigel^{TM} (BD Biosciences, 354234) was also added to the culture. Cells were allowed to attach and outgrow for 4 days, then mechanically disaggregated into small clumps using a drawn-out Pasteur and moved to fresh DIA-M feeders in RCGM.

2.7.4 Passaging rat ExS cells

Colonies of ExS cells were passaged in one of three ways described below:

2.7.4.1 Mouth pipetting (without trypsin).

This technique was used when there were only a small number of colonies (<10). Colonies were mechanically disaggregated into smaller pieces under a low-power microscope, using a drawn-out Pasteur and fine gauge syringe needle.

2.7.4.2 Mouth pipetting (with trypsin).

This technique was used when there were only 10 to 20 colonies growing in culture. Colonies were removed from the DIA-M feeder layer using a mouth pipette and transferred sequentially to a well containing PBS, then a well containing Trypsin and incubated 37^{o}C 3-5 minutes. Finally, colonies were dispersed to a near-single cell suspension into a well containing RCGM using a fine Pasteur, then seeded into a fresh well.

2.7.4.3 Trypsinising colonies *in situ*.

This technique was used when there were a large number of colonies growing in culture (>20). Cells were washed three times with PBS then 200µl Trypsin added and incubated 37^{o}C for 3-5 minutes. Colonies were dispersed to a near-single cell suspension by pipetting, then transferred to a universal containing 5-10ml GMEM + 10% FCS and centrifuged at 300*g* for 3 minutes. The supernatant was carefully aspirated and the cell pellet resuspended in 200µl RCGM and plated at the appropriate dilution(s).

2.7.5 Freezing and thawing rat ExS cells

Cells were washed three times with PBS then 200µl Trypsin added and incubated 37°C for 5 minutes. The cells were dispersed to near-single cell suspension by pipetting, transferred to a universal containing 5ml GMEM + 10% FCS and centrifuged 300*g* for 3 minutes. The supernatant was aspirated and the cell pellet resuspended in 1ml RCGM and this divided equally into two cryovials**.** An equal volume (500µl) of 2x Freezing mix was added to each, mixed carefully, then transferred to -80°C immediately and to LN_2 the next day.

Cells were thawed into a universal tube containing 20ml pre-warmed (37°C) GMEM + 10% FCS by transferring warm medium back and forth between universal tube and vial until all the contents of the vial had been transferred to the universal. The cells were pelleted by centrifugation at 300*g* for 3 minutes. The supernatant was aspirated and the pellet resuspended in 200µl RCGM and plated equally over two wells of a 4-well plate in a volume of 500µl/well. The cells were allowed to adhere overnight before adding Matrigel the next day.

2.7.6 Derivation and culture of rat ES cells.

Rat ES cells were originally derived by Mia Buehr. Rat blastocysts were collected at 4.5d.p.c and the zona pellucida removed with acid Tyrode's solution (Sigma, T1788). Immunosurgery was carried out to remove the trophectoderm and isolate the ICM. Blastocysts were incubated for 1 hour at 37°C (no CO_2) in PB1 (filter sterilised) containing rabbit anti-rat serum antibody (Sigma, R5256) at a dilution of 4:1. The blastocysts were then rinsed three times in successive drops of PB1 (filter sterilised) containing 10% FCS before transferring to PB1 (filter

sterilised) containing rat complement serum (Sigma, S3394) at a dilution of 4:1. They were monitored frequently until the trophectoderm had lysed, then transferred to GMEM + 10% FCS for 30-60 minutes. The resulting ICMs were plated and cultured in 3i medium on a layer of gamma-irradiated DIA-M cells. Cells were allowed to attach and outgrow for between five and seven days, then mechanically disaggregated into small clumps using a drawn-out Pasteur and moved to fresh DIA-M feeders in 3i medium.

2.7.7 Passaging rat ES cells

2.7.7.1 Mouth pipetting

Colonies were disaggregated under a low power microscope by mouth pipetting using a drawn-out Pastuer pipette.

2.7.7.2 Dispersing colonies to single cells.

Cells were washed with 500µl PBS then 200µl Accutase (Sigma, A6964) added and incubated 37^{o}C for 2-3 minutes. Colonies were dispersed to single cell by pipetting and transferred to a universal containing 5ml GMEM + 10% FCS. The cells were pelleted at 300*g* for 3 minutes then the supernatant carefully aspirated. The pellet was finally resuspended in an appropriate volume of 3i medium for plating or freezing.

2.7.8 Freezing and thawing rats ES cells.

Cells were dispersed to a single-cell suspension with Accutase (Sigma, A6964) then pelleted in GMEM + 10% FCS at 300*g* for 3 minutes. The pellet was

resuspend in 3i medium to give a final volume of 500µl/vial. 500µl cell suspension was transferred to a cryovial then 500µl 2xFreezing mix (N2B27+20%DMSO+20%FCS) added and mixed carefully.

Cells were thawed into a universal tube containing 20ml pre-warmed (37^{o}C) GMEM + 10% FCS by transferring warm medium back and forth between universal tube and vial until all the contents of the vial had been transferred to the universal tube. The cells were pelleted by centrifugation at 300*g* for 3 minutes. The supernatant was aspirated and the pellet resuspended in 200µl 3i medium. The cell suspension was divided over two wells of a 4-well plate in a volume of 500µl/well.

2.7.9 Teratoma formation

Approximately 200-400 cells were injected under the kidney capsule of SCID mice (BALB/c JHan Hsd-Prkdc scid). Tumours were collected at various times, and their weight determined by weighing both tumour and kidney, then subtracting the weight of the contralateral uninjected kidney. Tumours were embedded in paraffin wax, sectioned, and stained with Masson's trichrome by Ron Wilkie.

2.7.10 Blastocyst injections and chimaera production

Blastocyst injections were performed, by Jan Ure or Renee McLay, based on protocols previously described (Bradley et al., 1984). Rat ES cells derived from DA and F344 rat strains were injected into the blastocoel cavity of F344 and DA blastocysts respectively. Blastocysts were usually cultured for 2-3 hours in KSOM^{AA} (Biggers et al., 2000) to allow the blastocoel cavity to become fully expanded. Rat ES cells were prepared for injection by transferring several large colonies from the

feeder layer to a well containing 400µl Accutase (Sigma, A6964), incubating at 37°C for 3 minutes before pipetting to single-cell suspension. The cell suspension was spun in 5ml of GMEM + 10% FCS at 300*g* for 3 minutes in an Eppendorph 5702 centrifuge and the resultant pellet resuspended in an appropriate volume of M2 (Sigma-Aldrich, #M7167) medium. 10-15 cells were injected into each blastocyst and approximately 8 injected blastocysts were transferred to the uterine horn of a pseudo pregnant recipient rat. Chimeric pups were initially identified by coat colour chimaerism and confirmed by microsatellite analysis.

2.7.11 Transfection of cells

2.7.11.1 Lipofection of rat ExS cells

Colonies were dissociated with Trypsin and the cells pelleted in GMEM + 10% FCS at 300*g* for 3 minutes. The pellet was then resuspended in RCGM and plated in a final volume of 400µl. Stably-expressing cell lines were generated by lipofection using Lipofectamine™ 2000 (Invitrogen, 11668-027) as detailed in the manufacturer's instructions. A mixture of 1µg *Sca*I-linearised expression vector DNA and 1µl Lipofectamine™ 2000 was added to the culture and incubated overnight. The reagents were removed after 18 hours and replaced with RCGM culture medium. Stable transfectants were selected in appropriate selection (1µg/ml Puromycin (Sigma, PA8833) and/or 20µg/ml Zeocin (Invitrogen, R250-01)) applied 48 hours after transfection and subsequently maintained in selection**.** Polyclonal lines were obtained by pooling several resistant colonies.

2.7.11.2 Lipofection of rat ES cells

Colonies were dissociated with Accutase (Sigma, A6964), pelleted in GMEM + 10% FCS then resuspended in 3i culture medium containing 10% FCS and plated in a final volume of 400µl. The cells were transfected using Lipofectamine™ LTX (Invitrogen, 15338-100) according to manufacturer's instructions. A mixture of 0.25µg *Sca*I-linearised pPYCAGgfpIP vector DNA, 0.25µl PLUS reagent (Invitrogen, 11514-015) and 0.625µl Lipofectamine™ LTX was added to the culture then incubated overnight. The reagents were removed after 18 hours and replaced with fresh serum-free 3i culture medium. Stable transfectants were selected in 0.5µg/ml Puromycin applied 48 hours after transfection and subsequently maintained in selection prior to injection**.** Polyclonal lines were obtained by pooling several resistant colonies.

2.7.11.3 Electroporation

$1x10^6$ 2i rat ES cells were electroporated in PBS or N2B27+10% FCS with 5, 20 or 50µg *Sca*I linearised pPyCAGegfpIP vector using the Bio-Rad Genepulser apparatus (0.8kV, 3µF). $6x10^5$ electroporated cells were plated into a $10cm^2$ well containing 2i medium + 5% FCS. The following day the medium was replaced with serum-free 2i. 1µg/ml puromycin selection was added 72h post-electroporation and the number of puromycin-resistant colonies counted 9 days post-electroporation.

2.7.12 Lentiviral shRNA infection

2.7.12.1 Construction of pSicoR Cdx2 shRNA lentiviral vectors

Cdx2 shRNA oligonucleotides were designed from rat Cdx2 cDNA sequence using pSicoOligomaker 1.5 (http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html). The oligonucleotides were produced by Integrated DNA Technologies (IDT®) with *Hpa*I and *Xho*I overhangs incorporated into the sequence (Table 2.3). 100μM each, of sense and antisense oligonucleotides, were annealed in a 100μl reaction with 25μl 2x annealing buffer using the following cycling conditions; 94°C for 4 minutes, then 70°C for 10 minutes, -1°C/cycle for a further 65 cycles. $1x10^{-1}$ pmoles of annealed oligonucleotides were ligated into $1.2x10^{-2}$pmoles *Hpa*I/*Xho*I digested and gel-purified pSicoR vector (kindly supplied by Professor Tyler Jacks, Massachusetts Institute of Technology, http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html) in a 20μl reaction using 400U T4 DNA Ligase (NEB, M0202S). 20μl of the ligation reaction was transformed into 50μl One Shot Stbl3 competent cells (Invitrogen, C7373-03). A selection of the resultant colonies were grown in LB containing 50μg/ml final Ampicillin and DNA purified using the Endofree Plasmid Maxi Kit (Qiagen, 12362) according to the manufacturer's instructions.

Table 2.7 Rat Cdx2 shRNA oligonuclotide sequences.

Cdx2 shRNA oligonucleotide	**Sequence**
1808 sense 5’	TGT ATG TCT GTG TCG TAA ATT TCA AGA GAA TTT ACG ACA CAG ACA TAC TTT TTT C
1808 antisense 5’	TCG AGA AAA AAG TAT GTC TGT GTC GTA AAT TCT CTT GAA ATT TAC GAC ACA GAC ATA CA
725 sense 5’	TGA AGG AAT TTC ACT ATA GTT TCA AGA GAA CTA TAG TGA AAT TCC TTC TTT TTT C
725 antisense 5’	TCG AGA AAA AAG AAG GAA TTT CAC TAT AGT TCT CTT GAA ACT ATA GTG AAA TTC CTT CA
2006 sense 5’	TGG AGG GGG ATA TCT GAA CAT TCA AGA GAT GTT CAG ATA TCC CCC TCC TTT TTT C
2006 antisense 5’	TCG AGA AAA AAG GAG GGG GAT ATC TGA ACA TCT CTT GAA TGT TCA GAT ATC CCC CTC CA
1808 mutated sense 5’	TGT CCG TCT GTA TCG TCA ATT TCA AGA GAA TTG ACG ATA CAG ACG GAC TTT TTT C
1808 mutated antisense 5’	TCG AGA AAA AAG TCC GTC TGT ATC GTC AAT TCT CTT GAA ATT GAC GAT ACA GAC GGA CA

2.7.12.2 Lentiviral stock production

Lentiviral stocks were produced using 293FT cells. These cells were derived from the 293-F cell line, which is a fast-growing variant of the 293 cell line. The 293 cell line was originally established from primary embryonal human kidney, transformed with sheared human adenovirus type 5 DNA (Graham, Smiley et al. 1977; Harrison, Graham et al. 1977). The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein. The 293FT cell line stably expresses the SV40 large T antigen from the pCMVSPORT6TAg.neo vector. Expression of the SV40 large T antigen is controlled by the human cytomegalovirus (CMV) promoter and is high-level and constitutive.

3µg of pSicoR lentiviral expression vector DNA and 9µg of the ViraPower™ Packaging Mix (Invitrogen, K4975-00) were diluted in 1.5 ml of Opti-MEM® I Medium (Invitrogen, 31985-062) without serum and gently mixed. In a separate tube 36µl Lipofectamine™ 2000 was diluted in 1.5ml of Opti-MEM® I Medium without serum and gently mixed then incubated for 5 minutes at room temperature. After the 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine™ 2000 (Invitrogen, 11668-027), gently mixed then incubated for 20 minutes at room temperature to allow the DNA/Lipofectamine™ 2000 complexes to form. While DNA-lipid complexes were forming, the 293FT cells were washed twice with PBS, trypsinised, spun for 3 minutes at 300*g* in DMEM. The pelleted cells were then resuspended in DMEM to a density of $1.2x10^6$/ml. After allowing the DNA/Lipofectamine™ 2000 complexes to form, they were added to a 10cm tissue culture plate containing 5ml of DMEM. Finally, 5ml (6×10^6 total cells) of the 293FT cell suspension was added to the dish containing the DNA/Lipofectamine™ 2000 complexes and mixed gently by rocking the dish back and forth then incubated overnight at 37°C in a CO_2 incubator. The next day, the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with DMEM containing 1mM sodium pyruvate (Invitrogen, 11360-039). 72 hours post-transfection, the virus-containing supernatant was harvested by removing medium to a 15ml sterile, capped, conical tube and centrifuging at 1400*g* for 15 minutes at 4°C to pellet cell debris. The supernatant was then centrifuged in an SW41 Beckman rotor at 107,000*g* for 90 minutes. The resultant supernatant was discarded and any excess removed by inverting the tubes on a paper towel for 10 minutes. 40µl cold PBS was added to the pellet and stored overnight at 4°C. The next day the PBS, now

containing the viral stock, was washed gently around the bottom of the tube by pipetting and transferred to a cryovial then stored at -80°C.

2.7.12.3 Titrating Lentiviral stock

$4x10^5$ 293FT cells/well were plated in a 6-well plate 12-24 hours prior to titering. A 10-fold serial-dilution of virus was prepared in 1.5ml DMEM containing 8µg/ml Polybrene (Hexadimethrine bromide, Sigma, H9268) to give 5µl, 0.5µl and 0.05µl virus/well. An additional negative control was set up using DMEM/Polybrene without virus. The cells were then incubated at 37°C overnight and the lentiviral-containing medium removed the next day and replaced with fresh DMEM. 72 hours after infection the cells were trypsinised, centrifuged in DMEM at 300*g* for 3 minutes, and resuspend in 500µl cold PBS containing 2% FCS for FACS analysis for GFP expression, and the percentage of GFP positive cells recorded. A well with 0.1% and 10% of cells expressing GFP was used to determine the titer.

Sample calculation

Assuming 1% infection from the well with 0.01µl of virus:

0.01 (percentage of cells that are EGFP positive) x 4 x 10^5 = 4 x 10^3 positive cells.

4 x 10^3 x 100 (dilution factor) = 4 x 10^5 viral particles/µl.

2.7.12.4 Lentiviral infection of rat ExS cells

Cells were trypsinised and plated 24 hours prior to infection. Lentiviral stock was added to the cells in a final volume of 500µl RCGM containing 8µg/ml Polybrene (Hexadimethrine bromide, Sigma, H9268). The lentiviral-containing medium was removed the next day and replaced with fresh RCGM.

2.7.13 LIF-independent self renewal assay

The LIF-independent self-renewal assay was facilitated by the production of *lifr* knockout (LRK1) mouse ES cells (Chambers et al., 2003). LRK1 cells eliminate the background of undifferentiated cells that occurs in the absence of exogenous LIF due to the production of LIF by differentiated cells. These cells can be maintained in the absence of LIF by activation of gp130 using interleukin-6 and soluble interleukin-6 receptor (IL6/sIL6R). $1x10^6$ LRK1 cells were transfected with 3µg circular vector DNA using 3µl Lipofectamine^{TM} 2000 (Invitrogen, 11668-027) as according to manufacturer's instructions. The reaction was allowed to proceed for 24 hours. Cells were then trypsinised and plated at $2x10^5$ and $5x10^4$ in duplicate into $9cm^2$ gelatinised dishes. 1µg/ml Puromycin selection was added and IL-6/sIL6R added to one of the two dishes. Cells were fed every second day and then stained for Alkaline Phosphatase (Sigma, 86R-1KT) after 14 days.

2.7.14 Alkaline phosphatase staining

Cells were washed *in-situ* with 5ml of PBS and fixed with 3ml of Fix solution (65ml Acteone, 25ml Citrate solution, 8ml 37% formaldehyde) for two minutes. The cells were then washed with 5ml distilled water before staining with 4.5ml Staining solution for fifteen minutes, at room temperature, in the dark. Staining solution was prepared by mixing 1ml Sodium Nitrite with 1ml FRV-alkaline solution and incubating at room temperature for two minutes before adding to 45ml distilled water and then adding 1ml Naphthol AS-BI alkaline solution (all reagents supplied with kit, Sigma, 86R-1KT). After staining, the cells were washed once with distilled water, air-dried and the colonies scored.

Chapter 3

Nanog expression pattern in rat ExS cells and the rat embryo

3.1 Introduction

Whilst the derivation of genuine rat ES cells has so far proved elusive, ES-like cells have been derived by several laboratories from a variety of species, including rat (Buehr et al., 2003; Iannaccone et al., 1994; Li et al., 2003; Ruhnke et al., 2003; Saito et al., 2002; Vassilieva et al., 2000; Verma et al., 2007; Yadav et al., 2005).

Rat ES-like cells have been derived in this laboratory from the ICM of E4.5 blastocysts and subsequently maintained *in vitro* (Buehr et al., 2003). These have been termed ExS cells due to their extra-embryonic identity. The cells are maintained on a feeder layer of mitotically-inactivated mouse C3H 10T1/2 fibroblasts which have been modified to express the matrix-associated form of LIF (DIA-M) in the presence of FCS, LIF and Matrigel^{TM}. Their morphology is similar to mouse ES cells grown on feeders (Figure 3.1A & B). However, although these cells are similar to mouse ES cells, in that they self-renew *in vitro*, stain positive for alkaline phosphatase and express SSEA-1, they fail to express the key functional ES cell transcription factor Oct4 and fail to generate chimaeras upon blastocyst injection (Buehr et al., 2003).

Loss of Oct4 expression in mouse ES cells results in loss of pluripotency and differentiation into trophectoderm (Nichols et al., 1998; Niwa et al., 2000). Although the expression pattern of Oct4 in the rat embryo is equivalent to that

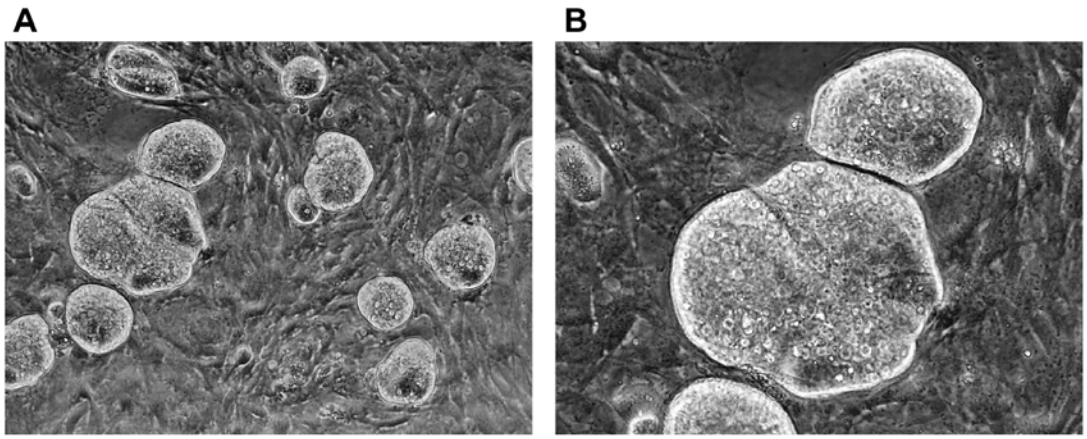


Figure 3.1 Rat ExS cells
Brightfield microscopic images of rat ExS cell colonies grown on DIA-M feeders at **(A)** x100 and **(B)** x200 magnification.

of mouse (Buehr et al., 2003), Oct4 is downregulated in rat ICM outgrowths within the first few days during derivation.

Nanog has been demonstrated to maintain mouse ES cell self-renewal in parallel with STAT3 activation via LIF-stimulation of gp130 (Chambers et al., 2003). In the absence of Nanog, mouse ES cells become committed to differentiate and pluripotency is compromised (Chambers et al., 2003; Mitsui et al., 2003). *In vivo*, Nanog expression is first observed within the central compartment of the compacted mouse morula then later restricted to the ICM and epiblast of the blastocyst. At implantation Nanog is downregulated, only to reappear as punctuate staining in E11.5 genital ridges, indicative of primordial germ cells. Although $Nanog^{-/-}$ embryos form an overtly normal blastocyst, by E5.5 the epiblast is indistinct and the embryo is composed mainly of disorganized extra-embryonic tissue. Furthermore, when the ICM of $Nanog^{-/-}$ blastocysts are cultured *ex vivo*, they fail to remain undifferentiated, but rather differentiate into parietal endoderm-like cells.

Although the expression pattern of Oct4 in the rat embryo is known, the expression pattern of Nanog in ExS cells and the early rat embryo remains to be determined. Subtle differences in the timing of Nanog expression during early development may indicate a more appropriate embryonic stage suitable for derivation. Therefore, the aim of the work described in this Chapter was to determine whether ExS cells express Nanog and to investigate the temporal and spatial expression pattern of Nanog in the rat embryo.

3.2 Nanog expression

Within the first few days during rat ExS cell derivation, Oct4 expression is quickly down-regulated such that it cannot be detected by RT-PCR (Buehr et al., 2003). Likewise, rat ExS cells fail to express Nanog (Figure 3.2A).

In order to determine if Nanog is actually expressed in the ICM prior to derivation, the rat Nanog transcript was isolated from E4.5 rat embryos by PCR using oligonucleotides designed to the predicted rat Nanog mRNA sequence XM_575662 (Figure 3.3). This transcript would also be utilised in an expression vector for investigation into genetic manipulation of rat ExS cells (see 3.3 and chapter 4). The restriction enzyme sites *Xho*I and *Not*I were incorporated into the 5' ends of the sequences of the oligonucleotides to facilitate subsequent cloning. The Kozak concensus sequence was also incorporated into the 5' end of the sequence of one oligonucleotide. The Kozak sequence is recognized as the translational start site by the ribosome and is required to initiate efficient translation (Kozak, 1987). The amplified cDNA was cloned into the TOPO pCRII vector (Figure 3.4A) and the sequence integrity confirmed. This suggested that Nanog is

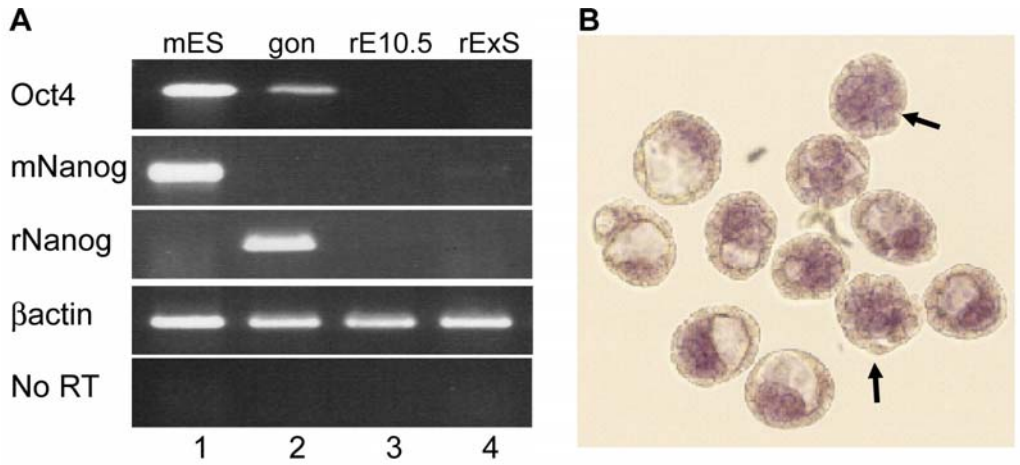


Figure 3.2 Expression pattern of Nanog in ExS cells and the rat embryo.
(A) RT-PCR analysis of (1) E14-Tg2a mESC, (2) E13.5 rat gonads, (3) E10.5 rat embryos and (4) rat ExS cells, using species-specific mouse (mNanog) and rat (rNanog) Nanog oligonucleotides.
(B) *In situ* hybridisation of rat blastocysts using a rat Nanog riboprobe. Arrows highlight morulae in which Nanog expression is confined to the central region that will become the ICM.

indeed expressed in rat blastocysts. To confirm this and to determine the spatial expression pattern of Nanog in rat blastocysts, *in situ* hybridisation was performed on E3.5/4.5 rat embryos using a rat Nanog riboprobe produced from the TOPO pCRII clone, as detailed in Chapter 2. It can be seen that the Nanog expression pattern in rat is similar to that in mouse at the equivalent stage of development (Figure 3.2B). Expression is restricted to the ICM and absent from the trophectoderm. In two cases, where the blastocyst cavity has yet to form, expression is confined to the central region of the morula, the region which gives rise to the ICM, and absent from the outer cells.

Figure 3.3 Rat Nanog mRNA PCR clone sequence.
Rat Nanog transcript isolated from E4.5 rat embryos by PCR using oligonucleotides (highlighted in boxes) designed to the predicted rat Nanog mRNA sequence XM_575662. Restriction sites and Kozak sequence were

3.3 Cloning of rat Nanog into expression vector.

The rat Nanog cDNA was successfully cloned, as discussed in Section 3.2, and an expression vector was constructed using this. The *Xho*I/*Not*I rat Nanog cDNA fragment was excised from the TOPO pCRII clone (Figure 3.4A) and used to replace the *Xho*I/*Not*I stuffer fragment in the pPyCAG-IP vector (Figure 3.4B) creating a pPyCAG-rat Nanog-IP expression vector (Figure 3.4C).

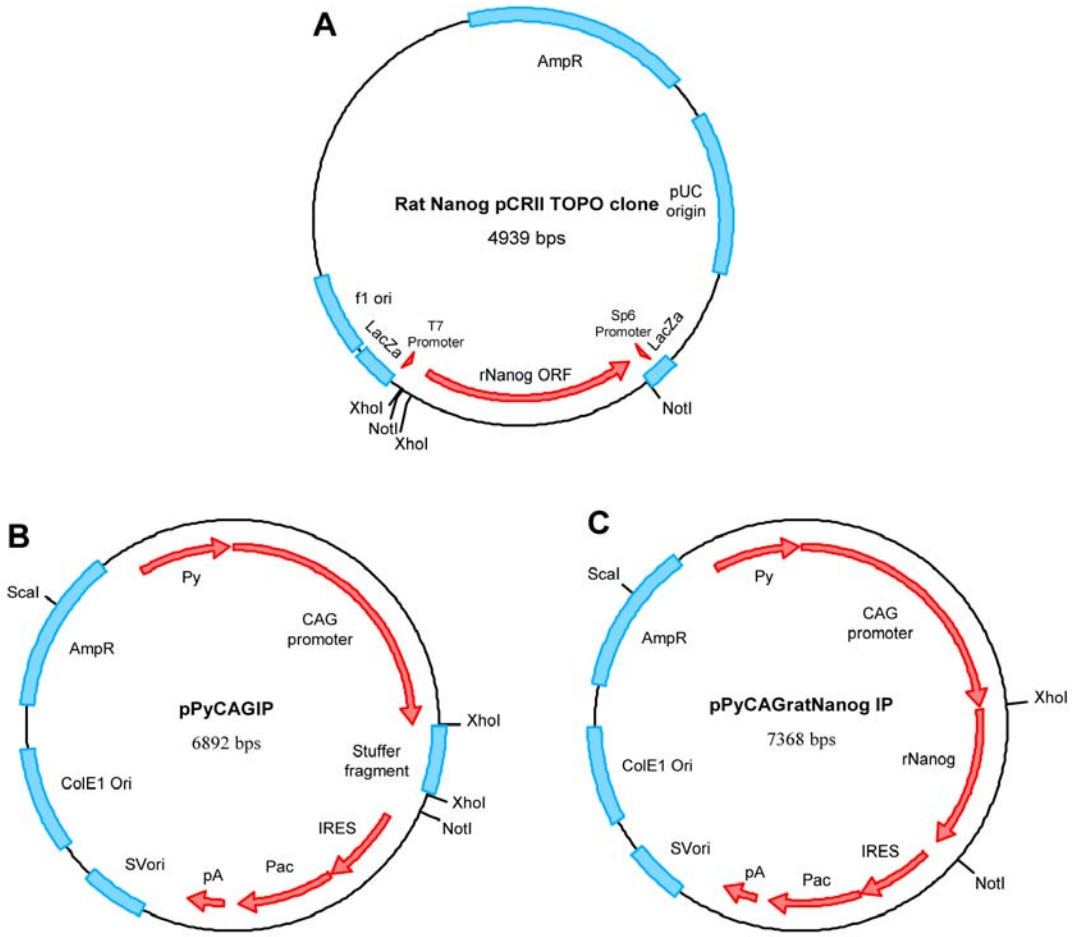


Figure 3.4 Construction of a Rat Nanog expression plasmid.
The rat Nanog transcript was cloned into the pCRII TOPO vector **(A)**. The XhoI/NotI rat Nanog cDNA fragment was removed and used to replace the stuffer fragment in the pPyCAGIP plasmid **(B)** to generate the rat Nanog expression plasmid pPyCAGratNanogIP **(C)**.

3.4 Functional assay of rat Nanog expression vector.

The ability of Nanog to mediate LIF-independent self-renewal was utilized in a functional assay to test the rat Nanog expression vector. However, due to the production of LIF by differentiated cells, a background population of undifferentiated ES cells persist, even in the absence of LIF (Rathjen et al., 1990). LIF acts via the LIFR/gp130 complex. In order to remove the undifferentiated background in the absence of LIF, mES cells with deletions of both alleles of the LIF receptor gene (*Lifr*$^{-/-}$) were utilised. These cells, called LRK1 cells, can be maintained in the absence of LIF by activation of gp130 using interleukin-6 and soluble interleukin-6 receptor (IL6/sIL6R). LRK1 cells also encode the Polyoma large T protein and were transfected, as described in Chapter 2, with vectors containing a Polyoma origin of replication and expressing either rat Nanog (Figure 3.4C) or mouse Nanog. The empty vector (MT) pCAG-IP (Figure 3.4B) was used as a negative control. All vectors contain a puromycin resistance gene. Cells were cultured for 14 days in medium containing puromycin and the resultant colonies (Figure 3.5A) were stained for alkaline phosphatase and scored as undifferentiated or differentiated (Figure 3.5B).

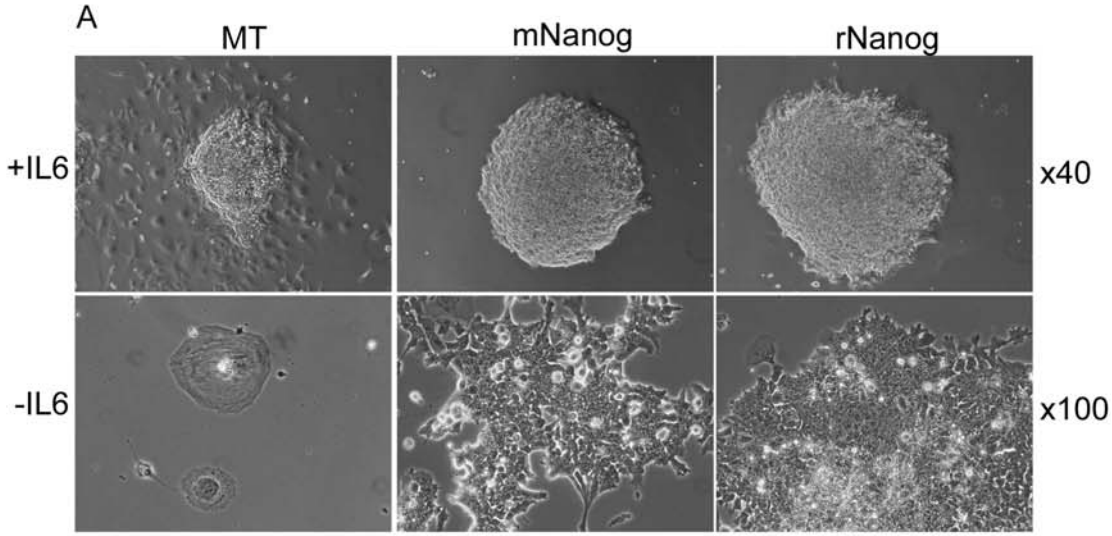
As expected, only those cells transfected with Nanog-expressing vectors could maintain ES cell self-renewal independent of gp130 stimulation. The MT vector control failed to produce undifferentiated colonies in the absence of gp130 stimulation (Figure 3.5C) and generated only flattened differentiated cells.

Interestingly, in the presence of cytokine, there were fewer colonies obtained with either Nanog expression vectors compared to the MT vector control. The presence of a transgene within the expression vector may interfere with the

transcription of the puromycin gene and thus fewer cells produce a sufficient amount of puromycin acetyltransferase to survive selection compared with the MT vector.

Nonetheless, both mouse and rat Nanog expression vectors maintained undifferentiated colonies in the absence of IL6/sIL6R. In both cases the number of undifferentiated colonies was fewer in the absence of cytokine than in its presence, probably because fewer cells expressed the required level of Nanog capable of maintaining self-renewal independent of gp130 stimulation. The colonies formed in the presence of cytokine were more compact and rounded compared to the flatter morphology observed in the absence of cytokine (Figure 3.5A) as previously observed (Chambers et al., 2003). Although the rat Nanog transcript generated fewer undifferentiated colonies than the mouse transcript, the relative percentage of undifferentiated colonies generated was comparable (Figure 3.5C). Furthermore, although less effective than mouse Nanog, rat Nanog was also capable of directing cytokine-independent self-renewal (Figure 3.5D).

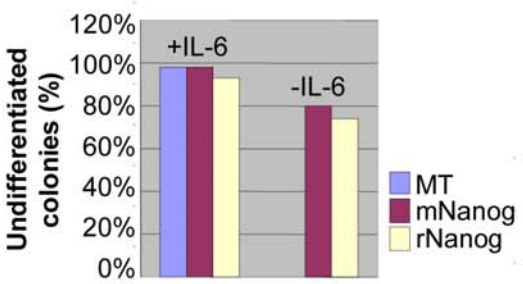
A



B

	MT		mNanog		rNanog	
	u	d	u	d	u	d
+IL-6	1270	32	585	12	138	10
-IL-6	0	0	380	94	20	7

C



D

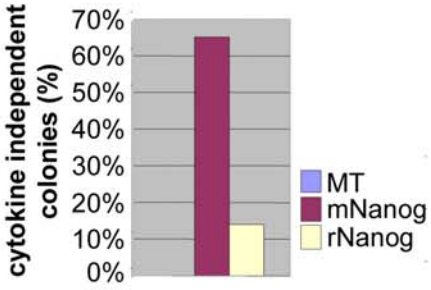


Figure 3.5 LIF-independent self-renewal assay.
LRK-1 cells transfected with empty vector (MT), pPyCAG-mouse Nanog-IP (mNanog) and pPyCAG-rat Nanog-IP (rNanog) expression vectors. $2x10^5$ cells were plated and selected for 14 days in 1µg/ml puromycin.
(A) Colony morphology of transformants cultured in the presence or absence of IL6/sIL6R (+/- IL-6).
(B) Number of undifferentiated (u) and differentiated (d) colonies, as determined by alkaline phosphatase staining.
(C) Percentage of undifferentiated colonies formed.
(D) Percentage of undifferentiated cytokine-independent colonies formed.

3.5 Discussion

Rat ExS cells and other rat ES-like lines fail to maintain significant levels of the key ES cell transcription factor Oct4. Oct4 is critical for specification of the pluripotent compartment *in vivo* as well as maintenance of ES cell pluripotency *in vitro* (Nichols et al., 1998; Niwa et al., 2000). The pattern of Oct4 expression *in vivo* is similar between rat and mouse. However, upon isolation and *in vitro* culture of the rat ICM, expression of Oct4 is quickly downregulated (Buehr et al., 2003).

However, little is known about the expression pattern of Nanog in rat ExS cells or the rat embryo compared with mouse. This is of interest because a common spatial expression pattern would suggest that Nanog plays a similar role in both mouse and rat, whilst a difference in the temporal expression pattern may highlight a more appropriate embryonic stage suitable for successful derivation. It was demonstrated that ExS cells do not express Nanog (Figure 3.1A). Also, the temporal and spatial expression pattern of Nanog in the rat embryo was shown to be restricted to the pluripotent compartments of the early embryo, namely, the inner cells of the morula and the ICM of the blastocyst, but excluded from the trophectoderm (Figure 3.2B). Expression was absent in the E10.5dpc embryo, but re-expressed in E13.5 gonads (Figure 3.2A). This indicates that rat Nanog is expressed in a similar pattern to mouse (Chambers et al., 2003). Furthermore, overexpression of rat Nanog in mouse ES cells is capable of mediating gp130-independent self-renewal (Figure 3.5), demonstrating a common function with its mouse orthologue and suggesting that it is likely to play a similar role.

Interestingly, although overexpressing rat Nanog in mouse LRK1 cells facilitated gp130-independent self-renewal, the efficiency was less compared with

the mouse equivalent (Figure 3.5D). This may simply be due to a difference in transfection efficiency or the level of transgene expression. However both the mouse and rat transgenes are contained within the same vector and driven by the same promoter, it is therefore unlikely that expression of either would differ significantly. The difference is more likely due to a variation in transfection efficiency. Only one transfection experiment was performed. Therefore performing replicates would provide more reliable data. Another consideration is that the ability of rat Nanog to function in mouse cells may be impaired. For example, the ability of human NANOG to mediate gp130-independent self-renewal in this system is reported to be considerably lower than for mouse (Chambers et al., 2003). Although the rat is more closely related to the mouse than the human, key differences in the functional domains may significantly affect cross-species functionality.

The Nanog protein can be roughly divided into 3 domains; the N-terminal domain, the homeodomain (HD) and the C-terminal domain. The C-terminal domain is further subdivided into the tryptophan repeat (WR) region which is flanked by 5' and 3' C-terminal sequence, CD1 and CD2 respectively (Pan and Pei, 2005) (Figure 3.6A). The overall homology between rat and mouse is 83%, compared with 58% with human. The highest homology exists within the DNA-binding HD (93%). This region is highly conserved and also contains a nuclear localization signal (NLS). A region of only six amino acids (aa) has been identified in the human NANOG HD and demonstrated to be responsible for exclusive nuclear localization (Chang et al., 2009). These six aa are conserved between mouse, human and rat (Figure 3.6B).

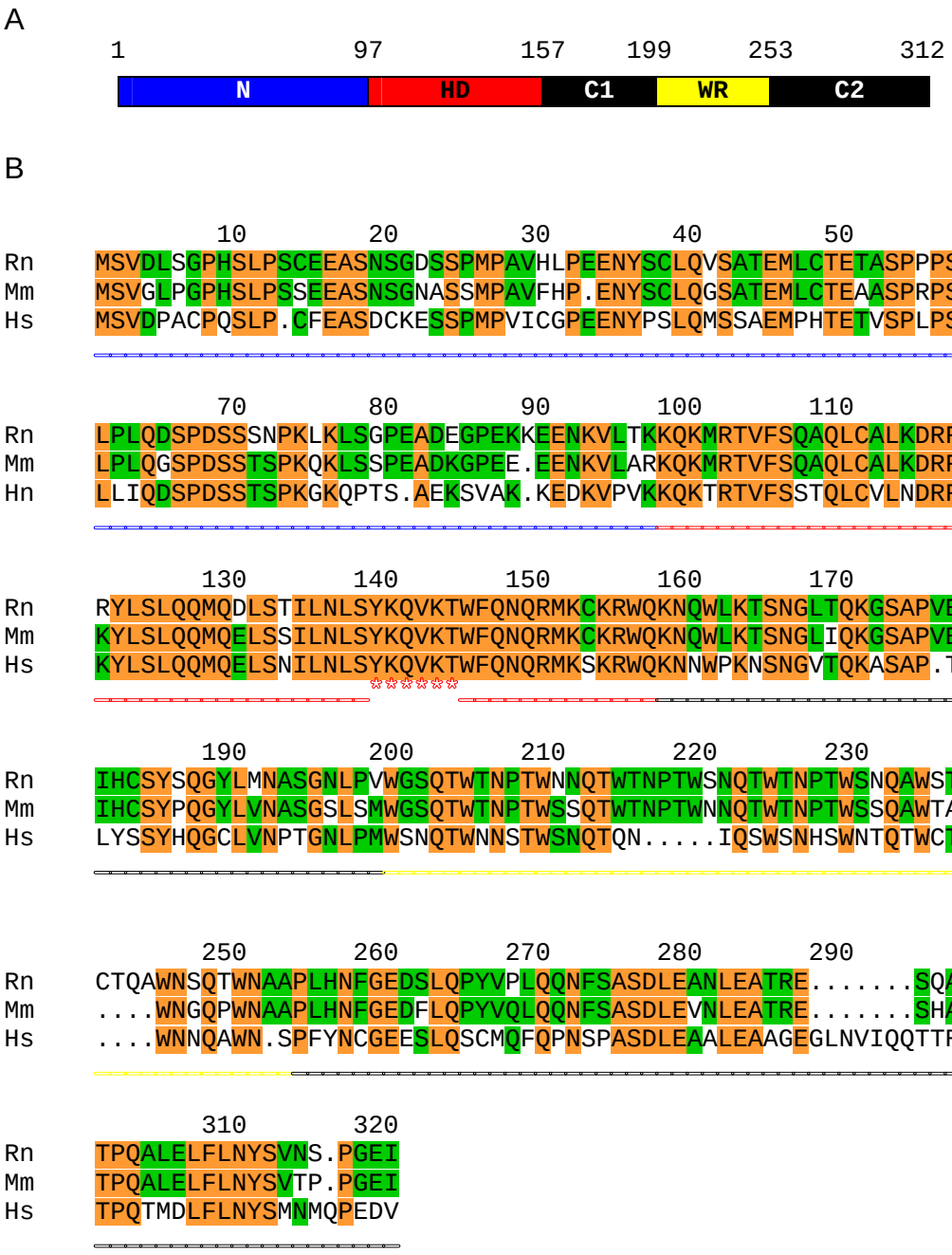


Figure 3.6 Nanog protein domains

(A) Summary of rat Nanog protein domains: Blue box (N), the N-terminal domain; red box (HD), homeodomain; black boxes (C1 & C2), C-terminal domain and yellow box (WR), tryptophan repeat region.

(B) Alignment of rat (Rn) protein sequence with mouse (Mm) and human (Hs) orthologues. The alignment was generated using Clustal W: orange, identical in all sequences; green, identical in two sequences. The domains are underlined: blue, N-terminal domain; red, homeodomain; black, C-terminal domain and yellow, tryptophan repeat region. The red astrisks represent the putative, conserved nuclear localisation signal. The yellow asterisk indicates the additional tryptophan insertion in the rat sequence.

This suggests that the HD is likely to be functional between species. Another key region, which is responsible for Nanog homodimerisation and its ability to direct LIF-independent self-renewal, is the WR region (Mullin et al., 2008; Wang et al., 2008). The lower degree of homology (76%) in this region is mainly due to the presence of an extra pentapeptide tryptophan repeat found in rat (Figure 3.6B). It is possible that both rat homodimers and rat/mouse heterodimers may form and in both cases the binding sites for Nanog targets may differ slightly from pure mouse homodimers, thus affecting binding and downstream activity.

3.6 Summary

The expression pattern of Nanog in rat ExS cells and the early rat embryo was investigated. Although rat ExS cells fail to express Nanog, the expression pattern in the embryo is comparable with that of mouse. Expression is restricted to the inner compartment of the compacted morula and ICM of the blastocyst and is downregulated by E10.5, but upregulated in E13.5 gonads. Furthermore, the rat Nanog transcript was isolated and cloned into an expression vector and demonstrated to facilitate LIF-independent self-renewal, a defining feature of Nanog overexpression (Chambers et al., 2003). Therefore, although both Oct4 and Nanog are expressed in the ICM of the rat blastocyst, unlike their mouse orthologues, they are both down-regulated *in vitro* during derivation. This suggests that differences exist either in the intrinsic properties of the rat and mouse ICM cells or in their response to unrefined extrinsic culture conditions.

Chapter 4

Reprogramming of rat ExS cells

4.1 Introduction

The ICM of both rat and mouse blastocysts express Oct4 and Nanog. However, during *ex vivo* culture, rat ICM cells fail to maintain expression of these key ES cell transcription factors.

In the absence of Oct4 or Nanog mouse ES cells become committed to differentiate and pluripotency is compromised (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000). Conversely, overexpression of Nanog is capable of maintaining Oct4 expression and LIF-independent self-renewal of mouse ES cells (Chambers et al., 2003). However, Nanog's ability to maintain LIF-independent self-renewal requires the continued presence of Oct4 suggesting that Nanog and Oct4 appear to act in concert (Chambers et al., 2003). Therefore, it was hypothesized that exogenous expression of Oct4 and/or Nanog from integrated expression vectors may facilitate reprogramming of ExS cells into an ES cell, pluripotent state.

The ability to reprogramme the nuclei of differentiated cells into a pluripotent state has been demonstrated previously by others using somatic cell nuclear transfer (SCNT) and cell fusion technologies. When enucleated Xenopus eggs were transplanted with the nucleus from keratinized skin cells of Xenopus they could support complex differentiation and development to the tadpole stage (Gurdon et al., 1975). Later it was demonstrated that the nuclei of adult sheep mammary gland cells

could be reprogrammed in a similar manner following nuclear transfer into enucleated sheep oocytes (Wilmut et al., 1997). This technology has also been applied successfully to rat fetal fibroblasts (Zhou et al., 2003). Establishing gene targeting technologies in rat fibroblasts or ExS cells would, when combined with SCNT technology, facilitate the generation of genetically modified rats. Animals that are born from SCNT exhibit 100% contribution from donor cell DNA. However, although this is an advantage over traditional ES cell based chimaera production, SCNT is labour-intensive and inefficient. Of 277 reconstructed sheep embryos only 29 developed to the blastocyst stage, from which one live birth was generated (Wilmut et al., 1997).

Successful reprogramming of central nervous system cells and bone marrow cells has also been demonstrated following spontaneous fusion with mouse ES cells (Terada et al., 2002; Ying et al., 2002; Zhou et al., 2003). Although, these hybrid cells resemble ES cells morphologically and demonstrate pluripotency, they are tetraploid, consisting of DNA from both the ES cell and the differentiated cell, and thus cannot contribute to the germline.

Collectively, these results demonstrate that the genome of differentiated cells has not undergone irreversible modifications, but rather, is amenable to reprogramming when exposed to the appropriate factors, such as those found within the cytoplasm of the oocyte or expressed by ES cells.

Therefore, the aim of the work described in this Chapter was to establish DNA transfection protocols for generating stable rat ExS cell transfectants. These methods were subsequently utilized for integrating Oct4 and Nanog expression vectors into ExS cells. Transgene expression was verified and the capacity of

exogenous Oct4 and Nanog expression to induce expression of endogenous ES cell markers and reinstate an ES cell identity was investigated.

4.2 Oct4 and Nanog expression vectors

The rat Oct4 cDNA, like the rat Nanog cDNA, was amplified from rat E4.5 embryos by PCR, using oligonucleotides designed to the predicted rat Oct4 mRNA sequence XM_228354. Likewise, the restriction enzyme sites *Xho*I and *Not*I were incorporated into the 5' ends of the sequences to facilitate subsequent molecular cloning, and the Kozak concensus sequence was also inserted into the 5' end of the sequence of one oligonucleotide. The amplified cDNA was cloned into the TOPO pCRII vector (Figure 4.1).

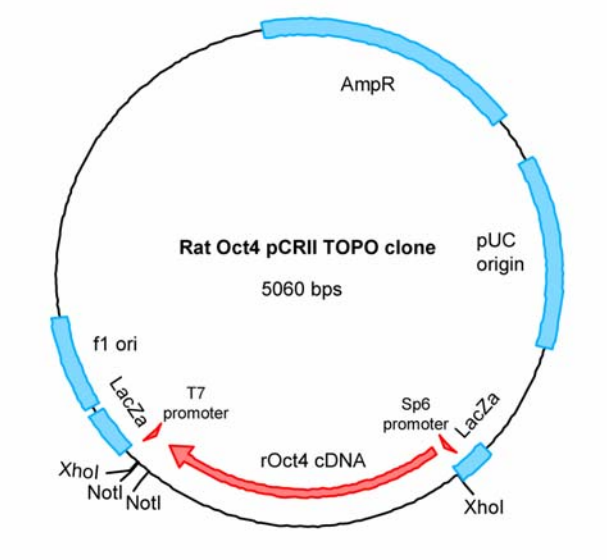


Figure 4.1 TOPO cloned rat Oct cDNA.

However, robust sequence integrity was never observed, with the best clone obtained containing an 'A' to 'T' mutation at position 130 and a 'T' deletion at position 1011 (Figure 4.2).

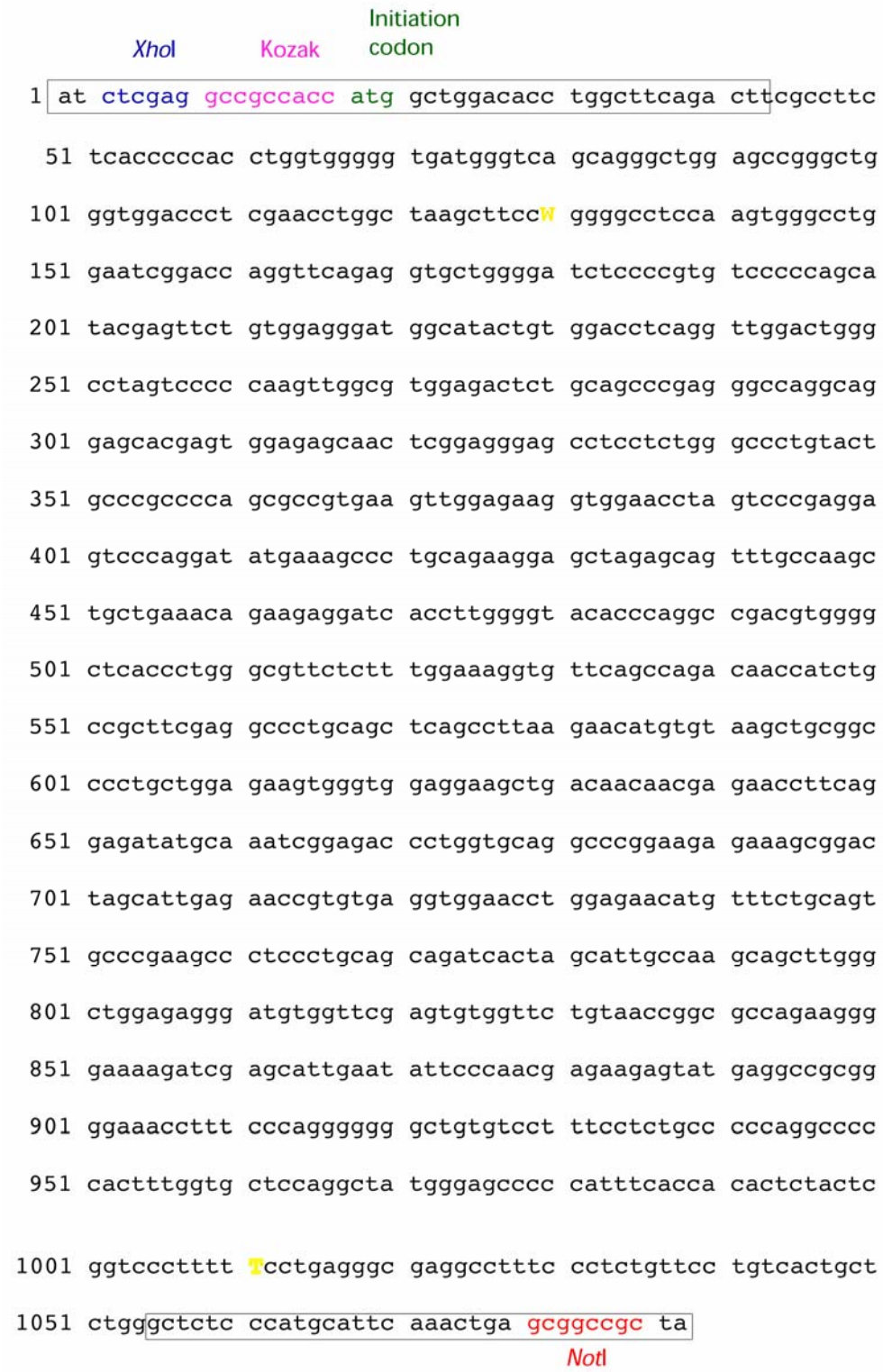


Figure 4.2 Mutated rat Oct4 mRNA PCR clone sequence.
Rat Oct4 transcript isolated from E4.5 rat embryos by PCR using oligonucleotides (highlighted in boxes) designed to the predicted rat Oct4 mRNA sequence XM_228354. Restriction sites and Kozak sequence were incorporated into the oligonucleotides and are indicated. There is an 'A' to 'T' mutation (W) at position 130, and a T' deletion at position 1011 (T).

As an alternative, mouse expression vectors were considered. The homology between rat and mouse Oct4 is 94% and for Nanog it is 87% (94% in the homeodomain). Therefore, it was likely that the mouse homologues would be functional in rat cells. As such, the mouse Oct4 (Niwa et al., 2002) and mouse Nanog (Chambers et al., 2003) expression vectors were utilised (Figure 4.3 B & D). These are episomal vectors that carry the Polyoma origin of replication (Py), allowing persistent extra-chromosomal expression for many generations in ES cells expressing the Polyoma large T protein (Gassmann et al., 1995). Co-expression of the puromycin-resistance gene (Pac) is facilitated by the presence of an IRES sequence (Mountford et al., 1994).

4.3 Transfection of rat ExS cells

Before integrating Nanog and Oct4 expression vectors into ExS cells, it first had to be determined whether ExS cells were amenable to transfection and generation of stable-expressing transgenic lines, and a suitable transfection protocol established.

4.3.1 Trypsinising rat ExS cells

Rat ExS cells grow on feeder cells as tightly packed colonies. The colonies are surrounded by a sheath-like coating and individual cells within the colony are not well defined. Colonies are typically expanded by trituration using a drawn-out glass Pasteur. This is a laborious method yielding only several small clumps of cells. This is not ideal for transfection, which typically requires a large number of cells (approximately 10^5) in a suspension or grown as a monolayer. Therefore, ExS cell

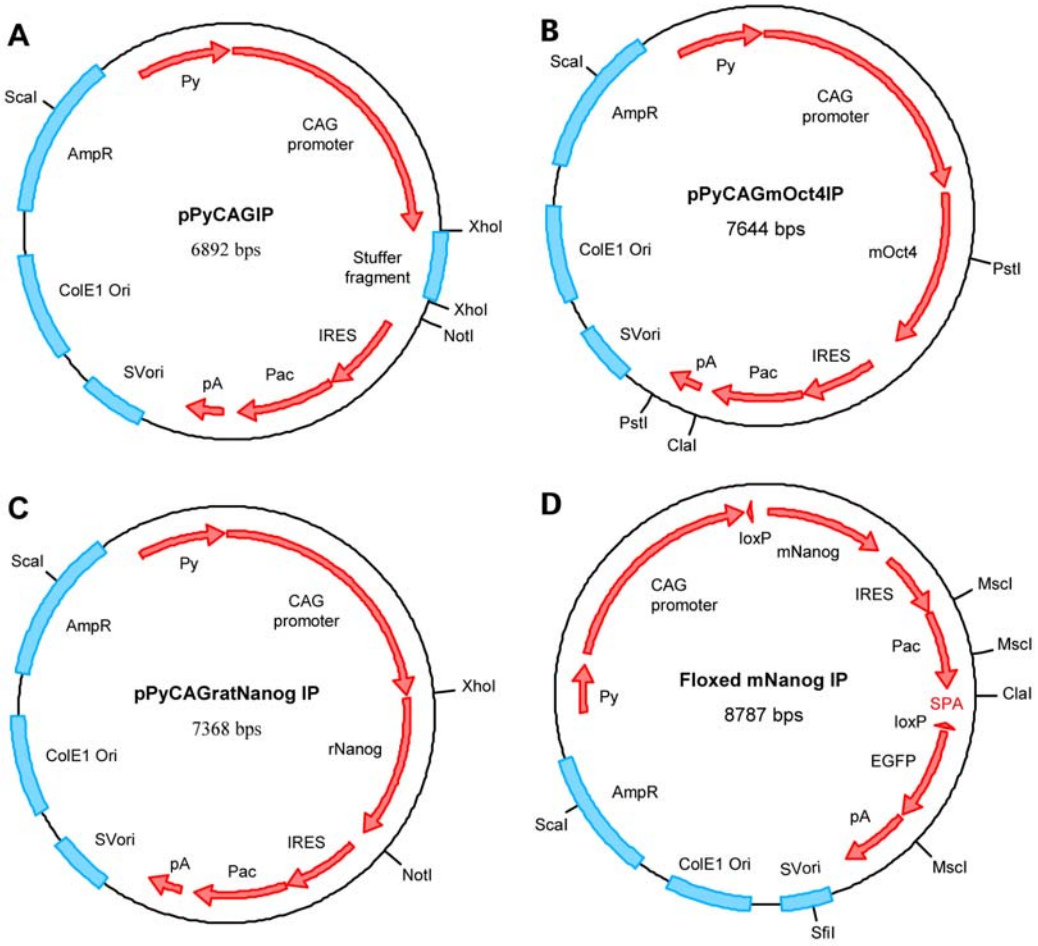


Figure 4.3 Oct4 and Nanog expression vectors.
Expression vectors carrying the promoter-enhancer-ori derived from PyFIOI (Py). cDNA expression is driven by a CAG promoter and co-expression of the puromycin-resistance gene (Pac) is facilitated by an IRES sequence.
(A) Control pPyCAG-IP empty vector.
(B) pPYCAG-mOct4-IP, mouse Oct4 expression vector.
(C) pPyCAG-rNanog-IP, rat Nanog expression vector.
(D) FloxedmNanog-IP, mouse Nanog expression vector in which the mouse Nanog cDNA, puromycin selection marker and synthetic polyA (SPA) are flanked by LoxP sites.

colonies were treated with trypsin. After attempting to disperse the colonies by pipetting, it was clear that the colonies were somewhat resistant to trypsin treatment. Colonies were then treated with a 4x concentrated stock of trypsin. This allowed the

colonies to be dispersed to a near-single cell suspension. When plated at various dilutions, large numbers of colonies began to appear after 3-4 days with no overt death or differentiation, although plating the cells too densely appeared to have an adverse effect on colony growth (Figure 4.4). This may be due to depletion of essential supplements from the medium or an increase in negative autocrine factors.

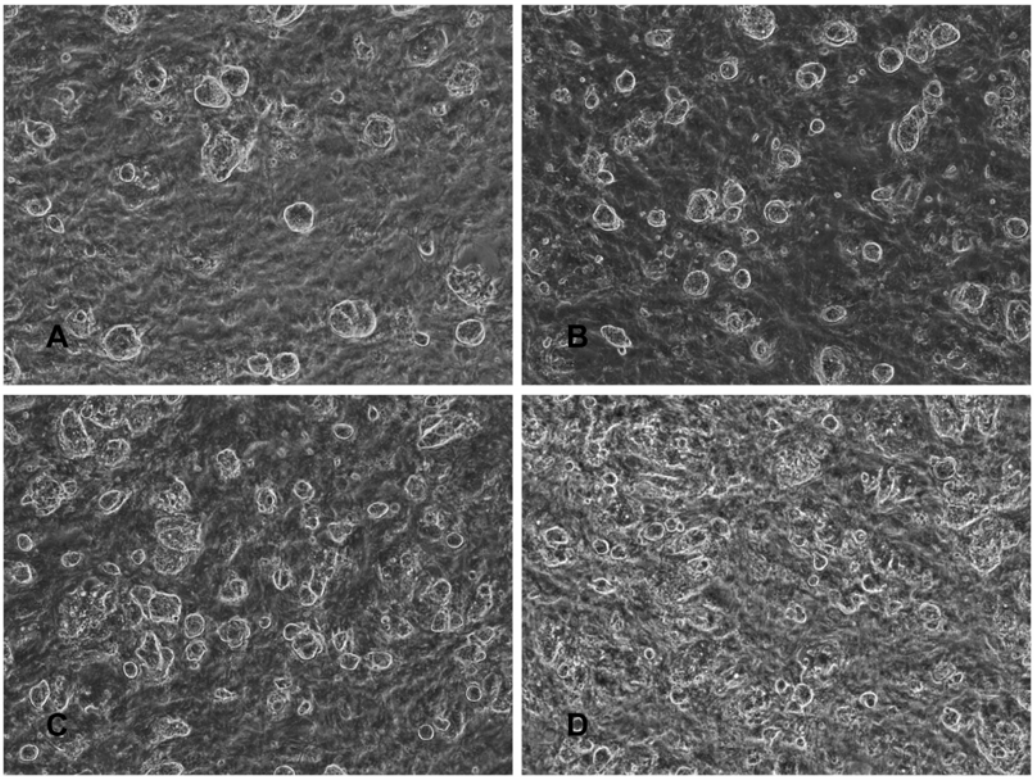


Figure 4.4 Trypsinised rat ExS cells
Trypsinised rat ExS cells trypsinised plated at various dilutions then cultured for 5 days. (**A)** 1 in 8, (**B)** 1 in 6, (**C)** 1 in 4 and (**D)** 1 in 2. (Magnification x40)

4.3.2 Lipofection

A vector expressing the green fluorescent protein from the jellyfish *Aequoria victoria* (Prasher et al., 1992), which had been altered to produce an enhanced fluorescence (Cormack et al., 1996), was employed as a reporter to determine the feasibility of transfecting rat ExS cells (Figure 4.5A). The *Sca*I-linearised pPyCAGegfpIP expression vector was transfected into ExS cells by lipofection as detailed in Chapter 2. Stable GFP-expressing cells were established and maintained in culture for over 20 passages (Figure 4.5B). Since several GFP positive colonies were pooled at each passage, and because trypsin treatment of ExS cells does not produce a purely single cell population, a Southern blot analysis was performed on restriction enzyme digested genomic DNA using a 783bp *Xho*I EGFP DNA restriction fragment probe to determine if the line was a homogeneous population or had multiple transgene copies integrated into the genome (Figure 4.6A & B). Two restriction fragments are detected in the *Bam*HI and *Xba*I samples. This data is consistent with there being either a single population containing more than one integrant or a double population each with a single integration. The larger than expected, ~5kb *Xba*I fragment suggests that at least one of the *Xba*I sites and some surrounding sequence are lost, indicating that one of the integrants may not be intact.

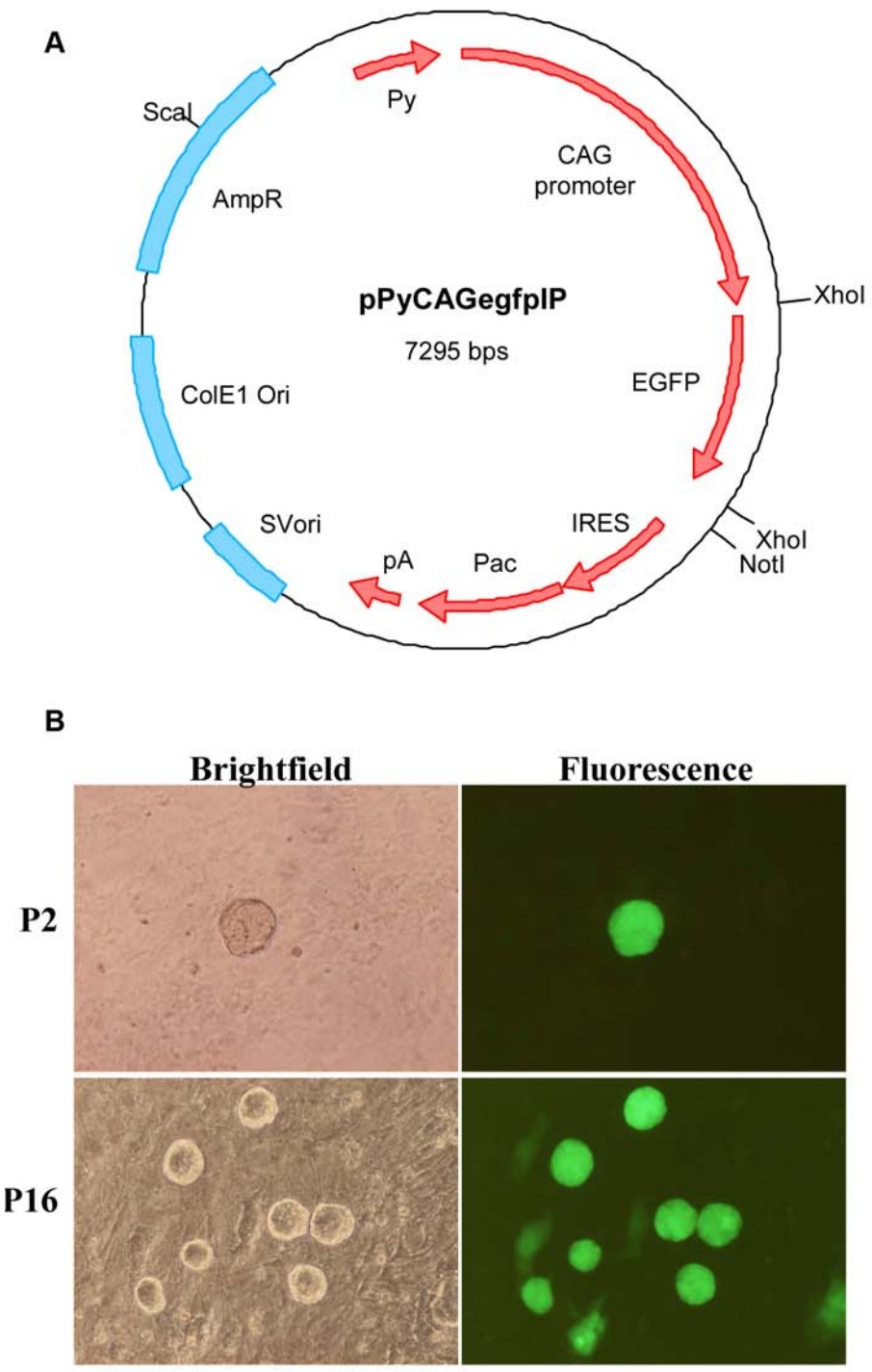


Figure 4.5 EGFP expressing rat ExS cells.
(A) EGFP expression vector driven by the CAG promoter. Co-expression of the puromycin (Pac) gene is facilitated by the inclusion of the IRES sequence. **(B)** Rat ExS cells expressing EGFP were maintained in culture and passaged for several generations.

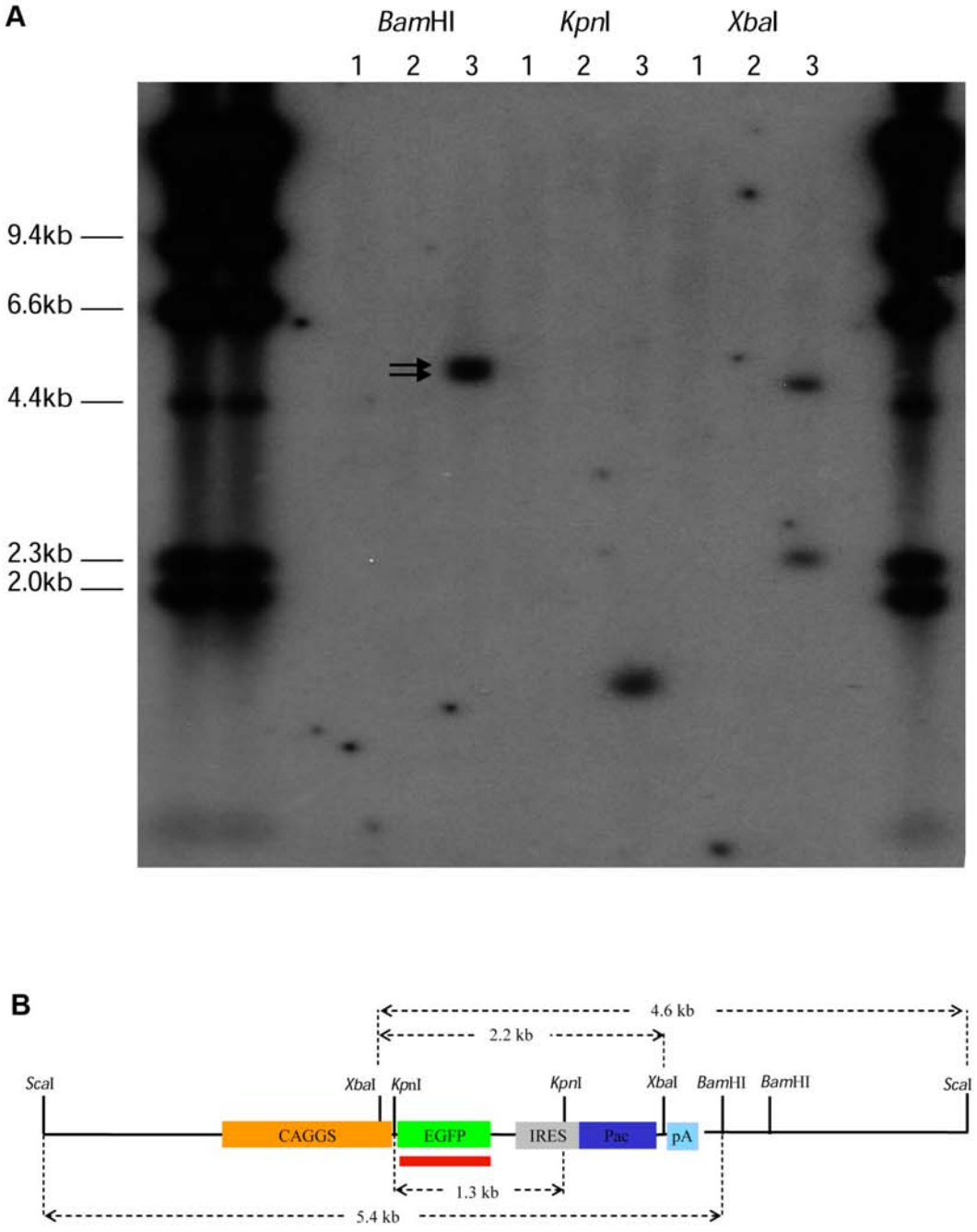


Figure 4.6 Copy number determination.
(A) Southern blot of *Bam*HI, *Kpn*I and *Xba*I digested genomic DNA from (1) E14-Tg2a mESC, (2) DIA-M and (3) EGFP-expressing rat ExS cells, probed with a 783bp XhoI EGFP cDNA fragment. The double arrows highlight two distinct *Bam*HI restriction fragments.
(B) Map of the *Sca*I linearised pPyCAGegfpIP expression plasmid. The minimal expected restriction fragment sizes are indicated by dashed arrows and the EGFP probe fragment by a red line.

4.3.3 Transfection of ExS cells with Oct4 or Nanog

A rat ExS cell line was transfected with *Sca*I linearised pPyCAG-mOct4-IP (Figure 4.3B), Floxed-mNanog-IP (Figure 4.3D) and pPyCAG-rNanog-IP (Figure 4.3C) expression vectors as detailed in Chapter 2. The pPyCAG-IP empty vector (MT) was used as a control (Figures 4.3A). Cell lines stably expressing the integrated transgene were selected with puromycin. These lines grew in a similar manner to the parental line. The morphology was also similar, with the exception of the mNanog line which had a more lobular appearance compared to the typical round, ball-like appearance (Figure 4.7).

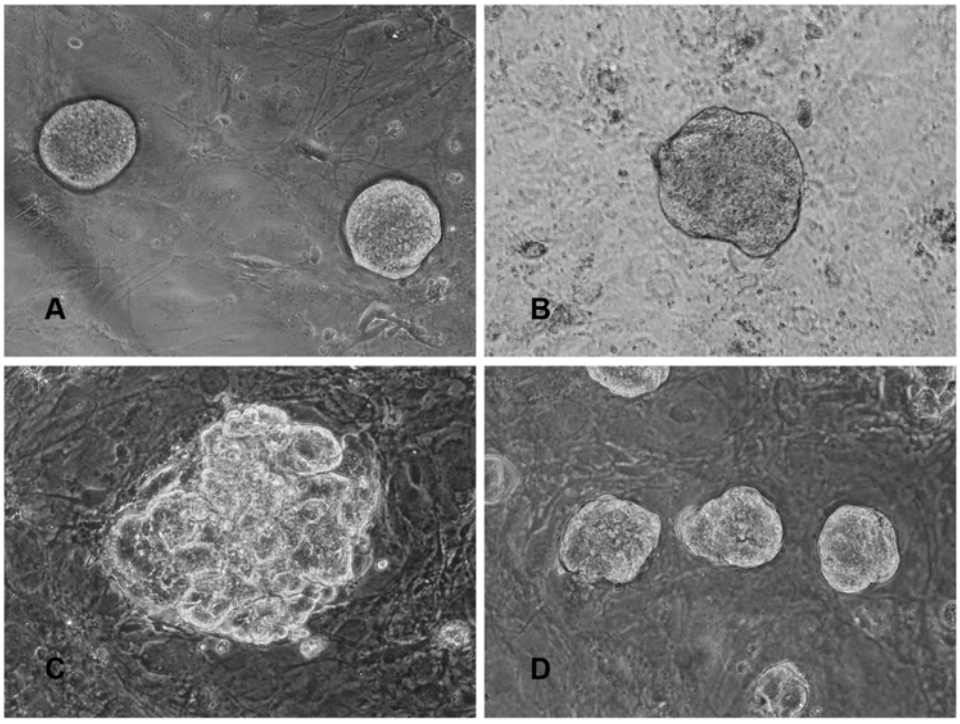


Figure 4.7 Morphology of Oct4 and Nanog transgenic rat ExS cells. Stable-expressing, puromycin-resistant rat ExS cell lines transfected with; **(A)** pPyCAG-IP [P2], **(B)** pPyCAG-mOct4-IP [12], **(C)** Floxed mNanog-IP [P5] and **(D)** pPyCAG-rNanog-IP [P8]. (Magnification x100).

4.3.4 Transgene expression

Transgene expression was confirmed in these lines by RT-PCR analysis (Figure 4.8A), and by *in situ* hybridization (Figure 4.8B) using riboprobes generated

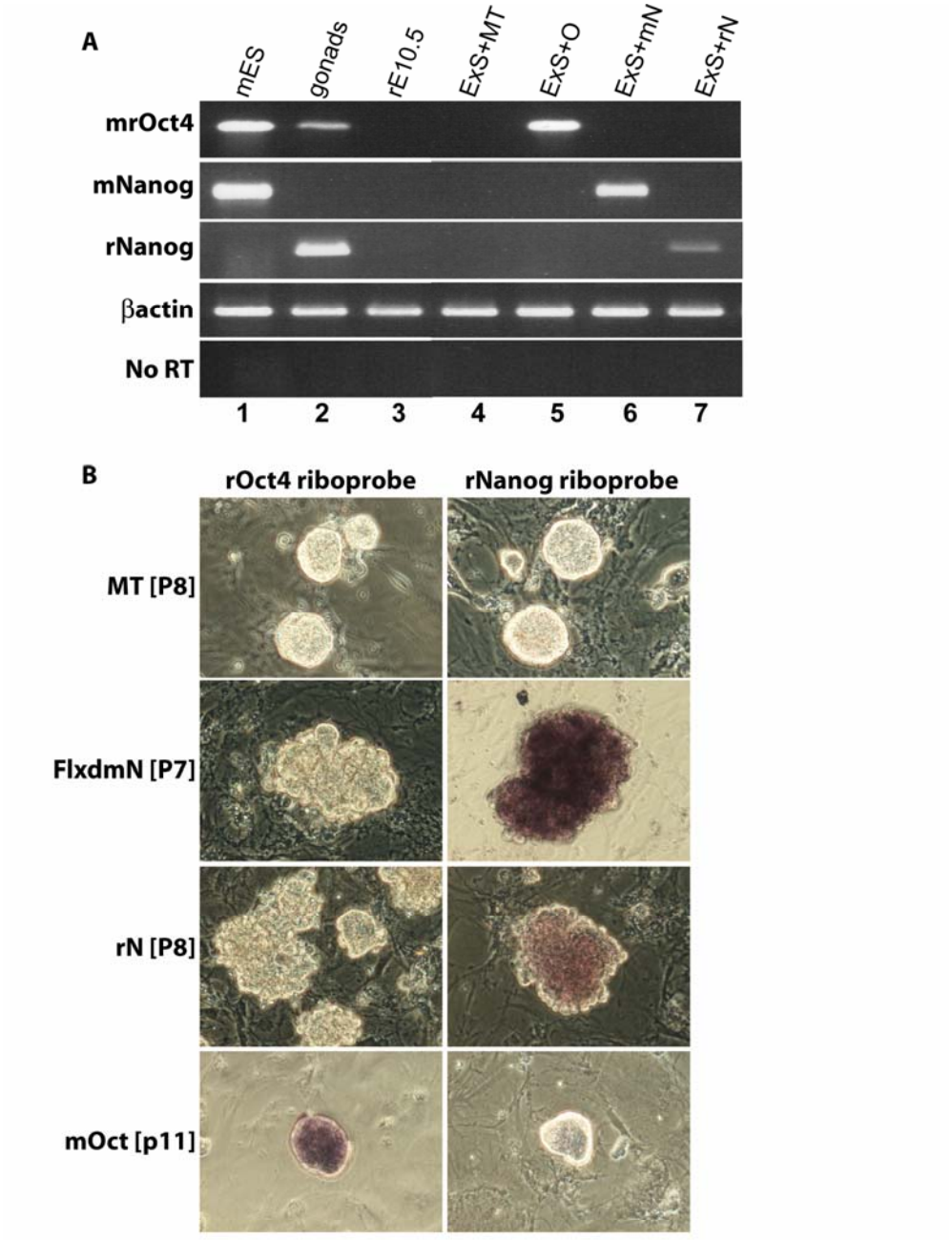


Figure 4.8 Transgene expression in rat ExS cells.
(A) RT-PCR analysis of (1) mESC, (2) E13.5 rat gonads, (3) E10.5 rat embryos, (4) ExS + MT [P6], (5) ExS + mOct4 [P9], (6) ExS + Flxd mNanog [P9] and (7) ExS + rNanog [P7], using species-specific mouse (mNanog) and rat (rNanog) Nanog oligonucleotides. mrOct4 detects both mouse and rat transcripts.
(B) In situ hybridisation of ExS lines stably transfected with empty plasmid (MT), mNanog (FlxdmN), rat Nanog (rN) or mouse Oct4 (mOct4) expression vectors, using rat Oct4 (rOct4) and rat Nanog (rNanog) riboprobes.

from rat Nanog and rat Oct4 pCRII TOPO clones (Figure 3.4A & 4.1). Protein levels were also determined by immunohistochemistry and Western blotting (Figure 4.9A-C).

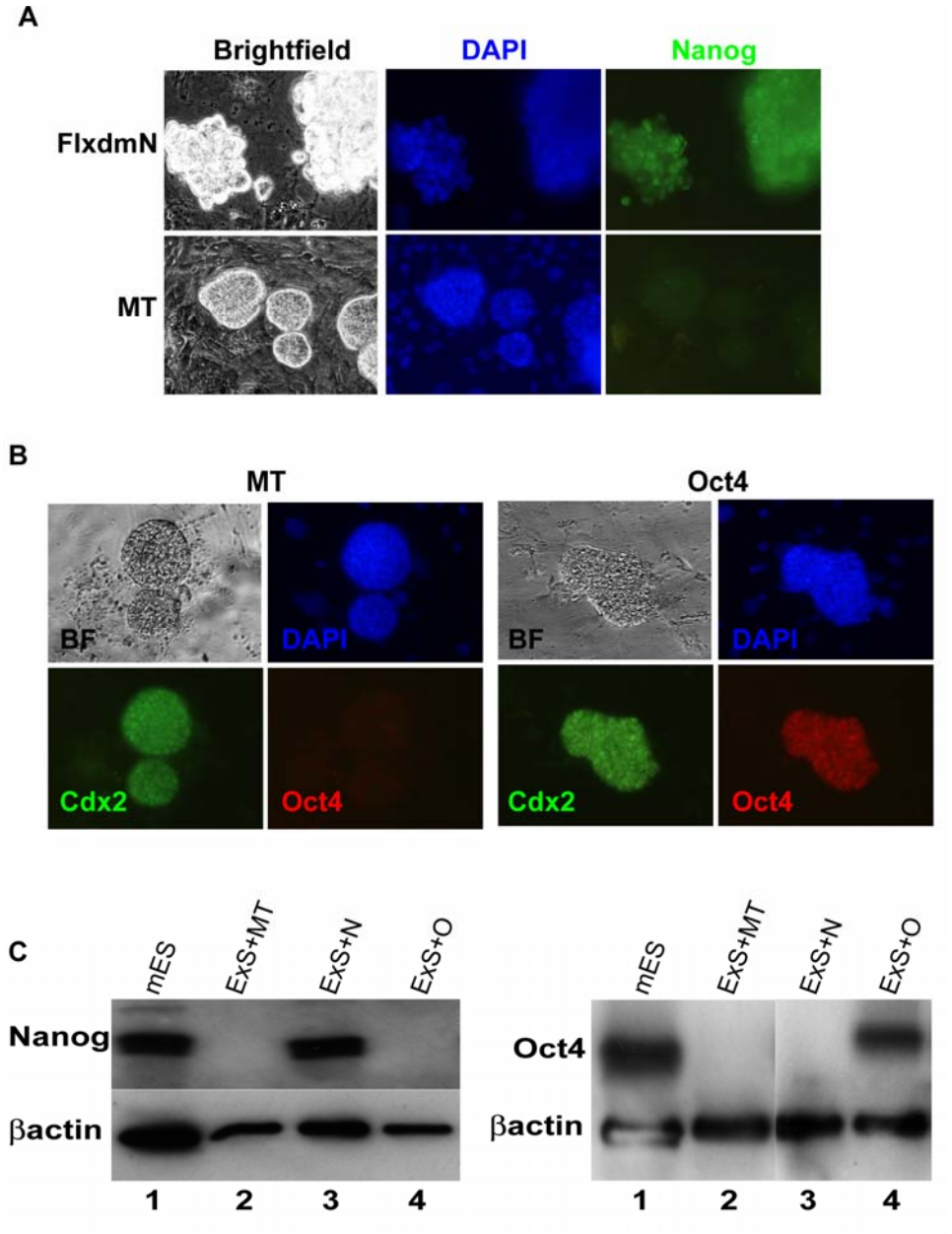


Figure 4.9 Transgenic Oct4 and Nanog expression at the protein level.
Brightfield and fluorescent antibody-stained images of rat ExS cells stably transfected with-
(A) pPyCAG-IP empty vector (MT) [P18] or Floxed-mNanog-IP expression vector (FlxdmN) [P14]. (Magnification x200).
(B) pPyCAG-IP empty vector (MT) [P16] or pPyCAG--mOct4-IP expression vector [P5]. (Magnification x200).
(C) Western blot of (1) E14-Tg2a mESC, (2) ExS + MT,
(3) ExS + Floxed-mNanog-IP and (4) ExS + pPyCAG-mOct4-IP.

4.3.5 Characterisation of transgenic rat ExS cells

Transgenic lines were maintained at least 30 days post-transfection prior to RT-PCR determination of ES cell marker expression (Figure 4.10). No reawakening of endogenous Oct4 or Nanog was observed (Figures 4.8A & B and Figure 4.9C). Likewise there was no significant up-regulation of the other ES cell markers Fgf4, Stella, Rex1 or Sox2. Furthermore, expression of the trophectoderm marker Cdx2 was retained (Figures 4.9B and 4.10).

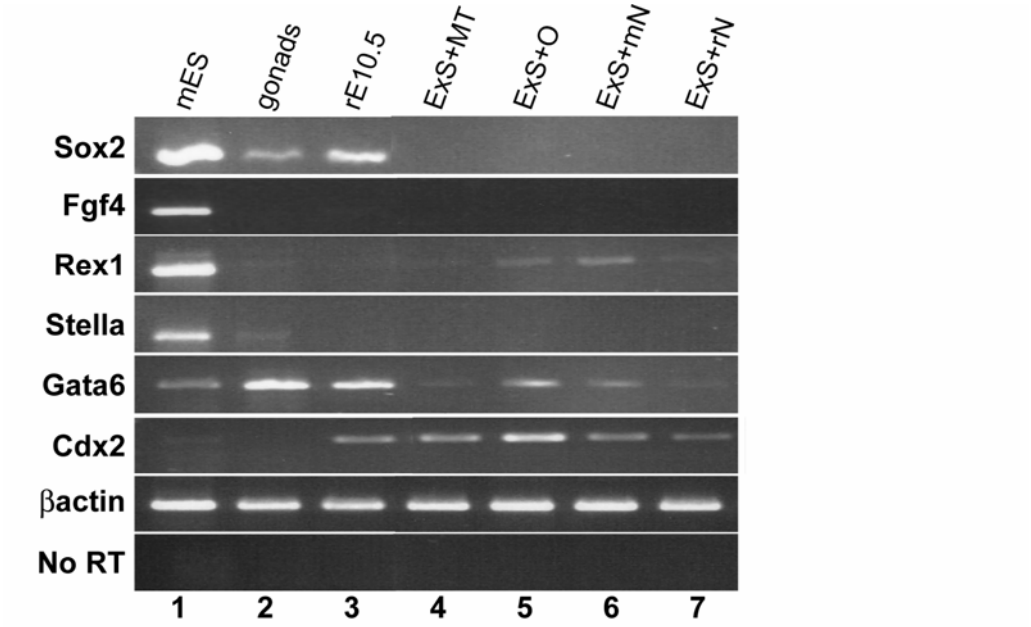


Figure 4.10 Expression profile of Oct4 and Nanog transgenic ExS cells. RT-PCR analysis of (1) E14-Tg2a mESC, (2) E13.5 rat gonads, (3) E10.5 rat embryos, (4) ExS + empty vector [P6], (5) ExS + mOct4 [P9], (6) ExS + Flxd mNanog [P9] and (7) ExS + rNanog [P7].

4.4 Generation of double transgenic rat ExS cells

Transgenic expression of Oct4 or Nanog in rat ExS cells failed to reawaken endogenous ES cell markers. Loss of either Oct4 or Nanog expression causes loss of pluripotency in mouse ES cells. Therefore, introduction of either transgene on its own is unlikely to be sufficient to induce transformation. It was also observed that

ExS cells fail to express the Sox2 (Sry-related HMG-box) transcription factor (Figure 4.10).

Sox2-null embryos die soon after implantation. Although they form a normal blastocyst, by E6.0 the embryo structure becomes disorganized, consisting mainly of extraembryonic cell types, such as trophoblast giant cells. Outgrowths from null blastocysts or isolated ICMs fail to produce ES cells and give rise mainly to trophoblast and primitive endoderm. Indeed, RNAi knockdown of *Sox2* resulted in ES cell differentiation, reduced AP staining and decreased Oct4 levels, but upregulation of trophectoderm markers. It has also been demonstrated that the enhancer regions of several ES cell-expressed genes contain both Sox2 and Oct4 binding motifs in close proximity to each other. These studies indicate the synergistic activity of Sox2 and Oct4 in regulating genes highly expressed in ES cells, such as *Fgf4*, *Utf1* and *Nanog* , as well as self-regulation (Tomioka, Nishimoto et al., 2002; Chew, Loh et al., 2005; Okumura-Nakanishi, Saito et al., 2005). Therefore, Sox2 plays an important role in maintaining ES cell pluripotency.

For these reasons, the mOct4 ExS transgenic line was transfected with either a mNanog or mSox2 expression vector to create double transgenic lines.

4.4.1 Zeocin selectable Sox2 and Nanog expression vectors

In order to select for cells carrying these second transgenes, a different antibiotic selectable marker had to be employed. Therefore, vectors were obtained that express mouse Nanog (gift from Jose Silva) or mouse Sox2 and also express the zeocin selection marker (Figure 4.11B & C).

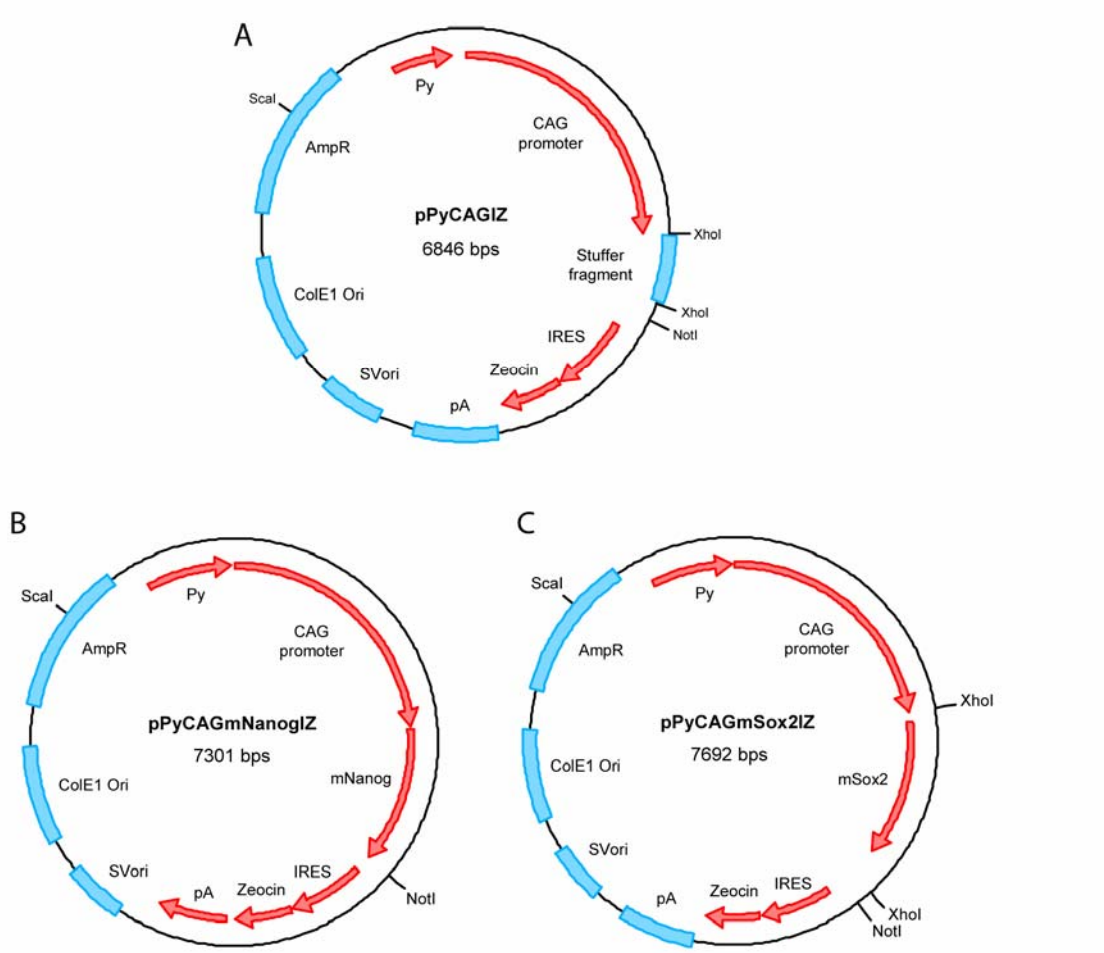


Figure 4.11 Zeocin-resistant expression vectors.
(A) Zeocin-resistant episomal expression vector which carries the promoter-enhancer-ori derived from PyFIOI (Py). cDNA is cloned directionally in place of the stuffer fragment. Expression is driven by a CAG promoter and co-expression of the zeocin-resistance gene is facilitated by an IRES sequence.
(B) pPYCAG-mNanog-IZ, mouse Nanog expression vector and
(C) pPyCAG-mSox2-IZ, mouse Sox2 expression vector.

4.4.2 Generating Zeocin-resistant DIA-M feeder cells

To allow selection of cells with zeocin in feeder-dependent culture, a zeocin-resistant DIA-M feeder layer was established. Neomycin/puromycin-resistant DIA-M feeder cells were transfected with a *Sca*I-linearised zeocin-selectable vector (Figure 4.11A) as detailed in Chapter 2. Transfected cells were plated at clonal density and selected in 20μg/ml zeocin. Individual clones were expanded and frozen. These were used in the usual manner for culturing ExS cells.

4.4.3 Plating ExS cells on gelatin and laminin

The ability of ExS cells to grow in feeder-free conditions was also investigated. If the requirement for the DIA-M feeder layer could be removed then this would also allow selection in zeocin. Therefore, rat ExS cells were removed from feeders by mouth pipetting, trypsinised then plated onto gelatin or laminin-coated wells (Figure 4.12).

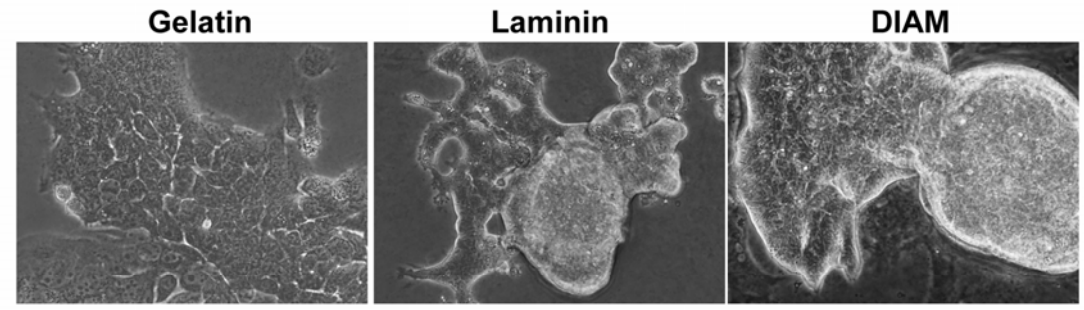


Figure 4.12 Gelatin and laminin cultures of mOct4ExS cells.
Rat ExS cells carrying the pPyCAG-mOct4-IP transgene were removed from the DIA-M feeder layer and cultured on gelatin (left panel) and laminin (middle panel) for 12 passages. Cells were also returned to DIA-Ms (right panel) after 10 passages on laminin. (Magnification x200).

Cells attached readily to laminin-coated dishes, but only poorly on gelatin. This made passaging on gelatin very inefficient. In both cases colonies appeared to flatten, resembling mES cells, although a population of compacted ball-like colonies remained. These did not represent a heterogenous population since passaging individual colonies still resulted in both colony types forming. Rat ExS cells could be maintained on laminin for at least twenty passages by trypsin dissociation every three days and returned to DIA-Ms without any morphological change.

4.4.4 Transfection of mOct4 rat ExS line

The mOct4 rat ExS cell line was maintained on laminin-coated plates prior and subsequent to lipofection. The cells were lipofected, as detailed in Chapter 2, with *Sca*I-linearised, zeocin-resistant mNanog-IZ or mSox2-IZ expression vectors carrying the zeocin resistance gene (Figure 4.11B & C). The pPyCAGIZ empty (MT) vector was used as a control (Figure 4.11A). Cell lines stably-expressing the integrated transgene were selected in 20μg/ml zeocin. These lines grew in a similar manner to the parental line and the mOct4 line. In general the morphology was also similar (Figure 4.13).

4.4.5 Transgene expression

Expression of the Nanog-IZ and Sox2-IZ transgenes was confirmed in these lines by RT-PCR analysis (Figure 4.14a) and Western blot (Figure 4.14b).

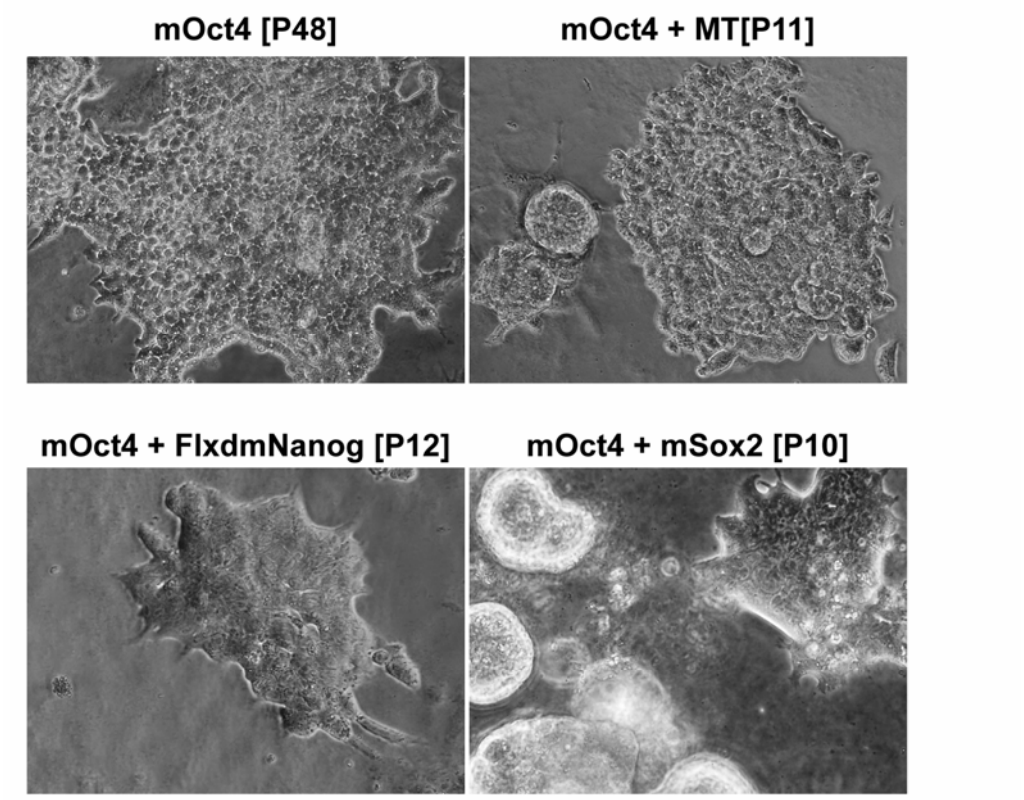


Figure 4.13 Morphology of double transgenic lines.
mOct4 transgenic ExS cells stably transfected with empty vector (MT), Floxed mNanog or mSox2 expression vectors, cultured on laminin. (Magnification x100).

4.4.6 Characterisation of double transgenic rat ExS cells

Double transgenic lines were maintained at least 30 days post-transfection. RT-PCR analysis was then performed to determine reactivation of ES cell markers (Figure 4.14c). Once again, there was no upregulation of endogenous Nanog, Sox2, FGF4 or Stella. Interestingly, Rex1 appeared to be up-regulated in the double transgenic lines, including the MT vector control (lanes 6, 7 & 8). This is likely due to plating on laminin rather than a direct effect of the transgenes. Furthermore, the endoderm marker GATA6 and the trophectoderm marker Cdx2 continued to be expressed.

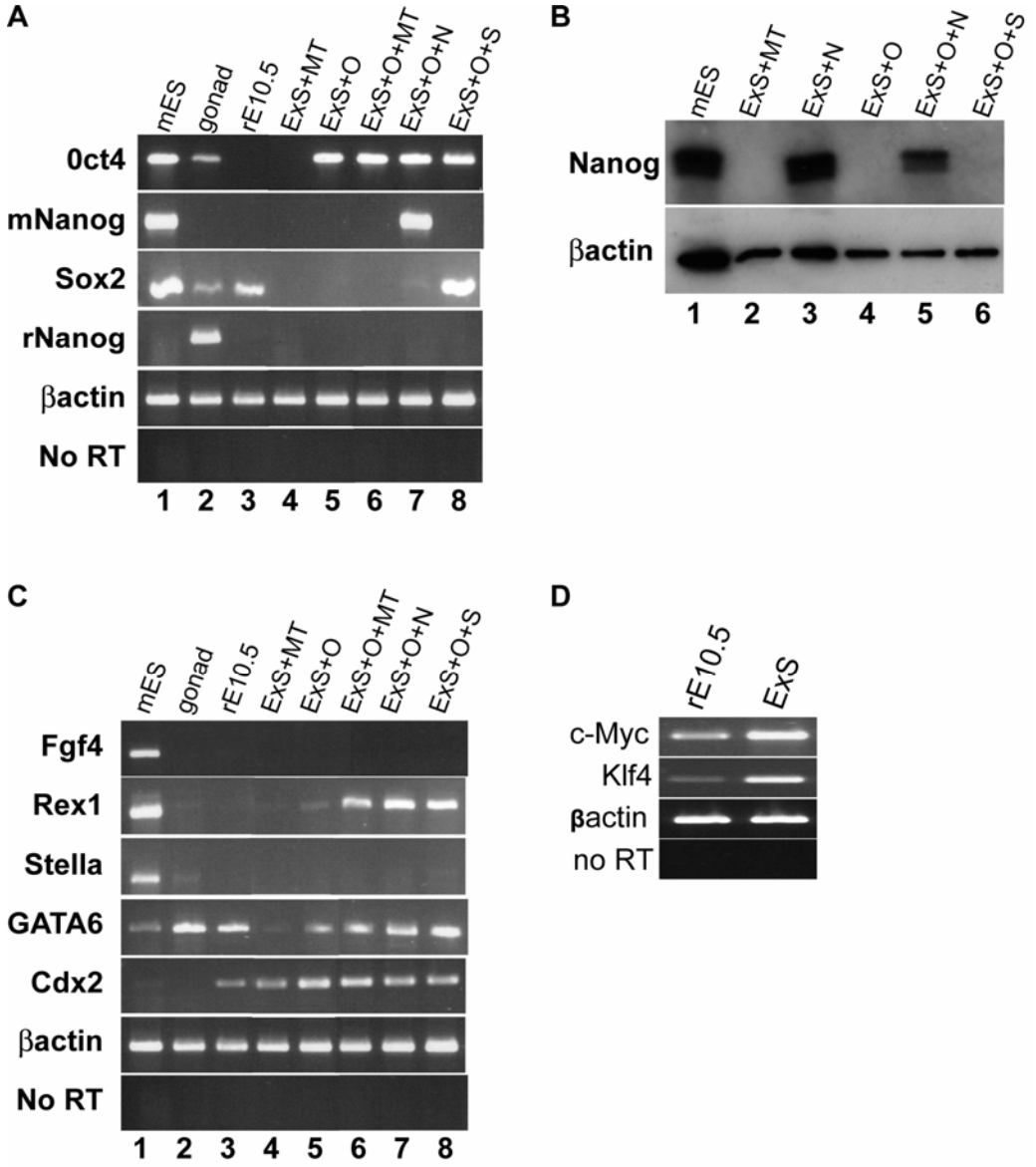


Figure 4.14 Transgene expression and characterisation of double transgenic rat ExS cells.

(A) RT-PCR confirmation of transgene expression; (1) mESC (E14-Tg2a), (2) E13.5 rat gonads, (3) E10.5 rat embryos, (4) ExS + MT [P6], (5) ExS + mOct4-IP [P9], (6) ExS + mOct4-IP +MT [P6], (7) ExS + mOct4-IP + mNanog-IZ [P7] and (8) ExS + mOct4-IP + mSox2-IZ [P12]. Species-specific mouse (mNanog) and rat (rNanog) Nanog oligonucleotides were used.
(B) Western blot of (1) mESC (E14-Tg2a), (2) rat ExS cells, + MT (3) ExS + mNanog-IP, (4) ExS + mOct4-IP, (5) ExS + mOct4-IP + mNanog-IZ, (6) ExS + mOct4-IP + mSox2-IZ.
(C) RT-PCR expression profile of double transgenic ExS cells. Loading as **(A).**
(D) Rat ExS cells express c-Myc and Klf4.

4.5 Discussion

Rat ExS cells stably expressing Oct4, either independently or in combination with Sox2 or Nanog, failed to upregulate the endogenous ES cell markers Nanog, Fgf4 or Stella. Rather, they continued to express GATA6 and Cdx2, suggesting that they have retained their extraembryonic state. Although the function of the expression vectors employed had been tested previously by others or as part of this project, their function in ExS cells has not been confirmed. Use of luciferase reporters containing the relevant enhancer binding motifs would confirm their activity.

Since the outset of this project several groups have demonstrated reprogramming of differentiated cells into pluripotent stem cells using defined factors. These have been termed induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). Twenty four factors were initially screened, using retroviral transduction, to investigate their ability to induce pluripotency in mouse embryonic fibroblasts. The factors were screened collectively and in pools, after withdrawal of individual factors. This study demonstrated that co-transduction of only four factors, Oct4, Sox2, c-Myc and Klf4, was sufficient to induce complete reprogramming. These iPS cells exhibit characteristics of mouse ES cells, such as expression of ES markers, differentiation into the three germ layers and contribution to adult chimaeras and the germline. Induction of pluripotency has now been demonstrated in several cell types and species, including terminally differentiated lines, as well as rat fibroblasts (Li et al., 2009) and human somatic cells. Successful reprogramming of differentiated cells has also been achieved using vector-based, CAG-driven expression, supporting the approach used in this project (Kaji et al., 2009). However,

the experiment by Kaji *et al* employed Nucleofection as a delivery system, whilst transfection of ExS cells utilized Lipofectamine, which is known to be less efficient. Furthermore, the efficiency may be hindered further due to the difficulty in generating a truly single cell suspension of ExS cells prior to transfection.

The efficiency of generating iPS cells is typically only 0.001% to 0.03%. However, efficiency varies depending on the delivery system employed, the number of factors transduced, the cell type transduced and the addition of chemical modifiers. It is also clear that the combination of factors used is important. In the original experiments, no iPS cells could be generated with only 2 factors. Even, with three factors, in the absence of c-Myc, the efficiency was very low and the reprogramming rate slower (Nakagawa et al., 2008). Successful reprogramming was eventually demonstrated with two factors or with Oct4 alone. However, these iPS cells were generated from neural stem cells (NSCs) rather than fibroblasts. NSCs express significant endogenous levels of Sox2, Klf4 and c-Myc compared to fibroblasts. This suggests that high levels of expression are likely to be an important driving force in initiating reprogramming. Interestingly, ExS cells express both c-Myc and Klf4 (Figure 4.14d), suggesting that in the presence of exogenous Oct4 and Sox2 expression, reprogramming could be possible. It would be interesting to determine the level of c-Myc and Klf4 expressed in ExS cells relative to NSCs. However, NSCs may possess a transcription factor profile or epigenetic state closely resembling that of ES cells that facilitates reprogramming in the absence of other exogenous factors.

It is surprising that Nanog is not one of the core factors required to generate iPS cells. Recently, however, it has been demonstrated that Nanog is necessary to

promote the formation of the pluripotent state following reprogramming of NSCs either by cell fusion with ES cells or by induction with exogenous factors. Although Nanog appears to be dispensable for the initial reprogramming process, in the complete absence of Nanog cells fail to become fully reprogrammed, but rather exist in a dedifferentiated state. This suggests that Nanog is essential for coordinating ES cell transcription factors into a functional network that facilitates the transition to the fully reprogrammed, pluripotent state (Silva et al., 2009).

The aim of this chapter was to generate stable over-expressing lines to examine the affect of these transgenes on ExS cells. Therefore, colonies were selected based on integration of the transgene rather than on upregulation of an ES cell-specific marker, such as Nanog, as a direct result of reprogramming, as used in iPS experiments. Nonetheless it is clear that Oct4, whether alone or in combination with Sox2 or Nanog, as used in this project, is unlikely to fully reprogramme ExS cells. Although high levels of expression could be expected from the CAG promoter, other factors are likely to be required either to promote survival or proliferation, or initiate chromatin remodeling to establish the correct epigenetic state. Also, the transgenes were introduced sequentially, rather than in combination. Any positive reprogramming function may be limited due to the absence of other factors whose activity may have been required previously or simultaneously. Indeed overexpressing any of the four factors in ES cells induces differentiation, senescence or apoptosis. Therefore, the balance and relative timing of expression throughout the reprogramming event may be important. Unsurprisingly, reprogramming is not instant, typically taking around 14 days for colonies to form that have a characteristic ES cell morphology and expression profile.

4.6 Summary

CAG-driven Oct4, Nanog and Sox2 expression vectors were intregrated into ExS cells to investigate the possibility of transforming these cells into ES cells. Stable lines were generated which express either Oct4 alone or in combination with Nanog or Sox2. However, no up-regulation of endogenous Oct4 and Nanog, or the other ES cell markers, Fgf4 and Stella, was observed. Rather, Cdx2 and GATA6 expression were preserved, suggesting that the cells have retained their extra-embryonic identity and overexpression of Oct4, Nanog or Sox2 is insufficient to downregulate expression of Cdx2 and GATA6. The recent studies generating iPS cells suggest that whilst Oct4 and Sox2 are necessary for reprogramming of somatic cells, they are not sufficient. Furthermore, the iPS reprogramming efficiency is relatively low, suggesting that the exact level of expression of the individual transgenes relative to each other may be important. Since ExS cells express Klf4 and c-Myc, exogenous expression of Oct4 and Sox2 may be sufficient for reprogramming should the relative levels be appropriate. However, the transfection method employed in this chapter may not yield sufficient numbers of transfectants to ensure those with the appropriate levels are produced.

Chapter 5

Characterisation of authentic rat embryonic stem cells derived in defined conditions

5.1 Introduction

Whilst mES cells are routinely cultured in feeder-free conditions, culture in the absence of both feeders and serum results in neural differentiation. In the presence of LIF, neural differentiation is reduced, but not blocked. However, Ying *et al* demonstrated that chimaera forming, germline competent mES cells could be derived and maintained in feeder and serum-free culture conditions in the presence of LIF when supplemented with bone morphogenetic protein (BMP4). BMP4 blocks neural differentiation by induction of Inhibitor of differentiation (*Id*) genes.

It has been demonstrated that differentiation of mouse ES cells occurs via autoinductive stimulation of Erk via Fgf4 signalling. Interestingly, neither LIF nor BMP4 inhibit Erk activation suggesting that they may act downstream of Erk. Recently, Ying *et al* have demonstrated that inhibition of FGF/Erk signaling by two small molecule inhibitors could sustain mouse ES cell self-renewal without requirement for LIF or BMP4. However, cell viability is compromised in such conditions where Erk activity is reduced. Robust maintenance could be achieved by addition of a selective GSK3α/β̃ inhibitor CHIR99021 (CHIR) (Murray et al., 2004). The effect of GSK3 inhibition is not mediated via STAT3 phosphorylation or upregulation of c-myc or anti-apoptotic factors. Although mouse ES cells cultured in

CHIR alone exhibit non-neural differentiation, in combination with FGFR/Erk inhibition, differentiation is blocked and growth enhanced. Indeed, both *Fgf4*$^{-/-}$ and *Erk2*$^{-/-}$ ES cells could be maintained in CHIR only. This, three inhibitor (3i) culture condition supported *de novo* derivation, clonal expansion and contribution to both chimaera formation and germline transmission. This suggests that maintenance of ES cell self-renewal does not require activation signals, but rather is the default state that can be maintained by elimination of differentiation signals.

The aim of the work described in this Chapter was to utilize this serum-free 3i culture condition to determine if established rat ExS cell cultures could be transformed into rat ES cells or if rat ES cell lines could be derived under these conditions.

5.2 Culturing rat ExS in 3i medium.

Rat ExS cells were passaged equally into four different culture conditions and cultured for 6 days, with media changes every second day (Figure 5.1). ExS colonies grew well in standard ExS cell culture conditions containing GMEM, serum, LIF and Matrigel. However, when cultured in 3i, cells stopped growing, no colonies formed and the cells appeared differentiated even in the presence of LIF or Matrigel. This indicates that blockade of FGFR signalling, pErk activation and GSK-3 signalling is insufficient to transform rat ExS cells in the absence of serum. Furthermore, either inhibition of one or more of these pathways prevents ExS cell self-renewal or, some factor(s) present in serum are required for ExS cell maintenance.

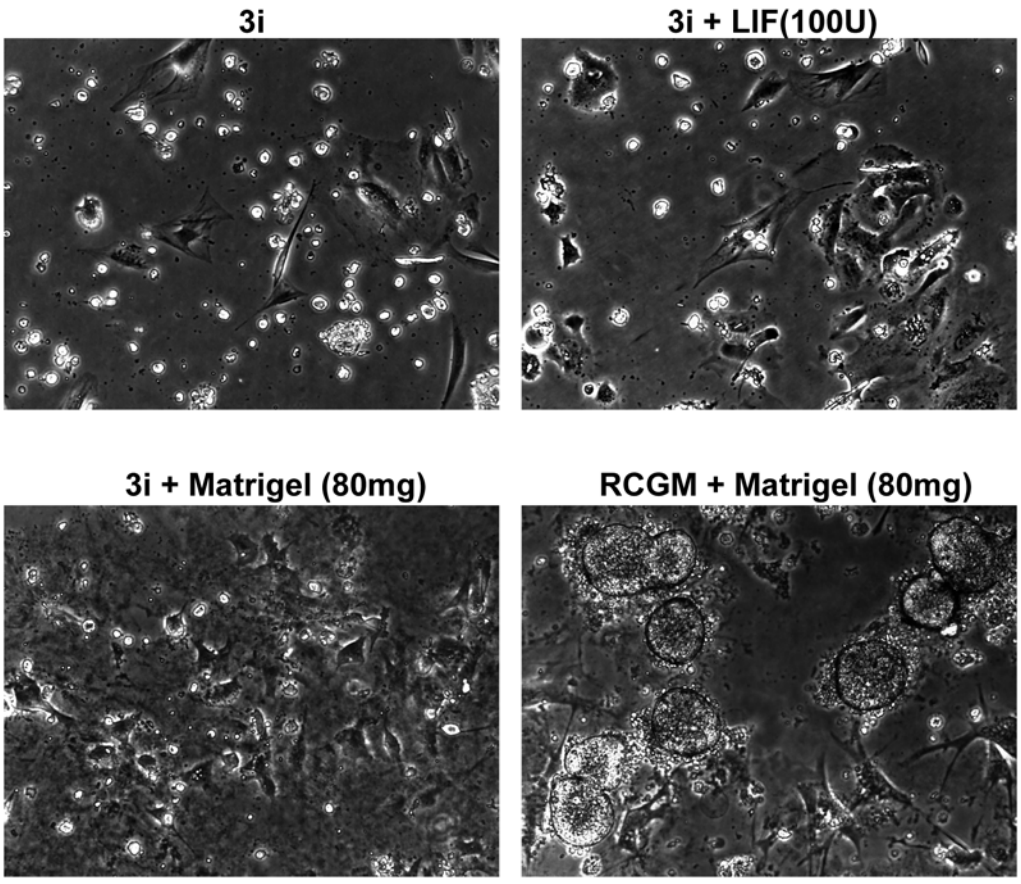


Figure 5.1 Rat ExS cells cultured in 3i medium.
Rat ExS cells cultured for 6d in 3i medium (top left panel), 3i +100U LIF (top right panel), 3i + Matrigel (bottom left panel) and RCGM + Matrigel (bottom right panel).

5.3 Derivation in 3i medium

Attempts were also made to derive cell lines in 3i from freshly isolated rat ICMs of Fischer and DA E4.5 rat embryos (derivations were carried out by Mia Buehr as described in Chapter 2).

These lines were maintained in order to assess if they possessed the key characteristics of ES cells. The cells were maintained on a DIA-M feeder layer and could be passaged by single cell dissociation using trypsin or Accutase™. These

cultures yielded colonies with a morphological resemblance to mES cells and were termed rat 3i cells (Figure 5.2).

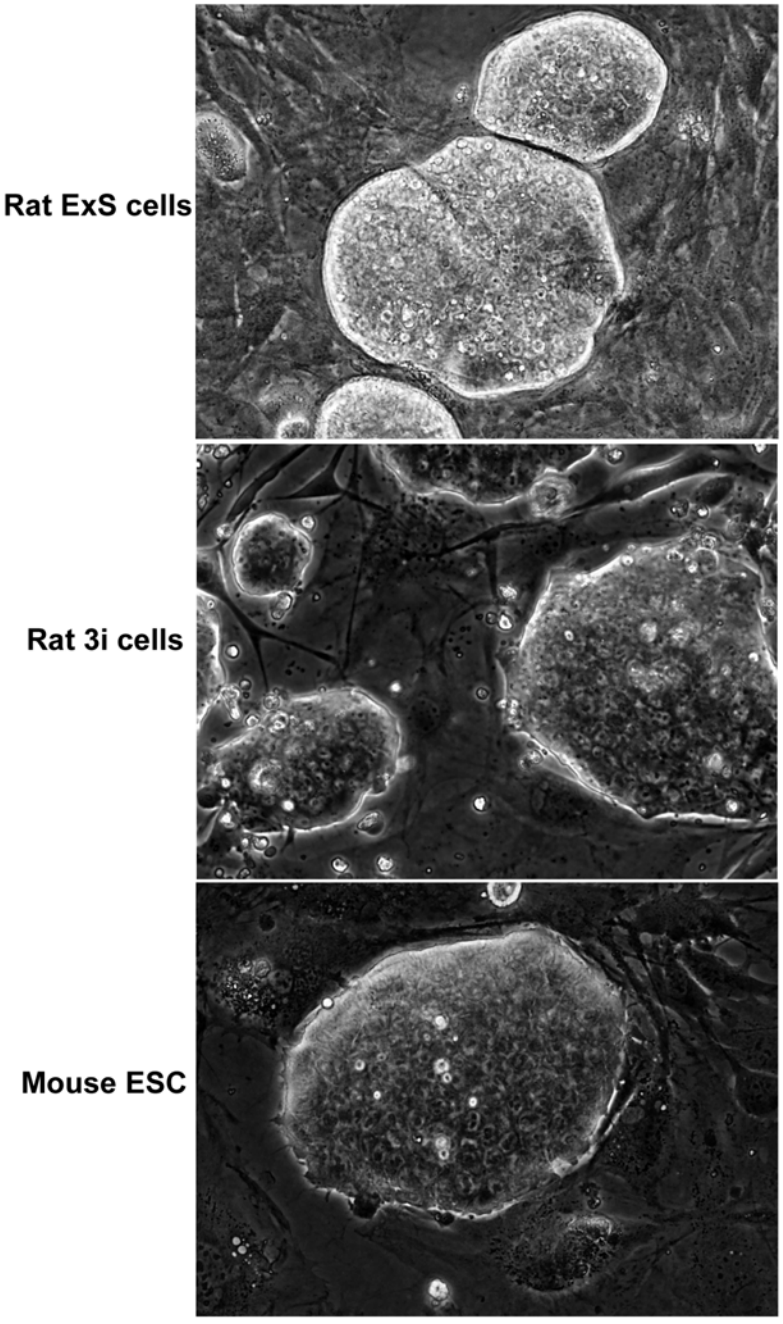


Figure 5.2 Rat 3i cell colony morphology.
Colony morphology of rat ExS cells (top panel), grown in GMEM containing serum, LIF and Matrigel, rat 3i cells (middle panel), grown in 3i medium and mouse ESC (bottom panel), grown in GMEM containing serum and LIF. All lines grown on DIAM feeder layer.

5.4 Rat 3i expression profile

RT-PCR analysis confirmed that rat 3i cells continued to express Oct4 and Nanog beyond the time when these markers are normally extinguished during the derivation of ExS cell cultures (Figure 5.3A). Furthermore species-specific Nanog oligonucleotides were used to indicate that only the rat transcript, and not mouse, could be detected in these cells. This data confirmed that these cells were indeed of rat origin and did not arise from mouse ES cell contamination.

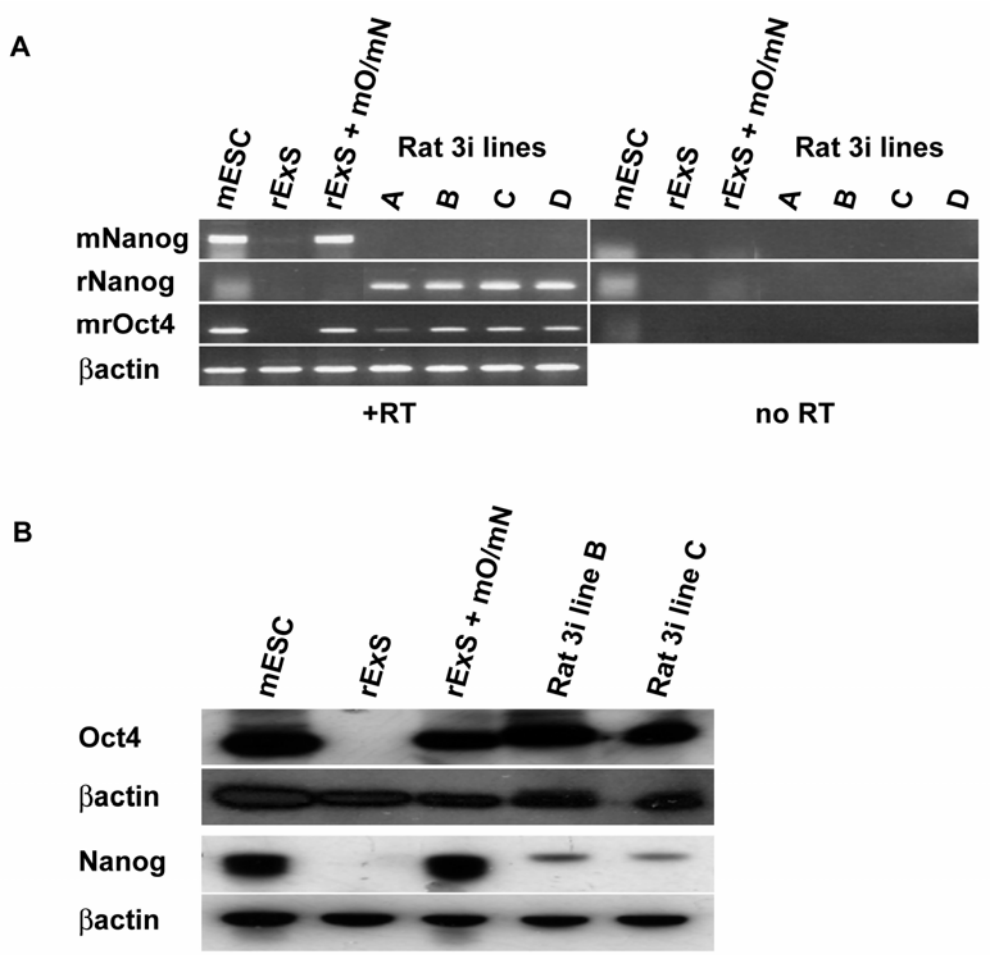


Figure 5.3 ICM-derived rat 3i cells express Oct4 and Nanog.
(A) RT-PCR analysis for Oct4 and Nanog (using rat and mouse specific Nanog oligonucleotides).
(B) Western blot for Oct4 and Nanog.
E14-Tg2a mouse ES cells (mESC), rat ExS cells (rExS), rat ExS cells expressing transgenic mouse Oct and mouse Nanog (rExS + mO/mN).

Expression of Oct4 and Nanog was also confirmed at the protein level by Western blotting. The Oct4 protein level is similar to mES cell levels (Figure 5.3B). However, the level of Nanog in rat 3i cells appears considerably lower than that for mES cells. Furthermore, at least two bands are detected in samples expressing mouse Nanog, but only one band in the rat 3i cell samples. The antibody used is polyclonal and was raised against amino acids 2-16 of mouse Nanog, in which there are two amino acid differences with rat Nanog. The differences in intensities observed are most likely due to differences in epitope specificity between mouse and rat.

Further expression analysis, performed by RT-PCR, demonstrated that rat 3i cells expressed similar levels of other markers commonly found in mES cells, such as Sox2, Rex1, Eras, Stella, Klf4, Errβ and the Oct4/Sox2 target Fgf4 which, like Oct4 and Nanog, is not expressed in ExS cells (Figure 5.4A). c-Myc was also expressed, but at a level lower than typically seen in mES cells (discussed in Chapter 6). Furthermore, the expression of differentiation markers was not detected (Figure 5.4B).

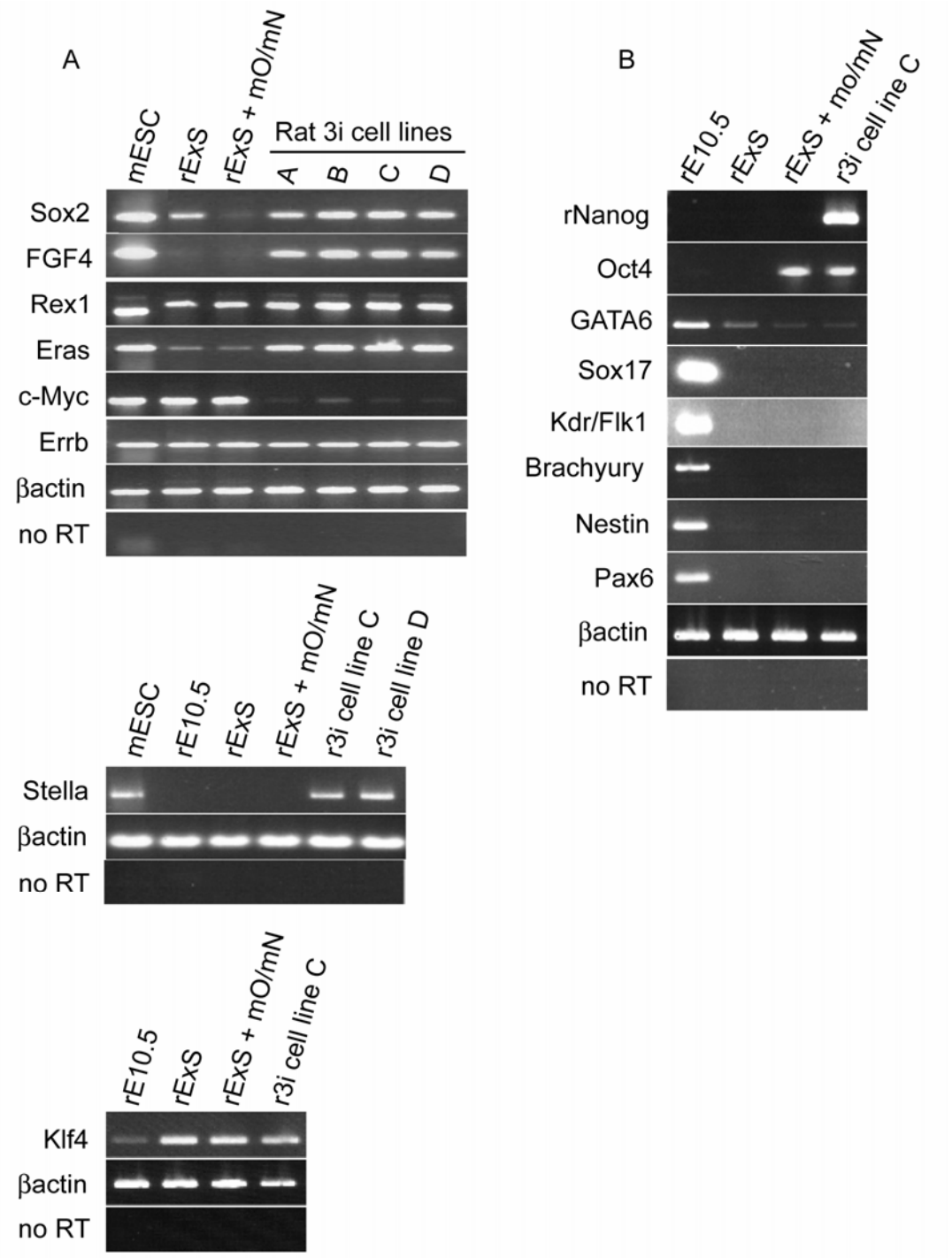


Figure 5.4 Characterisation of rat ES cells.
RT-PCR analysis of **(A)** ES cell markers and **(B)** markers of differentiation in rat E10.5 embryo (rE10.5), rat 3i cells, E14-Tg2a mouse ES cells (mESC), rat ExS cells (rExS), rat ExS cells expressing transgenic mouse Oct and mouse Nanog (rExS + mO/mN), using oligonucleotides designed to rat mRNA.

5.5 Differentiation capacity

The differentiation potential of rat 3i cells was assessed by teratoma formation and chimaera colonization.

5.5.1 Teratoma formation

The pluripotential capacity of rat 3i cells was examined by injecting cells, from two independent rat 3i lines (lines B and C, derived from Fischer and DA rats respectively), under the kidney capsule of SCID (Severe Combined Immuno-deficiency) mice (injections were performed by Renee McLay). The teratomas formed (Table 5.1) demonstrated multi-lineage differentiation as determined by histological analysis (Figure 5.5) (histological analysis was performed by Ron Wilkie). Differentiated cell types and structures, such as striated muscle, keratinized epithelium and gut epithelium, could be identified.

Fischer line B

Sample	Tumour Weight (g)	Days post-injection
1	1.5	40
2	3.7	42
3	4.2	55
4	0.01	55
5	1.7	55

DA line C

Sample	Tumour Weight (g)	Days post-injection
1	3.5	30
2	0	40
3	3.4	42
4	0	55
5	0	55
6	0	55

Table 5.1 Teratoma formation.
Age and weight of teratomas produced by injection of rat 3i cell lines B and C under the kidney capsule of SCID mice.

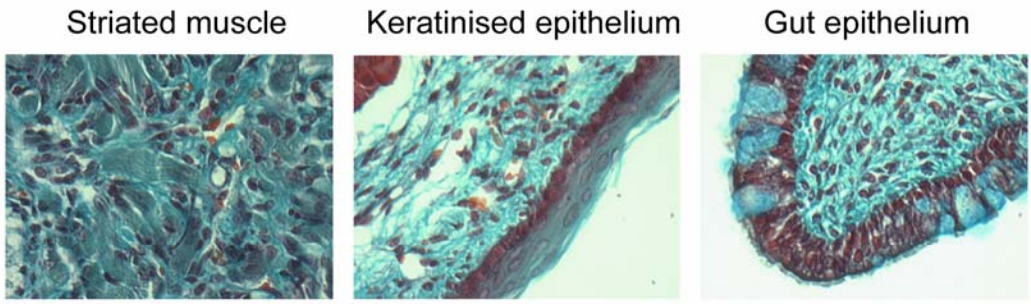


Figure 5.5 Differentiation potential of rat 3i cells in teratomas.
Masson's trichome-stained cryostat sections of a teratoma formed under the kidney capsule from rat 3i line C.

5.5.2 GFP-expressing rat 3i cell line

The developmental potential of ES cells is best defined by their capacity to colonise host embryos and contribute to all three germ layers of the developing embryo. Therefore, to track their distribution in midgestaion embryos following blastocyst injection, rat 3i cells were transfected with a pPyCAGegfpIP expression vector to generate stable GFP-positive cells. Initial attempts to transfect rat 3i cells using standard lipofection reagents, such as Lipofectamine 2000™, Fugene ™ and Optifect™ were unsuccessful. It was hypothesized that in serum-free conditions cells may be sensitive to the disruptive nature of lipofection. The presence of serum may protect against this or enhance the recovery of the cells post-transfection. Therefore, transfection was performed in the presence of serum using Lipofectamine LTX ™ (Invitrogen, 15338-100) - a reagent that is optimized for sensitive cells. The serum and reagent were removed 16 hours post-transfection. Puromycin selection (1µg/ml) was added 48 hours post-transfection and maintained for at least one week. GFP-

positive, puromycin-resistant colonies were obtained and pooled (Figure 5.6A). Stable GFP-expressing cells were injected into E4.5 d.p.c rat embryos.

5.5.3 Mid-gestation and neonatal GFP Chimaeras

The presence of a stable GFP reporter in rat 3i cells circumvents the need to use specific strains of embryo for injection. Therefore, cells were injected into both Fischer and DA blastocysts (injections were performed by Renee McLay and Jan Ure). Injected blastocysts were transferred into pseudo-pregnant recipients and embryos harvested at E10.5-14.5 d.p.c. No chimaeras were observed using the Fischer-derived cell line from a total of forty-five injected embryos (Table 5.2).

Cell Line	Strain	Sex	Transferred	Midgestation chimaeras
B-GFP	Fischer	F	45	0
D-GFP	DA	F	50	5
DA3-GFP	DA	F	80	1
DA2-GFP	DA	M	19	0

Table 5.2 Summary of rat 3i cell blastocyst injections and midgestation chimaera production.

However, two DA-derived lines (D and DA3) produced a total of six GFP-positive mid-gestation chimaeras that demonstrated widespread distribution throughout the embryo (Figure 5.6B & C).

Line DA3 also generated five postnatal chimaeras, three of which died neonatally, whilst two had to be sacrificed due to jaw deformity (Table 5.3).

Cell Line	Strain	Sex	Transferred	Born	Term Chimaeras
C*	DA	F	64	44	2 male
D	DA	F	44	6	0
DA3	DA	F	24	5	0
D-GFP	DA	F	105	18	0
DA3-GFP	DA	F	75	16	5[b]
B*	Fischer	F	60	20	0
E*	Fischer	F	55	20	0
F*	Fischer	M	12	10	0
G*	Fischer	F	27	16	0
4.1	Fischer	F	87	13	0

Table 5.3 Summary of rat 3i cell blastocyst injections and term chimaera production.

*Naturally mated recipients were used for these lines.

bThree died neonatally and two were sacrificed at 25 days due to jaw deformity.

Examination of these also demonstrated contribution in several tissues including, skin, liver, heart and lung (Figure 5.6D). GFP expression was also observed in the testis of a P25 chimaera (Figure 5.6E).

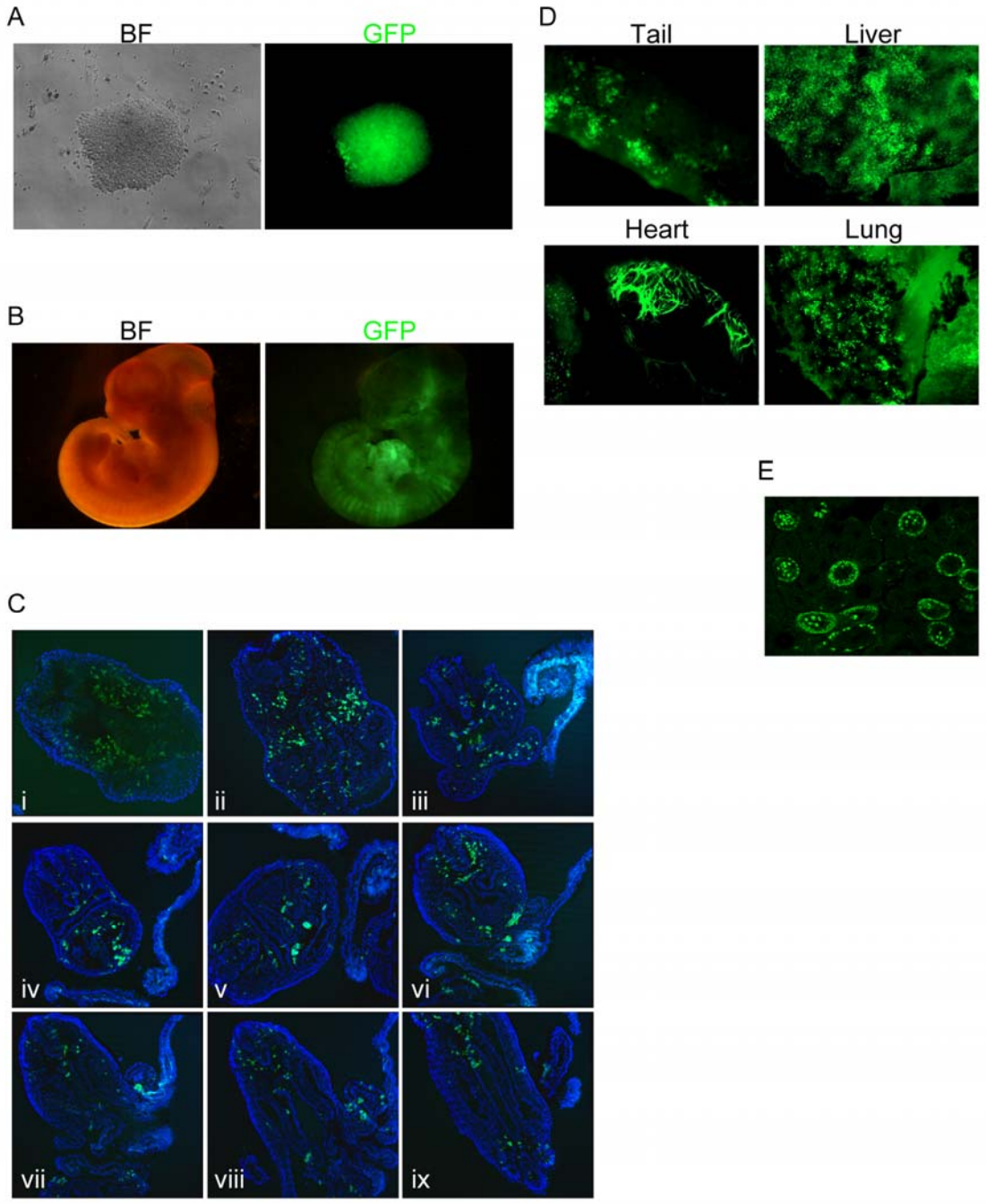


Figure 5.6 Stable GFP-expessing rat 3i cells generate mid-gestation chimaeras.

(A) Brightfield and fluorescent images of stable GFP rat 3i cell line D.
(B) Brightfield and fluorescent images of E13.5 chimaeric embryo.
(C) Fluorescent images of head-to-tail transverse vibratome sectioned (i-ix) E10.5 chimaeric rat embryo generated from GFP-expressing rat 3i cells (line D) shown in green, with DAPI nuclear staining in blue.
(D) GFP expression in whole tissues from neonatal chimaera.
(E) GFP expression in vibratome section of testis from P25 chimaera.

5.5.4 Adult chimaera formation

ES cell contribution to the germ cells is critical for germline transmission and generating rat disease models. For this purpose attempts were made to generate adult chimaeras that would be bred to demonstrate transmission of the ES cell genome through the germline.

At the time of the initial injections, vasectomised male rats for generating pseudo-pregnant female recipients were unavailable. As an alternative, naturally mated recipients were used. In order to identify chimaeras by coat colour, Fischer (albino coat colour) blastocysts were injected with DA (agouti coat colour) cells then transferred to naturally mated DA recipients. Conversely, DA blastocysts were injected with Fischer derived cells and subsequently transferred into Fischer recipients (injections were performed by Renee McLay and Jan Ure).

Five independent Fischer-derived cell lines failed to produce any chimaeras from a total of 241 injected embryos (Table 5.3). However, DA-derived cell line C produced two, coat-colour, male chimaeras from a total of sixty-four injected embryos (Figure 5.7A).

The relatively high coat-colour transmission of the chimaeras generated suggested that a 100% coat-colour contribution could not be excluded. A 100% coat-colour transmission would not be detected within a background of naturally born littermates. Therefore, genotyping using microsatellite PCR analysis of tail, ear and blood samples was routinely employed.

Analysis of microsatellite regions D1Rat122 and D3Rat17 by conventional agarose gel (Figure 5.7B) and fluorescent ABI-capillary detection (Figure 5.8) confirmed coat-colour chimaerism. However, although both chimaeras had a similar

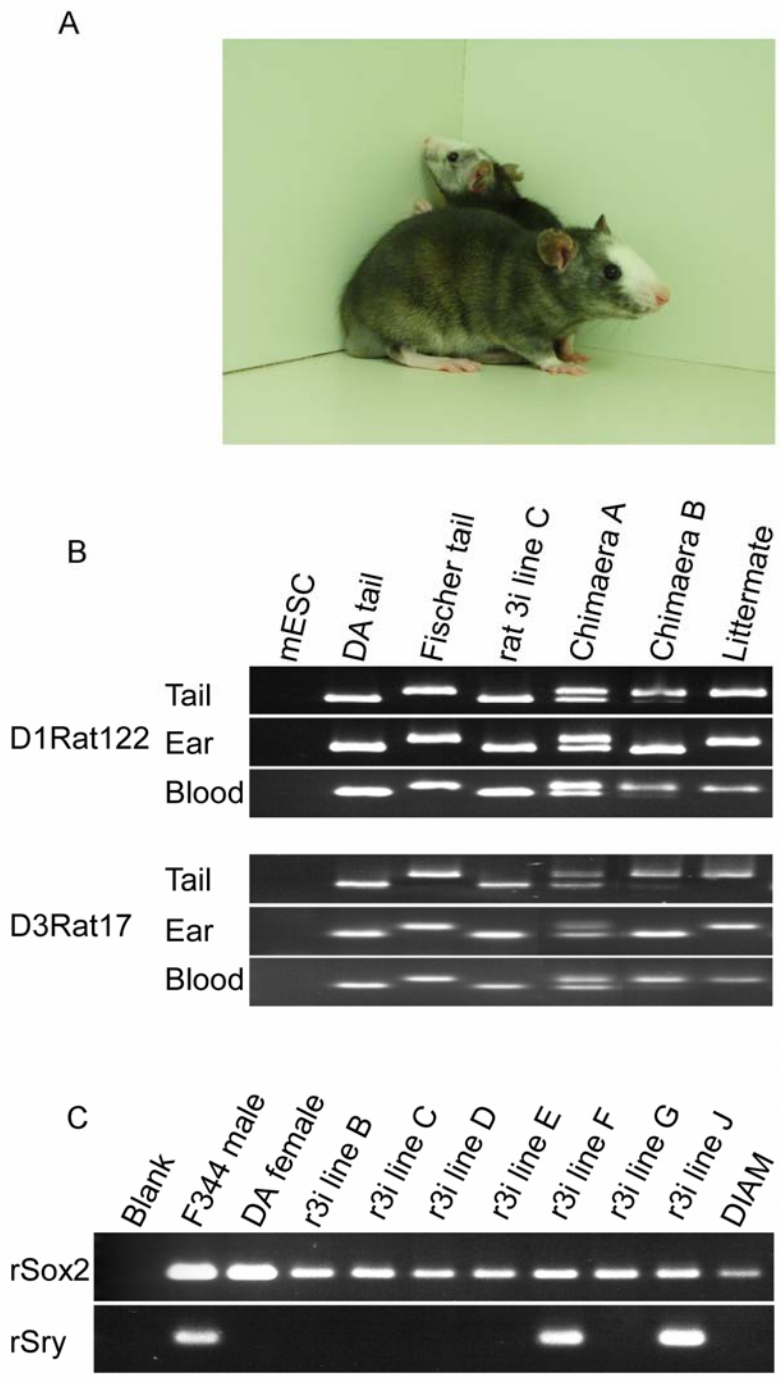


Figure 5.7 Rat 3i cells contribute to adult chimaeras.
(A) Two rat chimaeras generated from rat 3i line C (DA-agouti) injected into Fischer-albino blastocysts. **(B)** Confirmation of high chimaeric contributions using PCR genotyping of rat microsatellite regions D1Rat122 and D3Rat17 from genomic DNA of tail, ear and blood using conventional (agarose gel) detection method. **(C)** Sex determination of rat 3i lines by genomic DNA PCR with Sry oligonucleotides designed to rat sequence.

contribution as determined by coat colour, the contribution was significantly different when other tissue samples were analysed. Chimaera 'A' typically had a 60:40 (Fischer:DA) ratio for tail, ear and blood, whereas Chimaera 'B' was approximately 90:10. The degree of chimaerism varied according to the tissue assayed, with Chimaera 'B' demonstrating 10% chimaerism in tail and blood, but 100% in ear. This skewed coat colour contribution could be explained by the influence of the hooded gene (Yamamura and Markert 1981). Presence of the hooded gene within melanoblasts retards their migration from the mid-brain region of the developing neural crest. Therefore, in this case, the melanoblasts from the F344 albino hooded host embryo migrate slowly and become restricted to the head region. Whereas, the DA agouti non-hooded-derived ES cells migrate normally and preferentially contribute to the posterior of the rat.

Once vasectomised males became available injections were repeated in pseudo-pregnant recipients. However, no further chimaeras were obtained using either DA or Fischer-derived cell lines.

Sex determination was performed on the rES cell lines by PCR analysis of genomic DNA using oligonucleotides designed to the Sry gene (Figure 5.7C). Sry is a sex-determining gene linked to the Y-chromosome that triggers male development (Sinclair et al., 1990). Detection by PCR demonstrates the presence of the Y-chromosome. The results indicate that only Fischer-derived lines F and J are male.

Therefore, because the injected cell line (C) was female and the chimaeras produced were male, they will not support the development of the gametes and germ-line transmission is not possible.

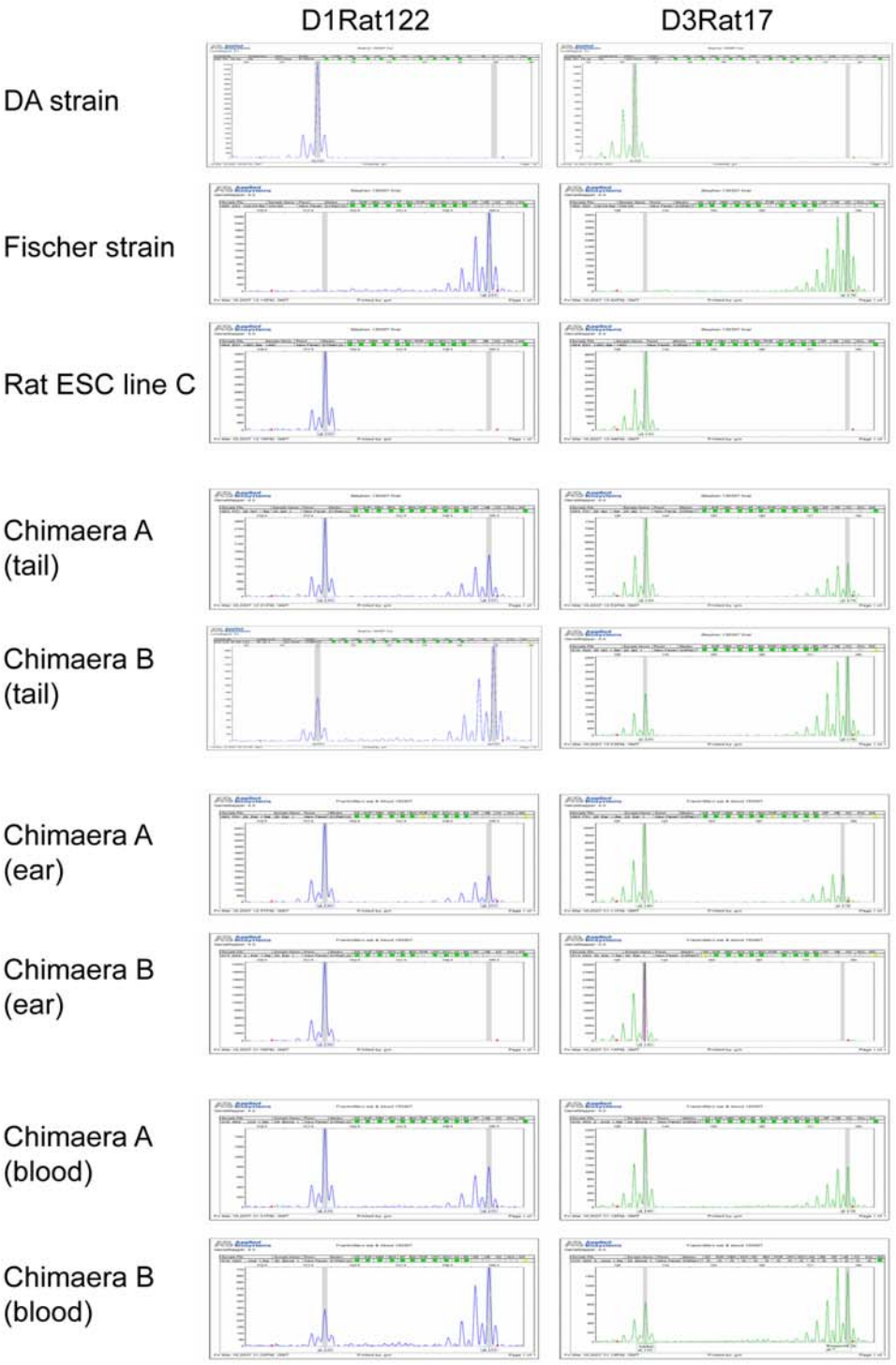


Figure 5.8 Fluorescent-based microsatellite analysis of rat chimaeras. Confirmation of high chimaeric contributions by cultured rat 3i line C using PCR genotyping of rat microsatellite regions D1Rat122 and D3Rat17 from genomic DNA of tail, ear and blood using fluorescent detection methods.

It had previously been demonstrated with mouse ES cells, that both the FGFR inhibitor SU5402 and MEK inhibitor PD184352 could be replaced by a more potent MEK inhibitor PD0325901 (Ying et al., 2008). Inhibition of FGFR is likely to have other downstream effects other than reducing phosphor-Erk levels. This modified culture medium, called 2i, suggests that MEK inhibition is the important downstream effector of FGF signalling necessary for ES cell maintenance. Therefore, lines were derived in 2i from the ICM of Fischer, DA and Sprague Dawley E4.5 rat blastocysts (derivations were carried out by Mia Buehr as described in Chapter 2).

Cell Line	Strain	Sex	Transferred	Born	Term Chimaeras
DA7-GFP	DA	F	90	11	0
DA8-GFP	DA	F	83	10	2M, 1F
DA8	DA	F	15	0	0
DA13	DA	F	21	6	1F
DA15	DA	F	22	11	1M, 1F
SD2	SD	M	37	6	1M

Table 5.4 Summary of rat 2i cell blastocyst injections.

5.6 Characterisation of rat 2i cells

Rat 2i cells are morphologically similar to rat 3i cells and could be maintained in the same way (Figure 5.9A). Immunohistochemical and RT-PCR analysis confirmed expression of Oct4 and Nanog (Figure 5.9B & C).

The capacity of rat 2i cells to contribute to chimaera formation was tested by blastocyst injection (injections were performed by Renee McLay and Jan Ure). Four

lines generated six chimaeras from a total of 163 injected blastocysts (Table 5.4). Three female chimaeras were produced by three independent female DA lines.

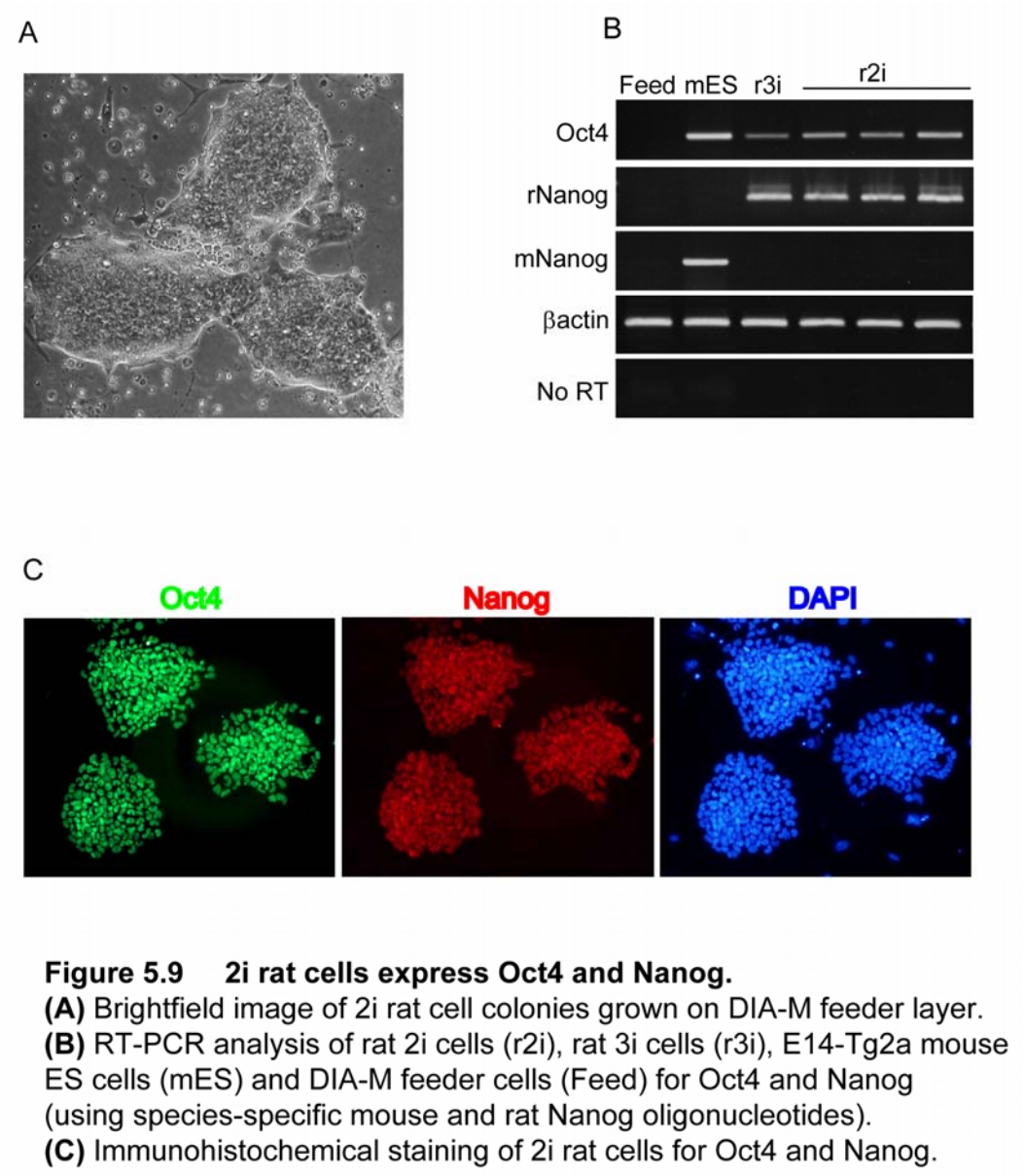


Figure 5.9 2i rat cells express Oct4 and Nanog.
(A) Brightfield image of 2i rat cell colonies grown on DIA-M feeder layer.
(B) RT-PCR analysis of rat 2i cells (r2i), rat 3i cells (r3i), E14-Tg2a mouse ES cells (mES) and DIA-M feeder cells (Feed) for Oct4 and Nanog (using species-specific mouse and rat Nanog oligonucleotides).
(C) Immunohistochemical staining of 2i rat cells for Oct4 and Nanog.

However, germline transmission has not been observed from breeding of these chimaeras. In contrast the male line SD2, isolated from the albino Sprague Dawley (SD) strain, produced one male chimaera, following injection into Dark Agouti (DA)

blastocysts, as determined by coat colour and microsatellite analysis (Figures 5.10A & B). Importantly, when this chimaera was crossed with a Fischer (F344) female it consistently produced litters with albino offspring (Figure 5.10B). Microsatellite analysis confirmed transmission of the ES cell genome to these offspring (Figure 5.10C). Interestingly, the F344 D2Rat250 microsatellite is undetectable in the littermate samples due to preferential amplification of the DA D2Rat250 microsatellite in F344:DA mixed samples (Figure 5.10D).

In summary, these findings demonstrate that rat 2i cells possess the key attributes of ES cells, namely, self-renewal, pluripotency and germline competency.

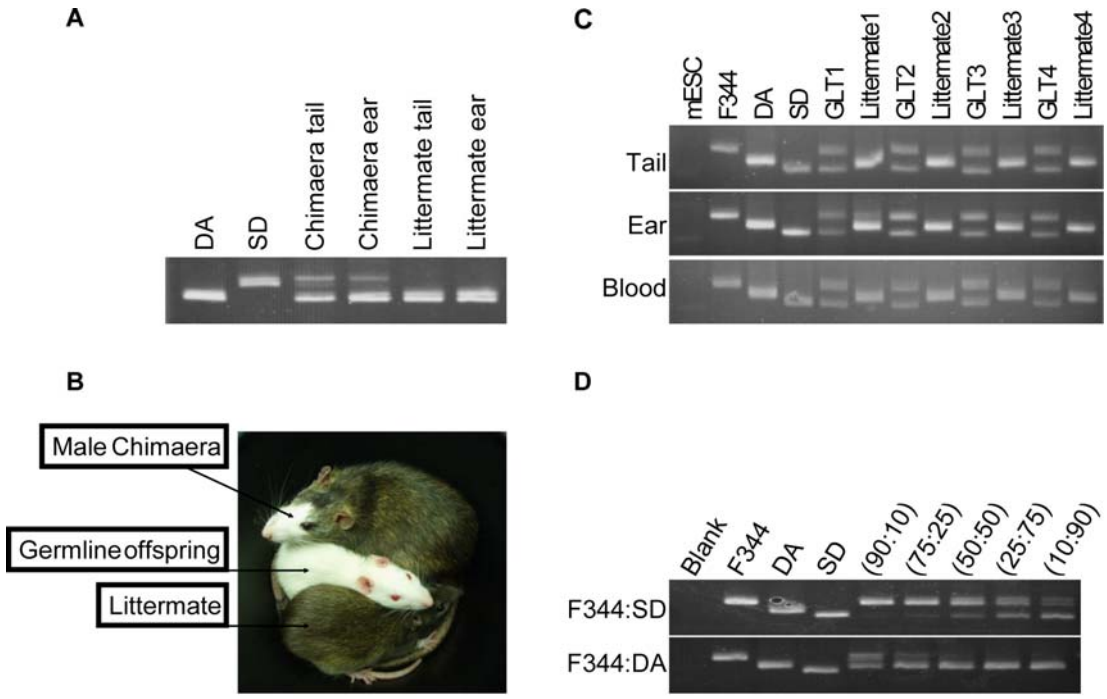


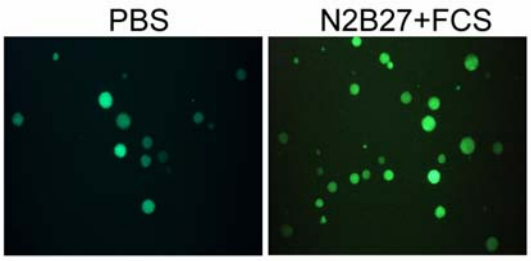
Figure 5.10 Rat 2i cells contribute to chimaeras and germline transmission.
(A) Microsatellite D3Rat17 analysis of tail and ear genomic DNA from chimaera and littermate generated from SD2 2i rat cells.
(B) SD2 2i male chimaera with albino germline offspring and agouti littermate.
(C) Microsatellite D2Rat250 analysis of tail, ear and blood genomic DNA from four germline transmitted albino offspring (GLT1-4) and agouti littermates (1-4).DNAs from mouse ES cells (mESC) and rat strains Fischer (F344), Dark Agouti (DA) and Sprague Dawley (SD) were used as controls.
(D) Microsatellite D2Rat250 titration analysis of mixed genomic DNA samples. In the F344:SD genomic mix, the band intensities are approximately as expected for each ratio. However, in the F344:DA genomic mix the F344 microsatellite is almost undetectable when in equal proportion, indicating that the DA D2Rat250 microsatellite is preferentially amplified in the presence of F344.

5.7 Electroporation of rat 2i cells

The isolation of authentic rat ES cells now provides a valuable tool for introducing genetic modifications into the rat genome and generating rat disease models for detailed phenotypical analysis. The conventional method employed for introducing gene targeting vectors to generate site-specific alterations by homologous recombination is electroporation. In this section I examine the feasibility of generating stable GFP-expressing rat 2i cell clones by electroporation.

Electroporations were performed as described in Chapter 2 with rat 2i cell line DA7 using *Sca*I linearised pPyCAGegfpIP expression vector. The transfection rate of human ES cells was improved when a protein-rich medium was used (Zwaka and Thomson, 2003). Furthermore, the addition of serum improves recovery of rat 3i/2i cells following freezing and lipofection (personal observations). Experiments were therefore performed to determine the effect of N2B27 and serum in the cell suspension medium as well as the effect of increasing DNA concentration. Surprisingly, in all cases there was no significant cell death 24 hours post-electroporation. GFP-positive, puromycin-resistant colonies were counted nine days post-transfection. The inclusion of N2B27 and FCS during the electroporation reaction increased the efficiency by around 40% over PBS (Figures 5.11A & B). There was also an obvious increase in efficiency with increasing concentrations of DNA, such that 50µg produced a stable transfection efficiency of 0.052% (Figure 5.11B). This is higher than that previously reported for human and monkey ES cells (Furuya, Yasuchika et al. 2003; Zwaka and Thomson 2003) and similar to that typically achieved in mouse ES cells.

A

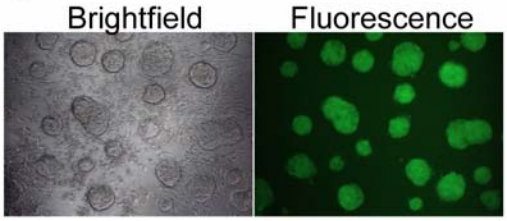


B

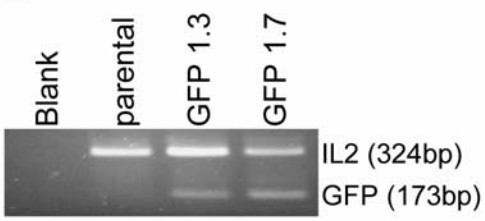
Cell suspension	PBS	N2B27 + FCS
GFP +ve colonies	75 (0.012%)	100 (0.017%)

DNA concentration	5µg	20µg	50µg
GFP +ve colonies	84 (0.014%)	215 (0.036%)	315 (0.052%)

C



D



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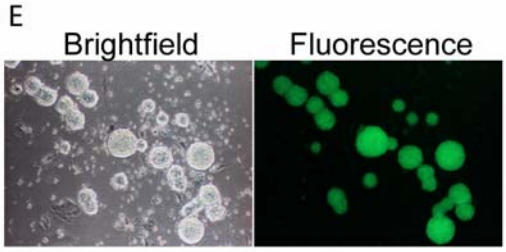

Figure 5.11 Electroporation of rat 2i cells.
(A) Fluorescent images of stable GFP+ve 2i rat cell colonies 7 days post-electroporation. Electroporated in PBS or N2B27+FCS. Magnification x40.
(B) Electroporation efficiency. Number of GFP+ve colonies obtained from $6x10^5$cells 9 days post-electroporation using different suspension media and DNA concentrations.
(C) Brightfield and fluorescent images of single GFP+ve clone 4 days after passaging into 96-well. Magnification x100.
(D) Genomic PCR confirmation of GFP transgene integration.
(E) Recovery of freeze/thawed GFP+ve clone 7 days post-thawing. (x100).

Individual colonies were picked, treated with Accutase™ and plated into DIA-M-coated wells of a 96-well plate (Figure 5.11C). The presence of the transgene was confirmed for two clones by genomic PCR (Figure 5.11D). Expanded clones could be cryopreserved and successfully recovered without loss of GFP expression (Figure 5.11E).

A notable concern of rat 2i cell culture is the tendency for colonies to detach from the feeder layer. This poses a significant practical problem when attempting to isolate individual clones. On this occasion individual colonies were removed by mouth pipette. Whilst this method is acceptable for small numbers of clones, improving cell attachment would allow colonies to be picked by hand pipette facilitating medium-throughput experiments.

5.8 Discussion

Since their derivation almost 30 years ago, it has become clear that the culture conditions established for mouse ES cells cannot be applied successfully to all species, or indeed, all mouse strains. The fact that other strains of mice are recalcitrant to the same culture conditions suggests that permissive mouse strains may be less susceptible to the differentiation cues that exist in such complex formulations.

Recently, the application of small molecule inhibitors of MEK and GSK3 was successful in establishing ES cells from both permissive and non-permissive strains of mice in defined serum- and feeder-free culture conditions (3i/2i). This suggested that inhibition of autoinductive differentiation signals is the limiting factor in capturing the pluripotent preimplantation epiblast cell state *in vitro*, and may be

the common requirement that will facilitate derivation from other species. In this chapter the application of 3i and 2i defined culture conditions to rat ES cell derivation was examined.

Utilising these defined culture conditions, rat cells that possessed the typical characteristics of ES cells could be isolated from the ICM of Fischer, DA and Sprague Dawley (SD) E4.5 embryos. These cells exhibit extensive self-renewal *in vitro* whilst maintaining expression of Oct4 and Nanog and retaining the capacity to differentiate into all three germ layers. Most importantly they could contribute to adult chimaeras and their genome was transmitted through the germline, demonstrating, for the first time, the isolation and expansion of authentic pluripotent, germline competent rat ES cells.

Their ability to generate chimaeras distinguishes rat ES cells from EpiSC (Brons et al., 2007). In addition, unlike EpiSC, rat ES cells express Klf4, Rex1 and Stella and can be routinely passaged by single cell dissociation.

Interestingly, the level of c-Myc transcript is lower for rat ES cells cultured in 3i medium compared with mouse ES cells in serum plus LIF. This was also observed for mouse ES cells cultured in 3i (Ying et al., 2008). Regulation of c-Myc occurs at both the transcriptional and post-translational level. It has been demonstrated that transcriptional regulation of c-Myc by STAT3 and suppression of GSK3-mediated T58 phosphorylation and subsequent degradation of c-Myc may play a role in mouse ES cell self-renewal (Cartwright et al., 2005). Erk is also involved in transcriptional upregulation of c-Myc as well as its post-translational stabilisation via S62 phosphorylation. The low level of c-Myc observed in 3i cultures is likely to be a consequence of phospho-Erk suppression. This suggests that high levels of c-Myc

are not essential for ES cell self-renewal in this defined, serum-free culture. However, CHIR99021 may compensate for the negative effects of Erk, by inhibiting GSK3-mediated degradation of c-Myc, thereby maintaining an appropriate level that may be necessary for maintenance of self-renewal.

Continued blockade of multiple signaling pathways may place unwanted selection pressures upon the cells. Whilst it is clear that blockade of Erk inhibits differentiation and facilitates self-renewal it is unclear what, if any, side effects this may have on the cell. The FGFR inhibitor SU5402 and the weaker MEK inhibitor PD184352 could be replaced with the more potent MEK inhibitor PD0325901. This not only simplifies the culture conditions, but also removes potentially undesirable consequences of FGFR inhibition, such as PI3K blockade. Whilst the capacity for prolonged self-renewal and maintenance of pluripotency are important characteristics, maintaining genomic integrity is essential for ensuring germline transmission (Liu et al., 1997). The capacity for germline transmission has been demonstrated for the rat ES cells isolated here, establishing their value as a research tool.

However, the jaw deformity observed in chimaeras from 2 independent lines, of which one demonstrated a trisomy for chromosome 9 (Buehr et al., 2008), raises concerns over the quality of some lines and the robustness of the culture system. Furthermore, there was also a female sex bias observed in the initial lines derived in 3i (Figure 5.7C). It is unclear whether all of these are genuine female lines or if some represent male lines which have lost their Y chromosome. Concern over germline potency, is also raised by Li *et al*. They have observed an increase in chromosomal

abnormalities with higher passages. Karyotypic analysis of the cells would confirm this.

Examination of birth rates and chimaera rates highlights other concerns (Table 5.5). Only 16-18% of transferred embryos produce liveborn pups. Such low efficiency would only be cost effective should the rate of chimaerism be high. This is not the case here where only 12-16% of the pups born are chimaeras. The work co-published by an independent laboratory, report a similar birth rate (Li et al., 2008). However, in contrast, 77% of their pups were chimaeric. Such differences may be explained by the quality of the cells injected. If the cells are in some way sensitive to these conditions then quality control of culture medium and routine cell maintenance is critical. Routine enzymatic dissociation may also be detrimental to the health of the cells.

Long-term propagation of rat ES cells in the absence of feeders has yet to be demonstrated. This suggests that cell attachment and/or factors generated by feeder cells may be necessary for additional support. Therefore, the type and density of the feeder layer used may contribute to the quality of the ES cells which they support. In the work described here, mouse 10T1/2 fibroblasts expressing matrix-bound LIF were used at a density of $0.75x10^4/cm^2$ whereas Li *et al* used primary mouse embryonic fibroblasts at $5x10^4/cm^2$. The quality of the embryos used for injection may also contribute to the integration of the donor cells.

Li et al demonstrated germline transmission using a donor cell:host embryo combination of DA:Fischer. Interestingly, germline transmission was not observed when the same cell line was injected into SD embryos, despite an equivalent

ISCR 3i

Number of lines	Embryos transferred	Born	Chimaeras (% of born)
3	312	56 (18%)[a]	7 (12%)

ISCR 2i

Number of lines	Embryos transferred	Born	Chimaeras (% of born)
4	231	38 (16%)	6 (16%)

Li, *et al* 3i

Number of lines	Embryos transferred	Born	Chimaeras (% of born)
3	95	13 (14%)	10 (77%)

Table 5.5 Comparison of birth and chimaera rates.
These tables compare the birth rate and chimaera rate between 3i and 2i cells and also between the work performed in this project with that of Li *et al, 2008*. Comparisons were made only between transfers from DA cell lines injected into Fischer blastocysts.

a Six litters were born from transfers into naturally-mated recipients. This figure was adjusted to include only pups born from transferred blastocysts (albino or chimaeric), but not those from the natural mating (agouti). The total born was eighty nine.

percentage of chimaeras being born. This suggests that specific combinations of donor cell and host embryo may be important for efficient germline transmission. However, the combination demonstrating germline transmission here was SD cells into a DA embryo suggesting that more than one combination is suitable. Collectively, this data demonstrates germline transmission of rat ES cells from both inbred (DA) and outbred (SD) rat strains.

Interestingly, no chimaeras were produced by Fischer cell lines, both in this work and in that of Li *et al.* It is possible that DA host embryos do not support efficient integration and development of Fischer-derived cells. However, it is also possible that the cells themselves have restricted potential to integrate or differentiate *in vivo*. Although Fischer cells demonstrated teratoma formation, the adult kidney provides quite a different environment from that of the early embryo. Injection into blastocysts requires the donor cells to respond in the appropriate manner and time alongside host cells. Fischer cells may possess subtle intrinsic differences, which alone or in combination with the culture conditions have an adverse effect on their potential. One possibility is that Fischer ES cells mey be more prone to develop aneuploidy in 2i/3i culture conditions compared with cell lines derived from DA. Detailed karyotypic analysis between several lines from different strains may highlight this.

A more specific problem may arise from continued culture in CHIR99021. During mitosis the chromosomes align on the metaphase plate of the mitotic spindle and the sister chromatids become attached to opposite poles. The sister chromatids are then pulled apart to opposite poles during anaphase. This bipolar orientation ensures equal segregation of genetic material to the daughter cells. The spindle is composed predominately of microtubules. During mitosis microtubules are constantly growing and shrinking. Careful control of such dynamics is necessary to ensure appropriate spindle formation and successful chromosome segregation during cell division (Compton, 2000). It has been demonstrated that GSK3 associates with both the centrosomes and microtubules during spindle formation (Wakefield et al., 2003). Interestingly, spindle-associated GSK3 activity is spatially regulated. Within

the centrosome active PKB phosphorylates and inactivates GSK3 whilst microtubule-associated GSK3 remains unphosphorylated and active. Inhibition of GSK3 using small molecule inhibitors causes spindle defects and chromosome misalignment. Microtuble dynamics are restricted resulting in suppressed chromosome movement and misalignment. This study was supported by others using a panel of GSK3 inhibitors, one of which was CHIR99021 (Tighe et al., 2007). Although the cells used in these experiments were predominantly cancer cell lines, and the concentration of CHIR99021 used was more than three times that used in this project it would be interesting to determine if potent GSK3 inhibition generates genomic instability in rat ES cells.

Furthermore, the ability to genetically manipulate rat ES cells whilst retaining germline potency is essential to be able to create rat transgenics with targeted genetic alterations. However, this first requires that suitable transfection protocols are established and optimized. In answer to this I have demonstrated that rat ES cells are transfectable by both lipofection and electroporation. The requirement for feeder cells complicates gene targeting by limiting the range of selection strategies that can readily be applied. Feeder cells resistant to the relevant drug-selection(s) would first need to be established. Furthermore, contact crossfeeding of toxic metabolites is likely to present a problem between the feeder cells and ES cells which differ in their resistance. Establishing feeder-free culture will further refine the culture conditions and remove technical limitations.

Whilst it is clear that further work is required to establish robust culture conditions, the isolation of authentic rat ES cells provides, for the first time, the necessary platform for precision genetic engineering in the rat, and the prospect of

creating knock-out rat models. Establishing efficient differentiation protocols will provide the necessary tools for drug discovery and cell replacement therapies. It will also be of interest if these defined culture conditions will serve as a universal solution for the isolation of ES cells from multiple species.

5.9 Summary

A defined serum-free culture condition employing small molecule inhibitors of MEK and GSK3 was applied to rat ICM cultures. Lines were established from Fischer, DA and Sprague Dawley rat strains that could be passaged extensively *in vitro* whilst maintaining expression of Oct4 and Nanog. The expression of Rex1, Stella and Klf4, together with the potential to contribute to chimaeric animals, distinguishes these cells from EpiSC. Furthermore, the transmission of the rat 2i cell genome through the germline of chimaeric animals fulfils the key criteria of ES cell identity. However, there are concerns regarding the genomic integrity of these cells. These will only become evident if more cell lines and chimaeras are tested. Further refinement and optimisation of both cell and rat embryo culture conditions could be required to ensure efficient and cost-effective production rat disease models. Nonetheless, this work has, for the first time, succeeded in establishing genuine self-renewing, pluripotent rat ES cells that have the capacity to generate germline competent chimaeras.

Chapter 6

Concluding Remarks

It has been almost 30 years since mouse ES cells were first generated. This discovery has, together with the application of sophisticated genome modification technologies, greatly facilitated the study of gene function and disease research. Despite these benefits, there are occasions where other species would serve as more relevant models, better suited to studies of physiology and other areas of biomedical research. The rat is one such species. Not only does its larger size facilitate experimental analysis and sampling, but it also possesses behavioural and physiological advantages which make it the preferred model of choice for basic research. A significant number of inbred disease models have been generated in rat over the last century which has led to a vast wealth of knowledge that can be applied to any new knockout rats. The generation of rat ES cells would therefore facilitate equivalent applications to rat genetics and provide another, yet more valuable, tool at the disposal of the researcher.

However, efforts to derive rat ES cells have demonstrated only limited success. As discussed earlier in this thesis, rat ES-like cells previously established in this laboratory, self-renew *in vitro* and resemble mouse ES cells morphologically, yet fail to express the key ES cell transcription factor Oct4, or generate chimaeras. The homeodomain transcription factor Nanog also plays an important role in ES cell pluripotency. Nanog is capable of maintaining LIF-independent self-renewal of mouse ES cells *in vitro* whilst *in vivo* it is required for the maturation of the ICM cells into pluripotent epiblast.

The work described in this thesis further demonstrated that whilst the expression pattern of Nanog during early development of the rat embryo was comparable to mouse, expression was absent in rat ExS cells. However, similar to its mouse orthologue, rat Nanog is also capable of facilitating LIF-independent self-renewal. These data suggest that Nanog is likely to play a similar role in the rat, and that its downregulation during derivation, along with that of Oct4, contributes to loss of pluripotency.

It was hypothesised that stably integrating Oct4 and Nanog expression into the ExS cell genome may facilitate in reprogramming these cells into an ES cell pluripotent state. However, despite exogenous expression of Oct4 and Nanog the cells failed to express typical ES cell markers or activate endogenous Oct4 or Nanog. Rather the cells appeared to retain their extraembryonic character. The key trophectoderm transcription factor Cdx2 is highly expressed in rat ExS cells and remains so, despite transgenic expression of Oct4 and Nanog or Sox2.

It has been demonstrated that repression of Oct4, to below 50% of endogenous levels, results in differentiation of mES cells into trophectoderm (TE) cells (Niwa, Miyazaki et al. 2000). This differentiation is also observed upon over-expression of Cdx2, suggesting that a reciprocal inhibition exists between Oct4 and Cdx2 (Niwa, Toyooka et al. 2005). Furthermore, in wild type mouse blastocysts Oct4 and Nanog are confined to the ICM, however in $Cdx2^{-/-}$ blastocysts, both Oct4 and Nanog are mis-expressed in the trophectoderm (Strumpf, Mao et al. 2005) suggesting that Cdx2 may repress Oct4 and Nanog.

Therefore, it was hypothesised that up-regulation of Cdx2 expression during rat cell derivation may be responsible for Oct4 and Nanog down-regulation and the

failure to establish rat ES cells in the presence of serum plus LIF. Therefore, a solution would be to reduce Cdx2 expression by RNA interference-mediated knockdown (Meissner and Jaenisch, 2006). However, because Cdx2 expression in ExS cells is not significantly altered by transgenic Oct4 and Nanog expression, it suggests that the fate of these cells may have become highly restricted early during derivation, and thus application of RNAi-mediated knockdown in established cells may not be sufficient to transform them into an alternative state. Therefore, Cdx2 knockdown may be required during the initial stages of the derivation process. One alternative would be to intervene during the first few days after plating the ICM, a point when Oct4 is still expressed and Cdx2 is likely to be up-regulated. The second alternative would be to induce knock-down in freshly isolated morulae, a point when Oct4 and Nanog are still expressed and prior to exposure to culture medium.

The failure to establish ES cells from a species so closely related to the mouse suggests that subtle intrinsic differences may impose sensitivity to complex, undefined culture conditions such that the pluripotent state of the ICM fails to be captured.

This project concluded by characterising ICM-derived rat cells that had been derived in a recently established and defined culture medium containing inhibitors of MEK and GSK3α/β. This work, for the first time, demonstrated the generation of self-renewing, pluripotent rat ES cells capable of germline transmission. The establishment of germline competent rat ES cells will allow technologies previously optimised in mouse, to be applied to the rat, providing a valuable functional tool to the researcher. As a first step toward this goal this project has successfully demonstrated stable integration of DNA into these rat ES cells by both lipofection

and electroporation. Furthermore, individual colonies could be isolated and expanded in a 96-well format and successfully freeze/thawed. This will facilitate attempts to generate rat genetic models by homologous recombination, making possible the study of gene function by constitutive or conditional gene knock-out. Considerations will also need to be given to the efficiency of gene targeting in rat ES cells. In the long-term future even more sophisticated genomic engineering strategies may be possible such as recombinase-mediated genomic replacement (RMGR) (Wallace, Marques-Kranc et al. 2007). This latter technology allows the replacement of large segments of DNA, of 100kb or more, at specific loci. This facilitates the replacement of a gene and all its regulatory elements in the model organism's genome with the syntenic region of the human genome. This humanisation of the mouse genome generates authentic mouse models of human disease and addresses instances where mutations in the mouse genome do not mimic that of the human condition. The generation of rat ES cells may therefore allow humanisation in a species with a greater scope for phenotypic analysis, and a better understood and more relevant physiology than mouse.

One concern regarding the generation of rat models via ES cells will be the time and cost required for breeding to obtain germline transmission and subsequently homozygosity of gene targeted mutations. This is also a concern with mouse, although, mice become sexually mature at 4-6 weeks, whilst rats take 8-12 weeks. Typically injection of ES cells into blastocyst-stage embryos yield chimaeric F0 animals with only partial contribution from the ES cell line. Assuming the ES cells have contributed to the germ cells these chimaeras can be bred to generate heterozygous F1 progeny, which when intercrossed can produce F2 homozygotes.

This process would therefore take around 6 months from birth of the chimaera to birth of the homozygote. A solution to this is to generate F0 rats that are completely ES cell-derived rather than chimaeric, thus allowing immediate phenotypic analysis without extensive breeding. This relies on the ability to generate developmentally compromised tetraploid embryos that can be generated by electrofusion of two 2-cell stage diploid embryos (Kubiak and Tarkowski, 1985). When mouse ES cells are aggregated with tetraploid embryos, the tetraploid cells fail to contribute to the embryo proper, but rather are restricted to the extra-embryonic lineages whilst the ES cells develop normally (Nagy et al., 1990). This approach was demonstrated to generate fully ES cell-derived F0 mice (Nagy et al., 1993). Recently, F0 mice fully derived from gene targeted ES cells was demonstrated following laser-assisted injection of ES cells into 8-cell embryos (Poueymirou, Auerbach et al. 2007). Unlike tetraploid complementation, this method yields equivalent numbers of healthy mice as traditional blastocyst injection, and thus is better suited to large-scale studies.

Efforts to establish these technologies in rat will be facilitated by the important resources that have been developed in the last decade, such as the sequencing of the rat genome, establishment of the rat genome database and BAC clone resources. At the same time, the generation of authentic rat ES cells will enhance the importance of such resources and hopefully fuel their continued support and development.

It is interesting why rat ES cells could not be derived using traditional mouse ES cell culture conditions. If 3i/2i medium acts by capturing the pluripotent cells that reside within the embryo, then application of this approach may prove successful in isolating ES cells from other species. This assumes that the mechanisms for

establishment and maintenance of pluripotency are conserved between species and that blockade of Erks in other species is sufficient to preserve pluripotency. Furthermore, it needs to be determined, for other species, the exact embryonic stage at which the pluripotent cells exist. By comparison to rodents, formation of the pluripotent cells in larger mammals, for example, may be transient or develop in a different manner which may make isolation with 3i/2i difficult. One limiting factor in assessing the effectiveness of 3i/2i on the derivation of ES cells from larger mammals is the availability of a suitable number of quality embryos. Other factors must also be considered regarding the *in vitro* culture conditions such as O_2 and CO_2 concentrations, pH and temperature, which may be required to be optimised for efficient survival and growth of the cells.

Nonetheless the generation of rat ES cells provides another valuable tool for disease modelling and also presents an opportunity to further investigate the common basis of pluripotency. Comparative analyses of rat and mouse ES cells, as wells as comparison of ES cell lines derived in LIF or 2i culture conditions, may highlight common genes and pathways involved in establishing pluripotency.

Appendix 1

Relevant publication

Buehr, M., Meek, S., Blair, K., Yang, J., Ure, J., Silva, J., McLay, R., Hall, J., Ying, Q. L. and Smith, A. (2008). "Capture of authentic embryonic stem cells from rat blastocysts." Cell **135**(7): 1287-1298.

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