UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

The Human Germ Cell Lineage: Pluripotency, Tumourigenesis and Proliferation

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ABSTRACT

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THE HUMAN GERM CELL LINEAGE: PLURIPOTENCY, TUMOURIGENESIS AND PROLIFERATION

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A pluripotent stem is characterised by indefinite self-renewal in culture, with the retained capacity to form derivatives of all three germ layers of the early embryo: endoderm, ectoderm and mesoderm. There are three clear sources of pluripotent stem cell; embryonic stem cells (ESCs), embryonal carcinoma cells (ECCs) and embryonic germ cells (EGCs). Human (h) ESCs are derived from the inner cell mass (ICM) of the early embryo, whereas hEGCs are derived from primordial germ cells (PGCs), the embryonic precursors of the gametes. hECCs also arise from the germ cell lineage, however, only after malignant transformation when they act as the stem cells of teratocarcinoma. Work described herein used these three human cell sources to investigate the regulators of pluripotency, the transcription factors OCT4, NANOG and SOX2.

OCT4 and NANOG were expressed by hESCs, hECCs and hPGCs/hEGCs. SOX2 was expressed by hESCs and hECCs and detected within mouse (m) PGCs. Surprisingly, SOX2 was absent from hPGCs/EGCs by RT-PCR, expression microarray analysis, immunohistochemistry and immunoblotting. Sox2 is required for mESC growth. Its absence may explain the difficulties of maintaining hEGCs in long-term culture. Consistent with this interpretation, Sox2-positive mEGC cultures are relatively easy to establish and maintain. Interestingly, this paradigm extends to germ cell tumour biology. Seminomas, which do not possess stem cells and resemble undifferentiated germ cells, lack SOX2 expression, whereas embryonal carcinoma specimens are SOX2-positive. This expression pattern is consistent with SOX2 playing important roles in pluripotency, and may indicate a determining role for SOX2 in gonadal tumour type.

Additional work undertaken within this thesis attempted to identify genes which could promote hPGC - and therefore hEGC - proliferation. Comparing gene expression levels by expression microarray and RT-PCR in developing ovary and testis highlighted a number of genes which are more highly expressed in ovary, and which also promote proliferation in other tissues. These data are informing ongoing work aimed at improving hPGC culture/hEGC derivation.

Finally, studies herein have demonstrated that expression of the OCT4-SOX2-NANOG transcription factor complex is not required for expression of target genes identified by biometric analysis. In addition, the potential shortcomings of the use of SSEA3, SSEA4, TRA-1-60 and TRA-1-81 to mark pluripotent cells have been identified. Differentiated cells - neuroprogenitors and stomach epithelial cells - also express these markers. Therefore their use to characterise stem cell lines is limited highlighting the necessity of a combination of functionally relevant markers for characterisation.

This study has unearthed a fundamental difference in gene expression between human and mouse germ cells, demonstrating the limitations of interspecies extrapolation. The data highlight SOX2 as an important transcription factor for further investigation in attempts to understand the relationship between human PGCs, GCTs, and the derivation, self-renewal, and pluripotency of human EGCs. The information is also instructive in attempts to generate human gametes from stem cell sources for ambitious fertility treatments.

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Abbreviations

ACF, APOBEC1 complement factor

AEG, anophthalmia-esophageal-genital

Akp2, alkaline phosphatase 2

AP, alkaline phosphatase

bHLH, basic-helix-loop-helix

BMP, bone morphogenetic protein

Brn2, brain 2

cAMP, cyclic adenosine monophosphate

CC, choriocarcinoma

CCKBR, cholecystokinin B receptor

ChIP, chromatin immunoprecipitation

CIS, carcinoma *in situ*

CK19, cytokeratin 19

CLC, cardiotrophin-like cytokine

CMV, cytomegalovirus

CNS, central nervous system

CNTF, ciliary neurotrophic receptor

CPA2, carboxypeptidase A2

CS, Carnegie Stage

CSF3, colony stimulating factor 3

Cx43, connexin 43

Cxcr4b, chemokine (C-X-C motif), receptor 4b

DAB, diaminobenzidine

DAPI, 4',6-diamidino-2-phenylindole

Dazl, deleted in azoospermia-like

ddH_2O, distilled deionised H_2O

Ddx, dead (Asp-Glu-Ala-Asp) box

DEPC, diethylpyrocarbonate

dhh, desert hedgehog

DMEM, Dulbecco's Modified Eagle Medium

DMSO, dimethyl sulphoxide

DNase I, deoxyribonuclease I

Dnd1, dead-end 1

dNTPs, deoxynucleotide triphosphate

dpc, days post-conception

dpp, decapentaplegic

DPPA4, developmental pluripotency associated 4

DTT, dithiothreitol

E, embryonic day of mouse development

EB, embryoid body

ECC, embryonal carcinoma cell

EGC, embryonic germ cell

EGF, epidermal growth factor

EMA1, embryonic mouse antigen 1

ESC, embryonic stem cell

Esg1, embryonal stem cell gene 1

Esrrb, estrogen related receptor β

Fancc, Fanconi anaemia complementation group C

Fbx15, F-box only protein 15

FCS, fetal calf serum

FGF, fibroblast growth factor

FISH, fluorescent *in situ* hybridisation

FITC, fluorescein isothiocyanate

FOXD3, forkhead box D3

FRAP1, FK506 binding protein 12-rapamycin associated protein 1

FRE, FGF response element

GAS11, growth arrest-specific 11

gbb, glass bottom boat

gcd, germ cell deficient

GFAP, glial fibrillary acid protein

GFP, green fluorescent protein

GI, gastrointestinal

gp130, glycoprotein 130

GRM4, glutamate receptor, metabotropic 4

GSK3, glycogen synthase kinase-3

H, human

H & E, haematoxylin and eosin

HBSS, Hank's Balanced Salt Solution

HMG, high mobility group

HPRT, hypoxanthine-guanine phosphoribosyl-transferase

HRP, horse radish peroxidase

HS, heparan sulphate

ICM, inner cell mass

Ifitm, interferon induced transmembrane protein

IGFII, insulin-like growth factor-II

IHC, immunohistochemistry

iPS cells, induced pluripotent stem cells

IL6, interleukin 6

IL6R, IL6 receptor

IL6ST, IL6 signal transducer

ISH, *in situ* hybridisation

ITGCN, intratubular germ cell neoplasia

Ki67, proliferation-related Ki67 antigen

Klf, Krüppel-like factor

LB medium, Lysogeny broth/Luria Bertani medium/Luria broth

LHX2, LIM homeobox protein 2

LIF, leukaemia inhibitory factor

LIFR, LIF receptor

LRH1, liver receptor homologue 1

M, mouse

MAPK/ERK, mitogen-activated protein kinase kinase/extracellular regulated kinase

miRNA, micro-RNA

mRNA, messenger RNA

Mvh, mouse *Vasa* homologue

NPY, neuropeptide Y

NPYR1, NPY receptor 1

OPC, oligodendrocyte precursor cells

OSM, oncostatin M

PACAP, pituitary adenylate cyclase-activating peptide

PAX, paired box

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PFA, paraformaldehyde

PGC, primordial germ cell

PI3K, phosphoinositide-3 kinase

PLAP, placental alkaline phosphatase

pMEF, primary mouse embryonic fibroblasts

pog, proliferation of germ cells

PolyA, polyadenylation

POU, Pit-Oct-Unc

PTEN, phosphatase and tensin homologue

REST, RE1-silencing transcription factor

Rex1, reduced expression-1

RISC, RNA-induced silencing complex

RNA, ribonucleic acid

RNase, ribonuclease

rRNA, ribosomal RNA

RT-PCR, reverse transcriptase polymerase chain reaction

SA, streptavidin

Sall4, sal-like 4

SCF, stem cell factor

SCO, Sertoli cell-only

Scp3, synaptonemal complex protein 3

SCYA20, small inducible cytokine subfamily A, member 20

Sdf1, stromal-cell derived factor 1

SEM, seminoma

SER, Sertoli

SF1, steroidogenic factor 1

siRNA, small interfering RNA

Smad, small mothers against decapentaplegic

SOB, super optimal broth

SOX2, SRY-like homeobox gene 2

SPC, spermatocyte

SPG, spermatogonia

SPT, spermatid

SRY, Sex determining Region of the Y chromosome

SSEA, stage-specific embryonic antigen 1

STAT, signal transducers and activators of transcription

Stra8, stimulated by retinoic acid gene 8

Tcf3, T-cell factor 3

Tcf8, transcription factor 8

TE, trophectoderm

TER, teratoma

TESPA, 3-aminopropyltriethoxysilane

TFAP2C, transcription factor activator protein-2C

TGCT, testicular germ cell tumour

TGF, transforming growth factor

Tiar, T-cell restricted intracellular antigen-related protein

TIN, testicular intraepithelial neoplasia

TNAP, tissue non-specific alkaline phosphatase

TNFα, tumour necrosis factor α

TR, Texas red

Utf1, undifferentiated embryonic cell transcription factor 1

UTR, untranslated region

Vrk2, vaccinia virus-related protein kinase 2

WNT, wingless-type mammary tumour virus integration site

wpc, weeks post-conception

YST, yolk sac tumour

Zfp, zinc finger protein

Zic3, zinc finger protein of the cerebellum 3

CHAPTER 1: Introduction

1.1 Pluripotent Stem Cells

A pluripotent stem cell is defined according to two characteristics: being able to reproduce indefinitely (undergo self-renewal); and to differentiate into any of the three germ layers of the embryo (so-called 'pluripotency') (Weissman 2000). The existence of stem cells was first demonstrated in 1963 (Becker *et al.* 1963; Siminovitch *et al.* 1963), and they can be derived from embryonic, fetal or adult tissues.

Of all three types, it appears that embryonic stem cells have the greatest differentiation capability (Verfaillie *et al.* 2002), although some fetal stem cells can differentiate into cells of a lineage other than that from which they were originally derived, a property termed 'transdifferentiation' or 'plasticity'. For example, Suzuki *et al.* (2002) isolated cells from mouse fetal liver that could be clonally expanded and could reconstitute liver and biliary epithelial cells and also epithelium of pancreas and the gastrointestinal (GI) tract. However, generating enough cells for therapeutic use in the pure form is difficult, reservations exist regarding collection and use of tissues from abortions and the problem of immune rejection needs to be overcome (O'Donoghue and Fisk 2004).

Of adult stem cells, those derived from the bone marrow (haematopoietic and mesenchymal stem cells [HSCs and MSCs, respectively]) show the greatest degree of plasticity. HSCs can generate all mature blood cell types of the haematolymphatic system, and in numerous studies have been shown to differentiate into cells of other lineages (reviewed in Bellantuono 2004). MSCs can differentiate into mesenchymal derivatives, as well as cells of other lineages (reviewed in Kemp *et al.* 2005). However, the numbers of MSCs obtained from bone marrow are small; 0.001–0.01% of mononuclear cells isolated from a ficoll density gradient of bone marrow aspirate are MSCs (Pittenger *et al.* 1999). Cells can be amplified *in vitro*, however spontaneous transformation of adult MSCs after long-term culture has been reported (Rubio *et al.* 2005). Therefore, whilst adult stem cells do offer some potential, and circumvent the issue of immunorejection, limitations remain: restricted differentiation potential and the inability to obtain large amounts of pure cells for transplantation.

Stem cells derived from the embryo overcome some of these restrictions as unlimited amounts can be grown in culture, and they are unequivocally able to differentiate to derivatives of all three germ layers. Three pluripotent cell types can be derived from the embryo: embryonic stem cells (ESCs), embryonic germ cells (EGCs) and embryonal carcinoma cells (ECCs) (Figure 1.1).

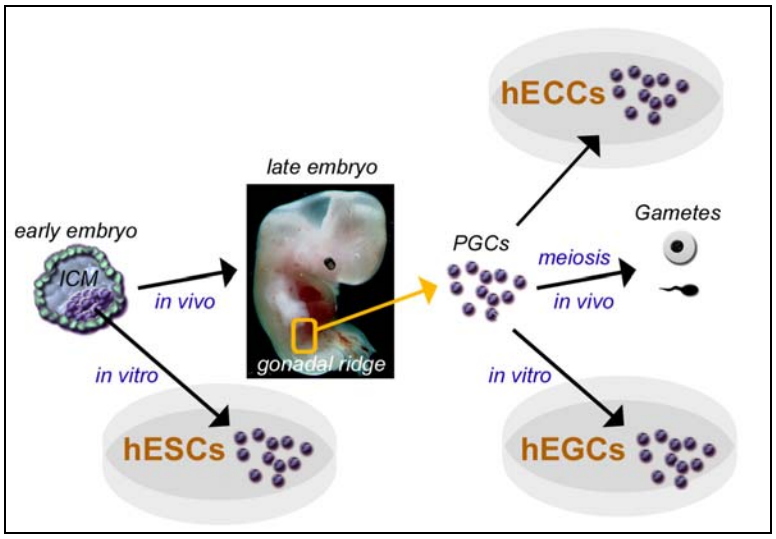


Figure 1.1 Pluripotent cells which can be derived from the human embryo
Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of the blastocyst, human embryonic germ cells (hEGCs) are derived from the precursors of the gametes, primordial germ cells (PGCs), and human embryonal carcinoma cells (ECCs) are transformed PGCs. [Reproduced from Turnpenny *et al.* (2006).]

In order to understand the origin and biology of these cells, it is first necessary to briefly review human embryonic development and differentiation.

1.2 Human Embryogenesis

Human embryogenesis (Figure 1.2) begins with fertilisation of an egg by a sperm, forming a zygote, which then undergoes a series of rapid divisions, known as cleavage, generating cells termed blastomeres. After the 8-cell stage, these cells undergo 'compaction', in which they bind tightly to each other, forming a compact sphere. The cells continue to divide, and, at the 16-cell stage, the embryo is termed a morula. The outer layer of cells, the trophoblast, secrete water into the morula, forming a central, fluid-filled cavity (blastocoel); a process termed cavitation. The outer layer of the embryo, the zona pellucia, begins to degenerate, and the embryo increases in volume. At this stage, four to six days after fertilisation, the embryo is termed a blastocyst. Cells then make their first cell fate 'decision', either becoming part of the inner cell mass (ICM), which will ultimately form the embryo, or part of the outer trophoblast, which will form the placenta. The ICM is composed of the epiblast, which will give rise to the embryo proper, and the hypoblast which will give rise to extraembryonic structures only, such as the lining of the yolk sac. Cells from the ICM are pluripotent; they can form cells of all three germ layers

and the germ line of the developing embryo, but they cannot give rise to the placenta and supporting tissues (Figure 1.2). Cells derived from the zygote and the first 2 to 16 blastomeres have the greatest developmental potential; these cells are termed totipotent as they can form both the embryo and the trophoblast of the placenta.

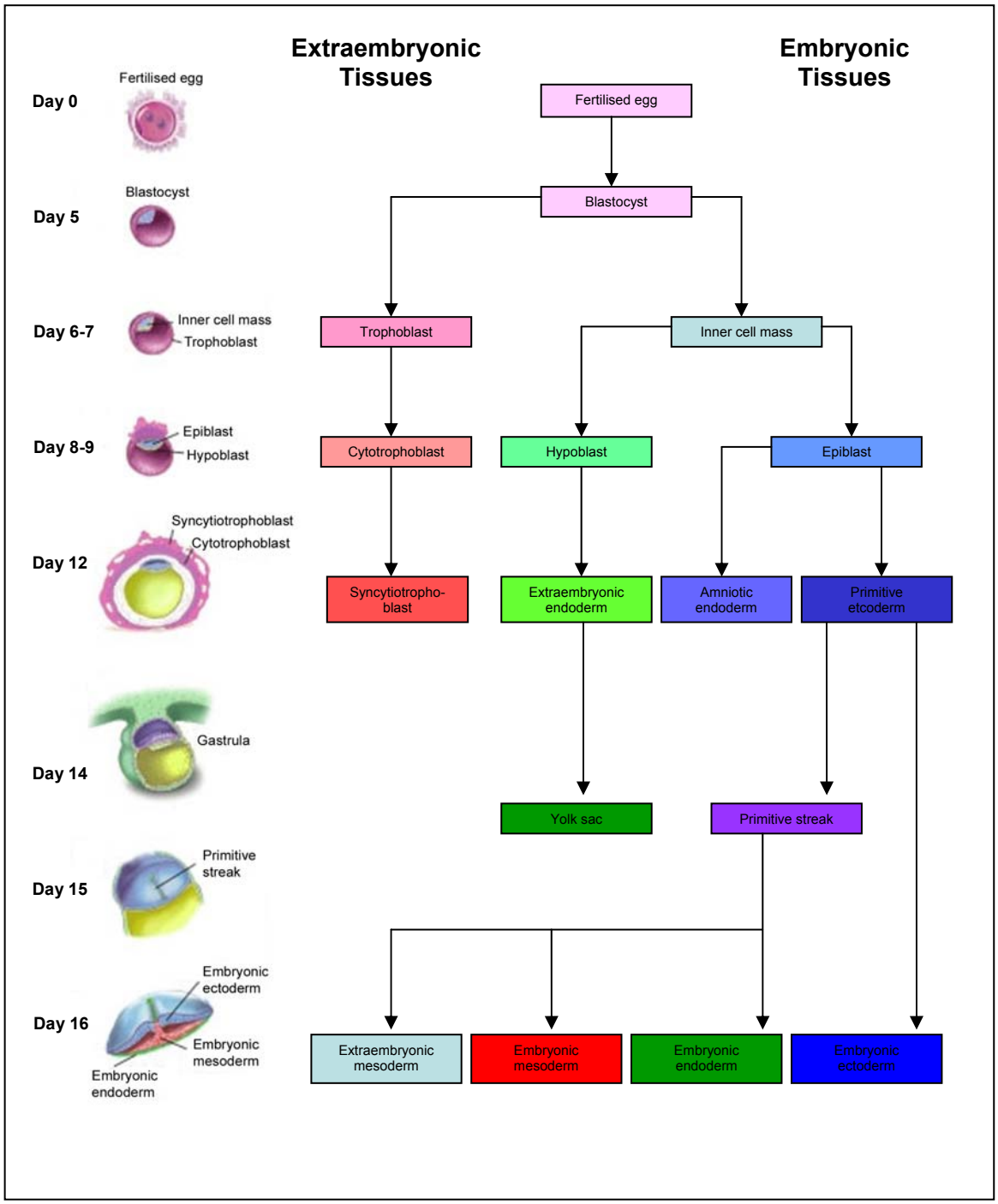


Figure 1.2 Development of the human embryo

[Adapted from National Centre for Biotechnology Information (NCBI) website: http://www.ncbi.nih.gov/]

The germ layers of the embryo (Figure 1.2 and 1.3) comprise the ectoderm, which will ultimately form cells of the skin and nervous system; the mesoderm, which will form the bones, most of the circulatory system, muscle, reproductive and urinary systems; and the endoderm, which will form the respiratory tracts and GI system, including the liver and pancreas. These layers arise during a process termed gastrulation.

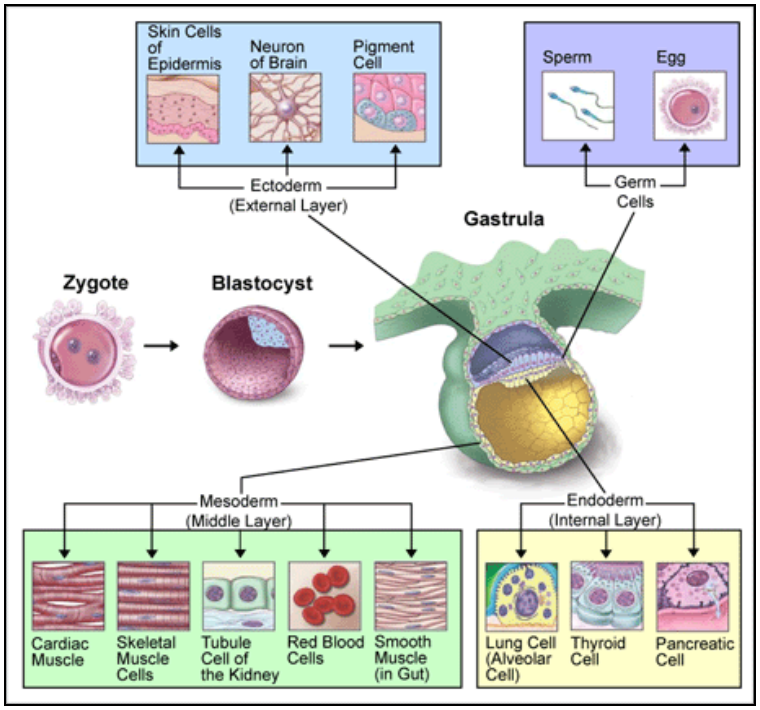


Figure 1.3 The three germ layers of the embryo, and the germline

[Reproduced from National Centre for Biotechnology Information (NCBI) website: http://www.ncbi.nih.gov/]

1.3 Embryonic Stem Cells

ESCs are, chronologically, the first stem cell that can be derived from the embryo (from the ICM). Mouse ESCs (mESCs) were first derived independently in 1981 by two groups (Evans and Kaufman 1981; Martin 1981). Evans and Kaufman used a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts (pMEFs) to derive mESCs from blastocysts, whereas Martin used conditioned medium from another pluripotent stem cell, embryonal carcinoma cells (ECCs; Section 1.5). To isolate ICM cells from a blastocyst, the trophectoderm (TE) is removed by immunosurgery, and the ICM plated onto a feeder layer of pMEFs. The cells attach and divide, and the outgrowth is disaggregated and plated onto another feeder layer. Colonies with an undifferentiated

morphology are individually dissociated and re-plated. These form colonies and are dissociated in the same way, generating ESC lines. They fulfill all criteria for pluripotency (Table 1.1). When injected into immunocompromised mice they form teratomas containing cells of the three germ layers, and when introduced into blastocysts, chimaeric mice are generated demonstrating that they can contribute to all cell lineages including the germ line (Bradley *et al.* 1984). They are pluripotent *in vitro* (Evans and Kaufman 1981; Wobus *et al.* 1984; Doetschman *et al.* 1985) and can develop into cells of the germ line (Hubner *et al.* 2003; Toyooka *et al.* 2003; Geijsen *et al.* 2004). Following a similar procedure to that for mESC derivation, Thomson *et al.* (1998) derived human ESCs (hESCs) from *in vitro* fertilised embryos. Again, these lines remained undifferentiated *in vitro*, and were able to form derivatives of all three embryonic germ layers.

Clonally derived cultures are capable of spontaneous differentiation into derivatives of all three germ layers *in vivo* (teratomas) or *in vitro*
When injected into blastocysts, contribute to all cell lineages in chimaeric mice, including the germ line
Maintain normal karyotype
Immortal and propagate indefinitely in the embryonic state

Table 1.1 Criteria for pluripotent stem cells

1.4 Embryonic Germ Cells

EGCs are derived from primordial germ cells (PGCs), the precursors of the gametes, and are defined as PGCs which continue to proliferate *in vitro* beyond the time point at which they would cease to do so *in vivo*. During embryonic development, PGCs migrate to the genital ridges of the developing gonads. If the gonads are dissected, and plated onto MEFs, these PGCs sometimes develop into EGCs. These were also first derived from the mouse, independently by two groups (Matsui *et al.* 1992; Resnick *et al.* 1992), and subsequently from the human (Shamblott *et al.* 1998). Whilst mEGCs unequivocally fulfill all criteria for pluripotency, hEGCs are somewhat less convincing.

1.5 Embryonal Carcinoma Cells

ECCs are transformed PGCs, originally isolated from mouse germ cell tumours (GCTs) (Kahan and Ephrussi 1970). These tumours occur spontaneously in the testes of mice (and men) and can be divided into two groups based on the degree and type of differentiation: seminomas, which are relatively uniform and resemble undifferentiated PGCs, and the more aggressive nonseminomas, which as well as containing an undifferentiated component also show varying degrees of embryonic and extraembryonic patterns of differentiation (Ulbright 1993; Chaganti and Houldsworth 2000). The nonseminomas are further subdivided into teratoma, embryonal carcinoma, teratocarcinoma, yolk sac carcinoma, and choriocarcinoma. Table 1.2 summarises the range of manifestations of GCTs (Andrews *et al.* 2001).

Histological type	Description	Comments
Teratoma	A tumour containing an array of differentiated somatic cell types.	The differentiated cells may be organised into well recognisable anatomical structures (e.g. teeth) or be haphazardly arranged. Often benign, but their malignant potential is well known. Most common form is benign 'dermoid cyst' of the ovary.
Embryonal carcinoma	'Undifferentiated' epithelial cells resembling embryonic cells of the ICM and the primitive ectoderm.	Highly malignant tumours; cells are regarded as stem cells with the ability to differentiate into a range of histological cell types.
Teratocarcinoma	Contains both teratoma and embryonal carcinoma.	Malignancy is due to the embryonal carcinoma component.
Yolk sac carcinoma	Cells resemble those of the extraembryonic 'yolk sac'.	In mouse may resemble parietal or visceral yolk sac; in humans no such clear distinction is evident.
Choriocarcinoma	Cells resemble cyto- and syncytiotrophoblast of the placenta.	Do not occur in GCTs of the laboratory mouse.
Seminoma	Relatively uniform cells resembling primordial germ cells.	A malignancy tumour that does not occur in the laboratory mouse; known as dysgerminoma in females.
Spermatocytic seminoma	Heterogeneous cells resembling a caricature of spermatogenesis.	A low malignancy tumour occurring in older men, and generally regarded as distinct from all other GCTs.

Table 1.2 Classification of germ cell tumours in laboratory mice and men
These classifications, while oversimplifications to experienced pathologists, represent common usage by biologists. [Adapted from Andrews *et al.* (2001).]

In humans, GCTs account for almost all testicular tumours and are always malignant. They typically occur in young post-pubertal men, with a peak incidence in the

third decade of life (Moller 1993). They appear to arise from a common precursor, the carcinoma *in situ* (CIS) cell within the seminiferous tubules (Skakkebaek 1972). Figure 1.4 depicts the varying morphology of GCTs.

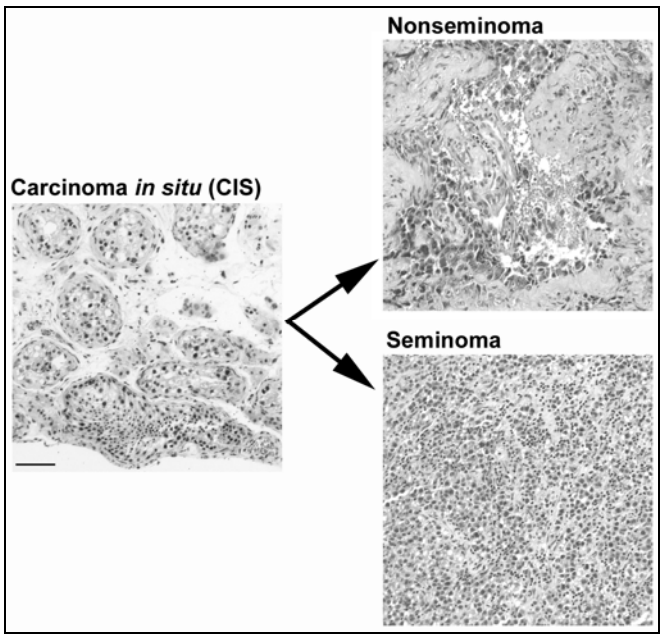


Figure 1.4 Testicular germ cell tumour morphology

Histological appearance of carcinoma *in situ*, nonseminoma (EC) and seminoma. The uniform undifferentiated appearance of the seminoma contrasts markedly with the differentiated cells within the nonseminoma. Size bar represents 100 μm.

Mouse ECCs are the progenitors of the differentiated elements of GCTs, i.e. they are the stem cells of the tumours (Kleinsmith and Pierce 1964). These cells can be expanded continuously *in vitro* in the undifferentiated state. They differentiate both *in vitro* and *in vivo*: the cells form benign teratomas or malignant teratocarcinomas when transplanted into extra-uterine sites of mice (reviewed in Andrews 1998); and when introduced into the ICM of early mouse embryos, some lines participate in embryonic development and contribute to a wide variety of tissues in the resulting chimaeric fetuses and mice (Brinster 1974). Therefore, ECCs fulfill all criteria for self-renewing, pluripotent stem cells (Table 1.1). Unlike mECCs, many established hECC lines show poor differentiation potential, with some being nullipotent, and almost all are aneuploid (Damjanov 1990; Harrison *et al.* 2007).

Gene expression patterns for hESCs, hECCs and GCTs are similar, indicating commonality in the mechanisms for maintaining proliferation and pluripotency in all (Sperger *et al.* 2003).

1.6 Characterisation of Pluripotent Cells: Surface Markers

Expression of particular cell surface antigens, namely stage-specific embryonic antigen 1 (SSEA1), SSEA3 and SSEA4, and TRA-1-60 and TRA-1-81[1], is often used to assist in confirming pluripotency. The experiments that led to the discovery of these surface antigens involved the immunisation of mice with syngeneic embryonic cells, generating antisera that recognised antigens on the embryo but not the adult (Artzt *et al.* 1973). TRA-1-60 and TRA-1-81 were identified as recognising distinct cell surface antigens on hECCs (Andrews *et al.* 1984). The antigenic determinant of the monoclonal antibody embryonic mouse antigen 1 (EMA1) shows a similar expression profile to SSEA1, on mESCs and EGCs (Hahnel and Eddy 1987). The surface antigens expressed differ between human and mouse ESCs, EGCs and EGCs (Table 1.3).

Marker	mESC	mECC	mEGC	hESC	hECC	hEGC	Comments
Alkaline phosphatase (AP)	Yes	Yes	Yes	Yes	Yes	Yes	Alkaline phosphatase activity
SSEA1	Yes	Yes	Yes	No	No	Yes	Lactoseries glycolipid; frequently appears during differentiation of hESCs/ECCs
EMA1	Yes	Yes	Yes	No	No	Yes	May recognise different parts or prefer different conformations of the same carbohydrate chain as SSEA1
SSEA3	No	No	No	Yes	Yes	Variable[2]	Globoseries glycolipid; down-regulated rapidly during differentiation of hESCs/ECCs
SSEA4	No	No	No	Yes	Yes	Yes	Globoseries glycolipid
TRA-1-60	No	No	No	Yes	Yes	Yes	Keratan sulphate (antibody recognises same epitope as GCTM2)
TRA-1-81	No	No	No	Yes	Yes	Yes	Keratan sulphate
GCTM2	No	No	No	Yes	Yes	Yes	Keratan sulphate

Table 1.3 Differential expression of characteristic surface marker antigens on mouse and human ES, EC and EG cells

[Adapted from Draper and Andrews (2004) and Turnpenny *et al.* (2006).]

[1] Named after the Battle of Trafalgar, not Tumour-Related/Rejection Antigen as sometimes stated (P. Andrews; pers. comm.).

[2] Detected weakly and inconsistently by some [Shamblott *et al.* (1998, 2004), Liu *et al.* (2004)] but not by others [Turnpenny *et al.* (2003, 2005), Pan *et al.* (2005)].

1.7 Maintenance of Pluripotency

The crucial molecules involved in pluripotency have been determined through studies in ESCs in the main, although much of this work was led by original discoveries that had been made in ECCs. Mouse and human ESC pluripotency is maintained by a network of transcription factors, of which OCT4 (also known as OCT3 or POU5F1[3]), SOX2 and NANOG are at the helm (Boyer *et al.* 2005; Loh *et al.* 2006). Homozygous knock-out of either *Oct4*, *Sox2* or *Nanog* in the mouse is embryonic-lethal, with no pluripotent epiblast being formed in either case (Nichols *et al.* 1998; Avilion *et al.* 2003; Mitsui *et al.* 2003).

1.7.1 OCT4

OCT4 is the most extensively studied of all the transcription factors involved in maintaining pluripotency. A member of the POU (Pit-Oct-Unc)[4] family of transcription factors, it binds to the octamer motif ATGC(A/T)AAT found in the regulatory domains of some genes. It is the earliest expressed gene known to encode a transcription factor which is developmentally regulated during mammalian embryogenesis. In the mouse, zygotic *Oct4* expression is activated at the four-cell stage (Yeom *et al.* 1991). Following cavitation, human and mouse expression is limited to the ICM and it is down-regulated in the differentiated TE (Okamoto *et al.* 1990; Rosner *et al.* 1990; Scholer *et al.* 1990; Hansis *et al.* 2000). Following implantation, expression is restricted to the epiblast (Yeom *et al.* 1996), and after gastrulation, PGCs are the only cells which retain *Oct4* expression (Ginsburg *et al.* 1990). *Oct4* is also expressed by pluripotent mouse and human ESCs, ECCs and EGCs (Pera *et al.* 1989; Okamoto *et al.* 1990; Rosner *et al.* 1990; Labosky *et al.* 1994; Yeom *et al.* 1996; Shamblott *et al.* 1998; Reubinoff *et al.* 2000). It is down-regulated upon differentiation (Scholer *et al.* 1989; Okamoto *et al.* 1990; Rosner *et al.* 1990; Brandenberger *et al.* 2004). *Oct4*-deficient mouse embryos die before implantation; the embryos develop to the blastocyst stage with normal cell numbers in the ICM; however, these cells can only form trophoblast cells (Nichols *et al.* 1998). Therefore, Oct4 is essential for the establishment of a pluripotent ICM.

[3] According to the The GDB Human Genome Database (http://www.gdb.org/), OCT3/4 should be referred to as POU5F1. However, this thesis uses the more familiar term, OCT4.
[4] Contain a POU domain, which was originally identified as a region of approximately 150 amino acids shared between the Pit-1, Oct-1, Oct-2, and Unc-86 transcription factors, hence the name 'POU'.

The exact level of *Oct4* expression is crucial in determining cell fate (Niwa *et al.* 2000): a decrease of 50% compared to endogenous level commits mESCs to the TE lineage; whereas a less than two-fold increase in expression causes differentiation into primitive endoderm and mesoderm. Oct4 prevents mESCs from differentiating into TE by blocking the expression and activity of the transcription factor Cdx2 (Niwa *et al.* 2005). Sustained expression of *Oct4* promotes neuronal differentiation of mESCs (Shimozaki *et al.* 2003), and high levels of *Oct4* expression were detected in primitive endoderm of the ICM at embryonic day (E) 4.5 (Palmieri *et al.* 1994). Therefore Oct4 can be regarded as a three-way switch to determine three different cell states – pluripotency, primitive endoderm and TE – in a dose-dependent manner (Niwa 2001).

1.7.2 SOX2

OCT4 often works in concert with another transcription factor, SOX2, a member of the SOX (Sry-related high mobility group [HMG] box containing) DNA-binding protein family. Proteins are grouped into the SOX protein family if they contain an HMG domain with strong amino acid similarity (usually greater than 50%) to the HMG domain of Sry (Sex determining region of the Y chromosome). SOX proteins bind DNA through this 79 amino acid HMG domain. In contrast to most DNA-binding proteins which bind to the major groove of DNA, the HMG box interacts with the minor groove, thereby introducing a dramatic bend in the DNA molecule. There are eight subgroups of SOX proteins, A-H, based on sequence similarity and genomic organisation (Schepers *et al.* 2002). Members within the same subgroup usually share greater than 80% amino acid identity within the HMG domain. SOX2 belongs to the SOXB subgroup, which comprises SOX1, 3, 14 and 21. SOX1-3 are transcriptional activators (SOXB1 subgroup), and SOX14 and 21 are transcriptional repressors (SOXB2 subgroup) (Uchikawa *et al.* 1999). The activator/repressor domain is found at the C-terminal of the protein.

Mouse *Sox2* expression is first detected at the morula stages, and in blastocysts it is specifically located within the ICM. It is expressed throughout the epiblast, but its expression becomes restricted during gastrulation. By E9.5, it is expressed throughout the brain, spinal cord, sensory placodes, branchial arches and gut endoderm, and in male and female germ cells (Avilion *et al.* 2003; Western *et al.* 2005). *Sox2*-deficient embryos fail to survive, dying shortly after implantation, at E6.0, *Sox2*-null ICMs contain only TE and extraembryonic endoderm, and *Sox2*-null mESCs cannot be derived. In addition, reduction

of *SOX2* expression in hESCs and mESCs results in a loss of the undifferentiated state and increased expression of TE markers (Chew *et al.* 2005; Fong *et al.* 2008). Small increases (two-fold or less) in Sox2 protein trigger the differentiation of mESCs (Kopp *et al.* 2008) and inhibit the expression of Sox2-Oct4 target genes (Boer *et al.* 2007).

Sox2 and Oct4 cooperatively activate *Fibroblast growth factor 4 (Fgf4)* transcription (Yuan *et al.* 1995); however *Fgf4* is expressed in *Sox2*-null embryos, which cannot be rescued by exogenous Fgf4 (Avilion *et al.* 2003). This indicates that *Fgf4* is not the crucial target gene of Sox2 in maintaining the pluripotent ICM. Maternal Sox2 protein persists throughout preimplantation development; it accumulates in the cytoplasm of growing oocytes and is present in all cells until at least the blastocyst stage (Avilion *et al.* 2003). This maternal Sox2 could account for *Fgf4* expression in the homozygous blastocysts, and may delay lethality until after implantation, when maternal Sox2 protein is diluted out due to growth of the embryo.

Both Sox2 and Oct4 are therefore required for the ICM epiblast lineage, and in their absence TE is formed. It is proposed that they cooperatively act on a number of target genes to maintain pluripotency within the epiblast.

1.7.2(i) SOX2/OCT4 Target Genes

Sox2 and Oct4 activate *Fgf4* transcription by forming a complex and binding to a distal enhancer located in the 3' untranslated region (UTR) of the *Fgf4* gene (Yuan *et al.* 1995). Sox2/Oct4 also cooperatively activate transcription of genes for the transcription factors *Undifferentiated embryonic cell transcription factor 1 (Utf1)* (Nishimoto *et al.* 1999; Nishimoto *et al.* 2005), *F-box only protein 15 (Fbx15)* (Tokuzawa *et al.* 2003), *Nanog* (Kuroda *et al.* 2005; Rodda *et al.* 2005), *Zinc finger protein 206 (Zfp206)* (Wang *et al.* 2007), *Developmental pluripotency associated 4 (Dppa4)* (Chakravarthy *et al.* 2008) and *Oct4* and *Sox2* themselves (Tomioka *et al.* 2002; Chew *et al.* 2005; Okumura-Nakanishi *et al.* 2005). Excluding *Fbx15*, whose function is unknown, these genes have all been demonstrated to function in ESCs, as detailed below:

- Fgf4 is involved in the patterning of the extraembryonic ectoderm by stimulating the proliferation of TE cells (Wilder *et al.* 1997; Tanaka *et al.* 1998). *Fgf4*-null embryos cannot form an egg cylinder and die soon after implantation. When these

embryos were cultured *in vitro,* proliferation of the ICM was severely impaired; growth and differentiation were rescued with exogenous Fgf4 (Feldman *et al.* 1995). However, *Fgf4*-deficient ESCs proliferate, suggesting Fgf4 is not required for ESC proliferation (Wilder *et al.* 1997).

- Utf1 is involved in the initiation of ESC differentiation (van den Boom *et al.* 2007); it is not involved in self-renewal or pluripotency *per se*, but appears to promote teratoma formation and proliferation (Nishimoto *et al.* 2005).
- The expression profile of *Fbx15* in mice is nearly identical to that of *Oct4*, restricted to ESCs, early embryos and testis tissue (Tokuzawa *et al.* 2003). Null mice are normal, however, and Fbx15 is not required for ESC pluripotency or differentiation. Although its exact function is unknown, it has been postulated to be an E3 ubiquitin ligase.
- *Zfp206* encodes a zinc finger-containing transcription factor which maintains pluripotency in mESCs. It enhances transcription from its own promoter as well as that of *Oct4* and *Nanog* (Wang *et al.* 2007).
- *Dppa4* is expressed in pluripotent cells and the germ line (Maldonado-Saldivia *et al.* 2007), and it plays an important role in the self-renewal of mESCs (Masaki *et al.* 2007).

1.7.3 NANOG

Nanog is a relatively new member of the pluripotency orchestra, named after the Celtic land of the ever-young, 'Tir nan Og'[5]. It is a dimer-active homeodomain protein (Mullin *et al.* 2008; Wang *et al.* 2008), whose over-expression confers constitutive self-renewal on mESCs, independent of LIF (leukaemia inhibitory factor)/STAT3 (signal transducers and activators of transcription 3) stimulation (Chambers *et al.* 2003). Over-expression of NANOG also allows feeder-free growth of hESCs (Darr *et al.* 2006); however, these cells down-regulate expression of markers specific to the ICM and acquire expression of a primitive ectoderm marker. Nanog is expressed by human and mouse ESCs, EGCs and ECCs, the ICM and PGCs (Hatano *et al.* 2005; Yamaguchi *et al.* 2005); however, it is absent from oocytes (Hyslop *et al.* 2005). *Nanog*-deficient ICM fails to generate epiblast and only produces parietal endoderm-like cells, and *Nanog*-deficient

[5] Located on an island far to the west of Ireland, beyond the edges of the map. It could be reached by either an arduous voyage or an invitation from one of its fairy residents.

mESCs lose pluripotency and differentiate into the extraembryonic endoderm lineage (Mitsui *et al.* 2003). Therefore Nanog appears to function at a later stage than that of Oct4, in the development of primitive ectoderm. Consistent with this notion, in hESCs, *NANOG* expression increases during early differentiation (Darr *et al.* 2006).

Nanog expression is up-regulated in mESCs by the binding of T (Brachyury) and Stat3 to an enhancer element in the *Nanog* gene. It acts to prevent mesoderm differentiation by interacting with Smad1 (Small mothers against decapentaplegic 1) and interfering with the recruitment of co-activators to the active Smad transcriptional complexes (Suzuki *et al.* 2006). Nanog represses the pro-differentiation activities of NFκB, and also cooperates with Stat3 (Section 1.8.1) to activate gene expression and promote self-renewal (Torres and Watt 2008). The role of Nanog in pluripotency *per se* has recently been questioned, as *Nanog*$^{-/-}$ mESCs can be established and retain pluripotency, albeit with an increased propensity to differentiate (Chambers *et al.* 2007). It is now proposed to act primarily in the construction of the ICM and germ cell states rather than in the housekeeping machinery of pluripotency, acting as a 'pluripotency rheostat', conferring a variable resistance to differentiation (Chambers *et al.* 2007; Rizzino 2008).

1.7.4 Mode of Action of OCT4, SOX2 and NANOG

OCT4, SOX2 and NANOG maintain pluripotency by activating transcription of genes required for pluripotency and repressing those which promote differentiation, in human (Brandenberger *et al.* 2004; Boyer *et al.* 2005) and mouse ESCs (Loh *et al.* 2006; Zhou *et al.* 2007). OCT4-SOX2-NANOG target genes have been identified in hESCs by using chromatin immunoprecipitation (ChIP) coupled with DNA microarrays (Boyer *et al.* 2005). Promoter regions were defined as being within 8kb upstream and 2kb downstream of the transcriptional start site. OCT4, SOX2 and NANOG were found to co-occupy a substantial proportion of their target genes, at least 353 (Figure 1.5).

Many of the target genes are transcription factors, some of which function in embryonic development. Some of the target genes identified are shown in Figure 1.6 and include *OCT4*, *SOX2* and *NANOG* themselves, as well as components of the WNT[6] and transforming growth factor β (TGFβ) signalling pathways, reported to play roles in

[6] The name Wnt was coined as a combination of Wg (wingless) and Int (Rijsewijk *et al.* 1987). The *wingless* gene was idenitifed in *Drosophila melanogaster* and functions during embryogenesis (Nusslein-Volhard and Wieschaus 1980). It is homologous to the vertebrate *Int1* gene, present near the integration sites of mouse mammary tumour virus (MMTV) (Nusse *et al.* 1984).

pluripotency and self-renewal in human and mouse ESCs (Sato *et al.* 2004; James *et al.* 2005; Sections 1.8.4 and 1.8.5) .

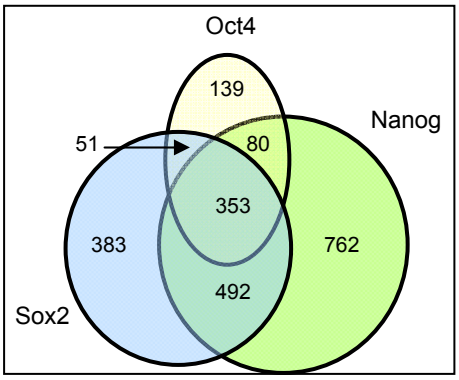


Figure 1.5 Venn diagram representing the overlap of OCT4, SOX2 and NANOG promoter bound regions

[Adapted from Boyer *et al.* (2005).]

The same research group has recently demonstrated that the transcription factor T-cell factor 3 (Tcf3), a terminal component of the Wnt pathway, co-occupies promoters throughout the mESC genome in association with Oct4 and Nanog (Cole *et al.* 2008). Tcf3 is highly expressed in mESCs, and is crucial for embryonic development (Korinek *et al.* 1998; Merrill *et al.* 2001); it may act to limit the actions of pluripotency-promoting transcription factors (Pereira *et al.* 2006; Cole *et al.* 2008; Yi *et al.* 2008). Most of the transcriptionally silent developmental regulators targeted by OCT4, SOX2 and NANOG are also occupied by polycomb proteins (Bernstein *et al.* 2006; Boyer *et al.* 2006; Lee *et al.* 2006). These are epigenetic regulators which facilitate the maintenance of cell state through gene silencing. Silent developmental genes co-occupied by OCT4, SOX2 and NANOG undergo an unusual form of transcriptional regulation, whereby the genes undergo transcriptional initiation but not productive transcript elongation (Guenther *et al.* 2007), presumably due to the presence of polycomb proteins at these promoters. In mESCs, Oct4 and Nanog associate with transcriptional repressor complexes on their target genes, and may thereby act to control ESC fate (Liang *et al.* 2008).

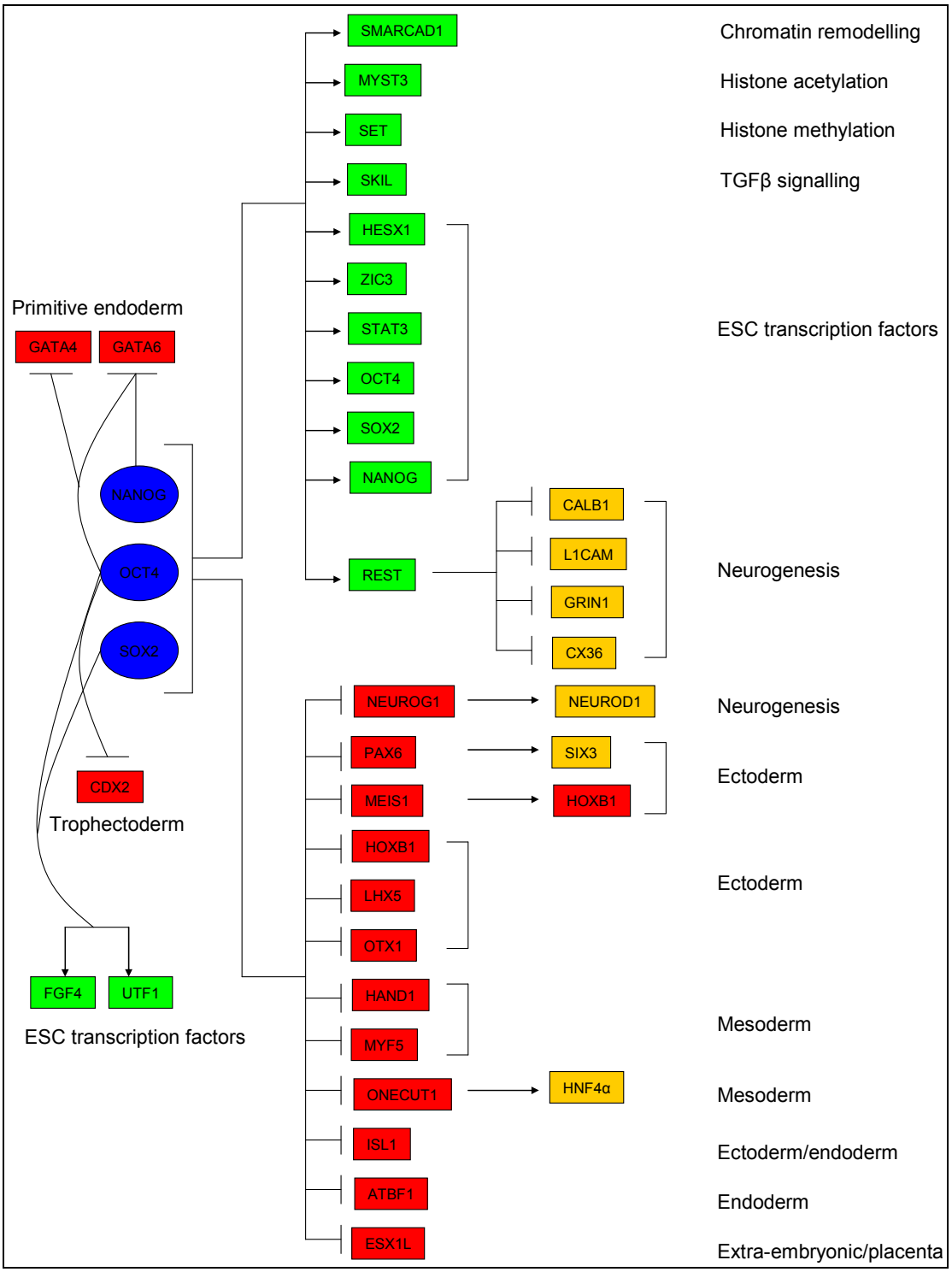


Figure 1.6 Core transcriptional regulatory network in hESCs

Constructed based on known targets of OCT4, SOX2 and NANOG and the binding of the transcription factors to the promoters of genes known to be expressed (green) and repressed (red) in hESCs. Regulators are represented by blue circles; gene promoters by rectangles; yellow boxes represent putative downstream target genes. OCT4, SOX2 and NANOG activate (arrows) transcription of genes required for pluripotency and repress (blocked lines) genes required for differentiation. [Adapted from Boyer *et al.* (2005).]

Interestingly, a study to identify Oct4 and Nanog target genes in mESCs found only 9% overlap between Oct4-bound genes and 13% overlap between Nanog-bound genes compared with hESCs (Loh *et al.* 2006). Therefore, there appear to be major differences in the regulation of pluripotency between species (Ginis *et al.* 2004; Sun *et al.* 2007). Of the 32 genes bound in both mouse and human ESCs, 18 encoded for transcription factors, including *Sox2* and *Nanog*. A further study analysing new and existing gene expression and location data has confirmed the key roles of Sox2, Oct4 and Nanog in mESCs, and identified coregulators, including Sal-like 4 (Sall4), Liver receptor homologue 1 (Lrh1), Stat3 and Estrogen related receptor β (Esrrb), allowing a regulatory network to be constructed (Zhou *et al.* 2007). The central role of these transcription factors is not exclusive to mammalian pluripotency: the Oct4 homologue PouV and Nanog regulate pluripotency in chick ESCs (Lavial *et al.* 2007).

In addition, a recent study has extended the core transcriptional network beyond that of Oct4, Sox2 and Nanog in mESCs (Kim *et al.* 2008). The target promoters of the genes for nine transcription factors, including Oct4, Sox2, Krüppel-like factor 4 (Klf4), c-Myc and Nanog, were identified. Promoters bound by a few of these transcription factors tended to be inactive or repressed, whereas those bound by more than four factors were expressed. In addition, targets of c-Myc generally fell into a different cluster than the other transcription factors, indicating a role in cell proliferation and the regulation of chromosomal accessibility rather than in pluripotency *per se*.

1.7.5 Additional Transcription Factors which Promote Pluripotency

1.7.5(i) RE1-silencing transcription factor

RE1-silencing transcription factor (Rest) is a major transcriptional repressor of neurogenesis (Ballas and Mandel 2005; Coulson 2005; Majumder 2006; Ooi and Wood 2007). It is expressed at high levels in mESCs (Ballas *et al.* 2005), where it acts to maintain self-renewal and pluripotency by suppressing the microRNA miR-21, resulting in a loss of miR-21 mediated repression of Oct4, Nanog and Sox2 (Singh *et al.* 2008).

1.7.5(ii) Zinc finger protein of the cerebellum 3

Zinc finger protein of the cerebellum 3 (Zic3) is a proposed target of Oct4, Sox2 and Nanog in human and mouse ESCs (Boyer *et al.* 2005; Loh *et al.* 2006; Lim *et al.*

2007). It is proposed to prevent endodermal marker expression, via direct or indirect regulation of Nanog, in mESCs (Lim *et al.* 2007).

1.7.5(iii) Sal-like 4

Sal-like 4 (Sall4) plays a critical role in maintaining mESC pluripotency by modulating *Oct4* expression (Elling *et al.* 2006; Zhang *et al.* 2006). It also interacts with Nanog and co-occupies Nanog genomic sites in mESCs; and Sall4 and Nanog regulate their own and each other's transcription (Wu *et al.* 2006), similar to the relationship between Oct4 and Sox2.

1.7.5(iv) Liver receptor homologue 1

Liver receptor homologue 1 (Lrh1) is expressed in the ICM and epiblast and knock-out is embryonically lethal with loss of *Oct4* expression. It activates *Oct4* reporter gene expression by binding to steroidogenic factor 1 (SF1) response elements in the proximal promoter and proximal enhancer of the *Oct4* gene (Gu *et al.* 2005).

1.7.5(v) Forkhead box D3

Forkhead box D3 (FoxD3), a member of the forkhead family of transcription factors, is expressed in human and mouse ESCs and in mouse epiblast and neural crest cells (Hanna *et al.* 2002). It is required for maintenance of the multipotent mammalian neural crest (Teng *et al.* 2008). *FoxD3*-null embryos die at E6.5 with a loss of epiblast and expansion of the proximal extraembryonic, endodermic and ectodermic tissues, and a lack of primitive streak. *Oct4*, *Sox2* and *Fgf4* expression are normal at E3.5; therefore, FoxD3 is thought to act downstream of Sox2 and Oct4. FoxD3 is not required for establishment of the ICM but for maintenance of the epiblast and the TE lineage in mouse embryos (Tompers *et al.* 2005). Oct4 inhibits FoxD3 activation of the *FoxA1* and *FoxA2* endodermal promoters *in vitro* (Guo *et al.* 2002).

1.7.5(vi) Krüppel-like Factors

Interest in Krüppel-like factors (Klfs) stems from the use of Klf4 in groundbreaking reprogramming experiments initiated by the group of Professor Yamanaka: somatic cells transduced with *Klf4*, *Oct4*, *Sox2* and *c-Myc* become pluripotent (Takahashi and Yamanaka 2006). However, Klf4 alone is not required for the maintenance of pluripotency in ESCs. It appears there is a degree of redundancy within the Klf gene family group: simultaneous depletion of Klf2, Klf4 and Klf5 leads to mESC differentiation (Jiang *et al.* 2008). These Klf proteins share many common target genes with Nanog, and regulate key pluripotency genes, including *Nanog* itself (Jiang *et al.* 2008). Klf4 is part of an extended pluripotency maintaining network identified in mESCs, where it is proposed to act upstream of Oct4, Sox2 and Nanog, and also occupy the c-Myc promoter (Kim *et al.* 2008).

1.7.6 MicroRNAs

MicroRNAs (miRNAs) are small (~21 nucleotide) RNAs which are processed from larger RNA molecules by the RNase III enzymes Drosha and Dicer. These small RNA molecules are then loaded into an RNA-induced silencing complex (RISC), where they induce mRNA degradation by base pairing with their complementary mRNA molecules. This degradation is catalysed by argonaute proteins - catalytically active members of the RISC complex. Thereby, genes containing sequences complementary to the small RNA molecules are silenced or down-regulated (Ruvkun 2001). Studies suggest that miRNAs play fundamental roles in ESCs. Human and mouse ESCs express a unique repertoire of miRNAs (Suh *et al.* 2004; Calabrese *et al.* 2007), and the miR-302 family in hESCs is orthologous to the zebrafish miR-430 family, expression of which is critical for development (Giraldez *et al.* 2006). Loss of Dicer causes differentiation defects in mESCs: the ESCs do not contribute to chimaeric mice or generate teratomas *in vivo,* and have limited differentiation *in vitro* (Kanellopoulou *et al.* 2005). The loss of Dgcr8, a protein involved in miRNA maturation, affects differentiation of mESCs: during retinoic acid-induced differentiation, cells do not fully down-regulate pluripotency markers and they retain the ability to produce ESC colonies (Wang *et al.* 2007). miRNAs also regulate *de novo* DNA methylation in differentiating mESCs (Sinkkonen *et al.* 2008).

1.8 Signal Transduction Pathways

1.8.1 LIF/STAT3 Pathway

STAT proteins play a role in transmitting signals from the membrane to the nucleus. When cytokines bind to their cell surface receptors, the receptor-associated JAK tyrosine kinases become activated and in turn phosphorylate a single tyrosine residue in the STAT molecule. Phosphorylated STATs enter the nucleus as dimers and bind to and activate transcription at gene promoters. STAT3 mediates signalling in response to the glycoprotein 130 (gp130) family of cytokines, which includes interleukin 6 (IL6), oncostatin M (OSM) and LIF. Disruption of the mouse *Stat3* gene is embryonic-lethal (Takeda *et al.* 1997). mESCs can be maintained under feeder-free conditions with the addition of LIF to the medium (Smith *et al.* 1988; Williams *et al.* 1988). LIF mediated self-renewal of mESCs requires *Stat3* activation (Niwa *et al.* 1998; Raz *et al.* 1999; Kristensen *et al.* 2005). However, LIF/STAT3 signalling fails to maintain self-renewal of hESCs (Daheron *et al.* 2004; Humphrey *et al.* 2004). This indicates that mechanisms governing pluripotency in mouse and human ESCs differ, and that there is an unidentified factor secreted by feeder cells which promotes hESC self-renewal. The LIF receptor (LIFR) consists of two subunits: gp130, which is common to all the cytokines from the IL6 family, and LIFRβ (or gp190), specific for LIF (Taga and Kishimoto 1997). The combination of IL6 and soluble IL6 receptor also interacts with and activates a homodimer of gp130 and has been used to maintain mESCs without involvement of the LIFR (Nichols *et al.* 1994; Yoshida *et al.* 1994).

1.8.2 MAPK/ERK Pathway

It appears that multiple signalling molecules may converge to activate MAPK/ERK (mitogen-activated protein kinase kinase/extracellular regulated kinase) signalling in ESCs. LIF treatment of mESCs increases MAPK activity (Boeuf *et al.* 1997) and induces phosphorylation of ERK1 and ERK2 (Burdon *et al.* 1999). The bridging factor between the LIFR and MAPK is the tyrosine phosphatase SHP-2, implicated in ESC proliferation and differentiation (Niwa *et al.* 1998; Qu and Feng 1998). In mESCs, MAPK/ERK signalling interferes with self-renewal, and mESCs show high ERK activity when they are stimulated to undergo differentiation (Burdon *et al.* 2002). MAPK/ERK kinases are targets of the FGF pathway in hESCs, and, in contrast to mESCs, high basal MAPK/ERK activity is required

to maintain pluripotency. MAPK/ERK signalling cooperates with PI3K/AKT signalling in maintaining pluripotency (Section 1.8.3). However, in contrast to PI3K/AKT signalling, it has no effect on regulating hESC proliferation or survival (Li *et al.* 2007).

1.8.3 PI3K/AKT Pathway

Phosphoinositide-3 kinases (PI3K) are a family of lipid kinases whose products PI(3,4)P2 and PI(3,4,5)P3 act as intracellular messengers and are implicated in a wide range of cellular processes, including cell proliferation and survival (Vanhaesebroeck and Waterfield 1999; Vanhaesebroeck *et al.* 2001). Akt is a serine/threonine kinase and acts downstream of PI3K. PI3K activation promotes mESC proliferation (Takahashi *et al.* 2003), and might also be crucial for self-renewal (Takahashi *et al.* 2005). Inhibition of PI3K and Akt induces differentiation of mESCs suggesting that PI3K/Akt signalling is necessary for mESC pluripotency (Paling *et al.* 2004). Activation of Akt signalling can maintain mESC pluripotency independent of Wnt/β-catenin signalling (Section 1.8.4) (Watanabe *et al.* 2006). PI3K-mediated signalling is also important for the maintenance of hESC pluripotency (Armstrong *et al.* 2006). A mechanism for PI3K signalling in pluripotency may relate, at least in part, to its ability to maintain *Nanog* expression (Storm *et al.* 2007). The growth factors LIF and FGF2, which are required for mESC pluripotency, activate PI3K/Akt signalling (Jirmanova *et al.* 2002; Xu *et al.* 2005). In addition, PI3K/Akt signalling suppresses apoptosis in mESCs (Gross *et al.* 2005) and hESCs (Pyle *et al.* 2006).

1.8.4 Wnt Pathway

Wnts are secretory proteins which regulate gene expression, cell differentiation and proliferation (Wodarz and Nusse 1998). Activation of the Wnt pathway by use of the glycogen synthase kinase-3 (GSK3) inhibitor 6-bromoindirubin-3 can maintain human and mouse ESC pluripotency independently of LIF/STAT3 signalling (Sato *et al.* 2004). However, Ogawa *et al.* demonstrated that Wnt signalling is not sufficient to maintain self-renewal but enhances the effect of LIF in doing so in mESCs (Ogawa *et al.* 2006). Conditioned medium from *Wnt3a*-expressing cells can maintain mESC pluripotency (Singla *et al.* 2006). In contrast to other signalling pathways, Wnt signalling appears to play the same role in human and mouse ESCs. Wnt signalling up-regulates *Stat3*

expression, and therefore may also converge on the LIF/Stat3 pathway (Hao *et al.* 2006). In addition, Wnt signalling up-regulates *c-Myc*, also a Stat3 target (Cartwright *et al.* 2005; Kristensen *et al.* 2005) implicated in mESC pluripotency (Kim *et al.* 2008). LIF elevates levels of β-catenin, a component of the Wnt pathway, which, via an interaction with Oct4, up-regulates *Nanog* (Anton *et al.* 2007; Takao *et al.* 2007).

1.8.5 TGFβ/Activin/Nodal Pathways

Several studies indicate that members of the TGFβ superfamily function in ESC pluripotency. The TGFβ family contains over 40 members, including TGFβ, Activin, Nodal and Bone morphogenetic proteins (BMPs) (Roberts *et al.* 1986; Massague 1998). Members signal by binding to a heteromeric complex of type I and type II receptors (Heldin *et al.* 1997). TGFβ and Activin have high affinity for type II receptors whereas BMPs have higher affinity for type I receptors (Valdimarsdottir and Mummery 2005). Binding causes the type II receptor kinase to phosphorylate the type I receptor, which activates intracellular signalling cascades, including Smad pathways. Activated Smads form complexes with a common partner Smad, i.e. Smad 4, translocate into the nucleus and regulate the expression of target genes in cooperation with various transcription factors.

Smad4-null mice display defective epiblast proliferation and retarded ICM outgrowth (Sirard *et al.* 1998). *Nodal*-null mice show reduced epiblast size with markedly reduced *Oct4* expression (Conlon *et al.* 1994; Robertson *et al.* 2003). TGFβ, Activin and Nodal activate Smad2/3 (Valdimarsdottir and Mummery 2005); and activation of Smad2/3 is required for maintenance of the undifferentiated state (James *et al.* 2005). *TGFβ* and correlated factors, *Nodal*, *Cripto*, *Lefty1* and *Lefty2*, are expressed at high levels in hESCs (Sato *et al.* 2003), as are genes downstream of TGF superfamily signalling (Zeng *et al.* 2004). In addition, Activin A may be sufficient to maintain pluripotency of hESCs without conditioned medium or STAT3 activation (Beattie *et al.* 2005; Xiao *et al.* 2006). Over-expression of *Nodal* in hESCs can block neuroectoderm differentiation, and its expression disappears upon differentiation (Vallier *et al.* 2004). Inhibition of the Activin/Nodal/TGFβ signalling pathway induces differentiation of hESCs (Vallier *et al.* 2005).

1.8.6 FGF2 Pathway

Human and mouse ESCs are routinely cultured in the presence of FGF2 (Evans and Kaufman 1981; Thomson *et al.* 1998). hESCs express four FGF receptors, particularly *FGFR1*, along with some components of their downstream cascades (Bhattacharya *et al.* 2004; Brandenberger *et al.* 2004; Dvash *et al.* 2004; Ginis *et al.* 2004). Modulation of FGF signalling via *FGFR1*- or *FGFR2*-null mice, or via *FGF2* mutants, alters blastocyst development (Deng *et al.* 1994; Chen *et al.* 2000; Esner *et al.* 2002), and inhibition of FGFR signalling causes hESC differentiation (Dvorak and Hampl 2005). hESCs are maintained with either 4 or 10 ng/ml FGF2. 40 ng/ml FGF2 combined with noggin, which inhibits a BMP4-like activity in Knock-Out Serum Replacement (Invitrogen), or 100 ng/ml FGF2 alone, maintains hESCs in the absence of feeders (Dvorak *et al.* 2005; Wang *et al.* 2005; Xu *et al.* 2005). FGF2 may function by mediating chromatin remodelling (Song and Ghosh 2004), or by acting on FGF response elements (FREs), which serve to integrate FGF signalling with that of other growth factors (Haremaki *et al.* 2003). FGF signalling is necessary, but not sufficient to maintain pluripotency, and effects depend on the TGFβ pathway (Vallier *et al.* 2005). FGF2 can inhibit BMP activity by preventing the nuclear translocation of phosphorylated Smad1 (Xu *et al.* 2005); however, this does not account for all of its effects (Vallier *et al.* 2005). FGF2 can also up-regulate telomerase activity in human endothelial cells (Kurz *et al.* 2003), which may explain another mode of action in hESCs; however, this is, as yet, undemonstrated. MEFs produce factors which maintain hESCs, evidenced by the fact that hESCs can be maintained by culture with MEF-conditioned medium. FGF2 treatment of MEFs is required for the production of these factors: a recent study has suggested that FGF2 has an indirect effect on hESCs by inducing the production of supportive factors from feeders, including TGFβ factors and IGFII (insulin-like growth factor-II), which then act directly on hESCs (Bendall *et al.* 2007). Another study showed that FGF2-regulated genes in MEFs include *inhibin β*, which leads to the assembly of Activin B, and *gremlin 1,* which encodes for an antagonist of BMPs (Diecke *et al.* 2008).

1.8.7 Heparan Sulphate Chains

A recent study has implicated heparan sulphate (HS) chains as a central mediator in self-renewal and pluripotency in mESCs, by responding to various extrinsic signalling factors. Wnt/β-catenin signalling mediated by HS chains regulates *Nanog* expression, and FGF and BMP/Smad signalling is regulated by HS chains (Sasaki *et al.* 2008).

The above regulatory factors and pathways have been demonstrated in ESCs, and in some cases ECCs, and while the mechanisms governing pluripotency are assumed to be similar in EGCs, this has not been proven. This is particularly true for hEGCs, which are notoriously difficult to derive and culture. This thesis describes work focussed on investigating the human germ cell lineage, in particular to determine whether the fundamental regulators of pluripotency and self-renewal are expressed in hEGCs. Herein follows a brief introduction to the germ cell lineage, followed by a more detailed description of EGCs and an overview of the questions addressed by this thesis.

1.9 The Germ Cell Cycle

Germ cells mediate the transmission of an individual's genome to future generations. In contrast to somatic cells, which make up an organism and die with it, germ cells form the organisms for subsequent generations (Figure 1.7). PGCs, the founder cells of the germ cell lineage, are usually established early during embryonic development. Specification can either occur through the inheritance of germ cell determinants already present in the egg, as in *Drosophila melanogaster* and *Caenorhabditis elegans*, or in response to inductive signals, as for mice and probably all mammals.

1.10 Origin of Primordial Germ Cells

In *D. melanogaster* and *C. elegans,* founder PGCs are set aside at the outset of embryogenesis from the totipotent cells and prevented from differentiating into somatic cells by repression of the global transcriptional machinery (Strome and Lehmann 2007). Determinants of somatic cells and germ cells are already segregated in specific regions of the oocyte. In mammals however, no such determinants are already set aside in the oocyte;

the germ cell fate is imposed on pluripotent cells of the epiblast, induced by signals from extraembryonic tissues.

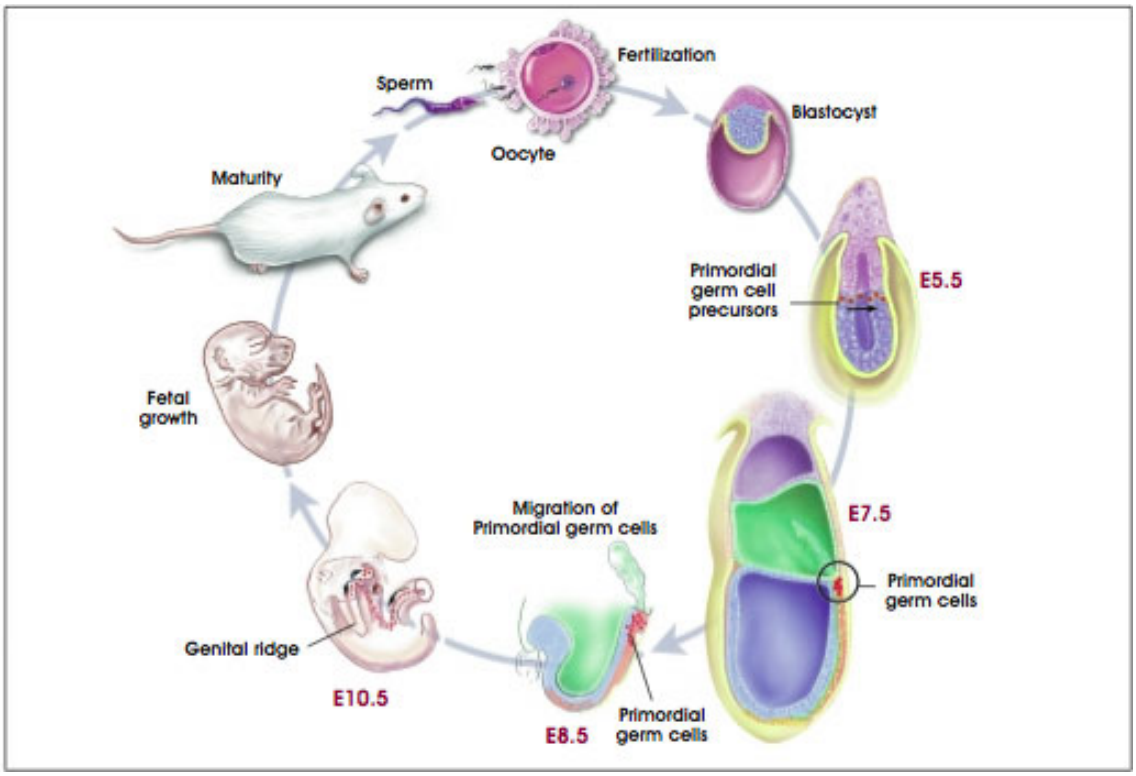


Figure 1.7 The germ cell life cycle

Fertilisation (*top*) is the point at which the gametes (sperm and oocyte) fuse; this generates a diploid zygote, which undergoes differentiation and mitosis, forms a blastocyst and implants into the uterine endometrium. During gastrulation, the process by which germ layers become positioned in the embryo, germ cell precursors (primordial germ cells) are set aside, then migrate to the gonads, undergo meiosis, mature, and eventually form haploid spermatozoa and ova in the mature organism. [Reproduced from the NIH Stem Cell Information website (http://stemcells.nih.gov/info/scireport/appendixA.asp).]

To describe PGC development, it is firstly necessary to briefly review early mouse embryonic development (aspects of which are depicted in Figure 1.8). At around the time of implantation (E4.0-4.5) the blastocyst is composed of trophoblast cells enclosing the undifferentiated ICM. The ICM subsequently differentiates into an inner epiblast, or embryonic ectoderm population, and an outer primitive, or extraembryonic visceral endoderm. After implantation, the trophoblast forms the extraembryonic ectoderm and the epiblast forms a ‘cup-shaped’ epithelium. Gastrulation, the formation of the germ layers

(Figure 1.3), begins around E6.0. Proximal epiblast cells move posteriorly, lose epithelial organisation, and give rise to unpolarised mesodermal cells. The first cells to delaminate from the epiblast give rise to extraembryonic mesoderm, whereas cells that drop out later give rise to embryonic mesoderm in the primitive streak [reviewed in Hogan (2001)].

Germ cells originate in the pluripotent proximal epiblast at about E6.0 (Figure 1.8) when cells respond to signals from extraembryonic tissues and begin to express *Fragilis/Interferon induced transmembrane protein 3 (Ifitm3)* (Saitou *et al.* 2002). *Fragilis* is an interferon-inducible gene, and was thought to mediate migration (Tanaka *et al.* 2005) until the demonstration that knock-out mice show normal germline development (Lange *et al.* 2008). It is only expressed transiently, being down-regulated as the cells migrate, although it is re-expressed later. At E6.25, about six of these cells begin to show expression of *Blimp1*: these cells are PGC precursors (Ohinata *et al.* 2005), although further cells are recruited to become PGCs before E7.25 (Saitou *et al.* 2002; McLaren and Lawson 2005; Ohinata *et al.* 2005). Blimp1 is a transcriptional repressor which is partially responsible for repressing the somatic program in PGCs while allowing establishment of germ cell character (Ohinata *et al.* 2005; Vincent *et al.* 2005). *Blimp1* expression confers competent cell lineage restriction on a PGC, but not yet specification. At around E7.0, the PGCs express *Stella*, which they retain during migration toward and into the genital ridges (Saitou *et al.* 2002).

1.10.1 Signals for Specification

BMP4 and 8b from the extraembryonic ectoderm and BMP2 from the visceral endoderm are critical for PGC specification: loss of any of these signalling molecules leads to partial or complete loss of PGCs (Lawson *et al.* 1999; Ying *et al.* 2000; Ying and Zhao 2001). BMP4 acts via Alk2, a type 1 BMP receptor in the visceral endoderm surrounding the proximal epiblast (de Sousa Lopes *et al.* 2004). Activation leads to the phosphorylation of serine residues on Smad1/5/8, causing translocation to the nucleus with Smad4. Loss of Smad1, 4 and 5 result in severe reduction in PGC number (Chang and Matzuk 2001; Tremblay *et al.* 2001; Hayashi *et al.* 2002). Along with the repression of somatic gene expression, the expression of genes which establish/maintain pluripotency are retained from the epiblast, including *Sox2*, *Nanog*, *Oct4* and *Embryonal stem cell gene 1 (Esg1)* (Ohinata *et al.* 2005; Western *et al.* 2005; Yamaguchi *et al.* 2005; Yabuta *et al.* 2006; Chambers *et al.* 2007). However, despite the expression of these key pluripotency genes,

mouse PGCs are nullipotent: unlike pluripotent ESCs they do not contribute to any tissues, either somatic or germ line if they are transferred into blastocysts (Durcova-Hills *et al.* 2006)

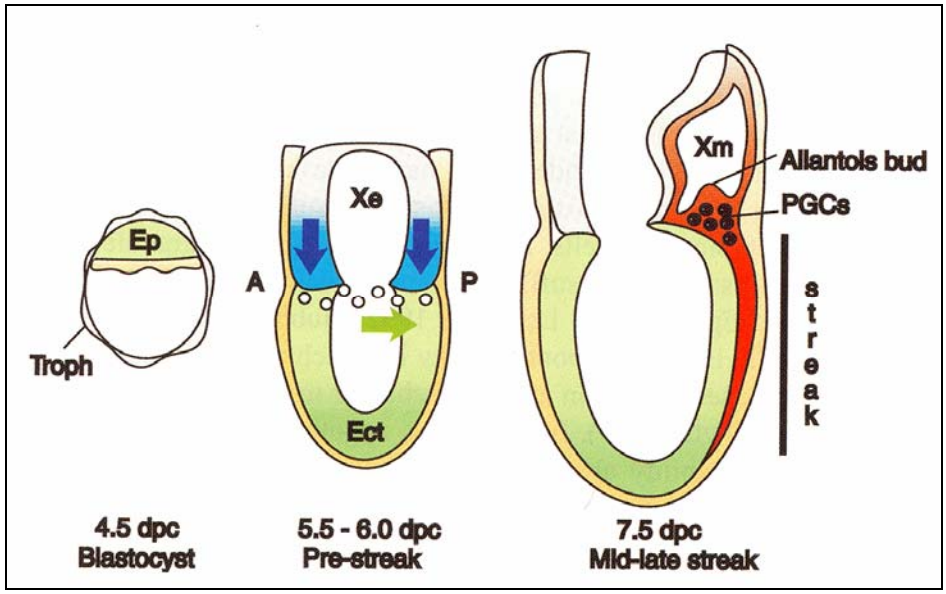


Figure 1.8 Model for the development of PGCs in the mouse embryo

At E4.5, the blastocyst consists of an outer layer of trophoblast (Troph) surrounding the epiblast (Ep; *green*) and the primitive or visceral endoderm (*orange*). By E5.5-6.0 the epiblast has given rise to the embryonic ectoderm (Ect) and the trophoblast has formed the extraembryonic ectoderm (Xe). Signals from the Xe, including bone morphogenetic protein 4 (BMP4) and BMP8b (*blue arrows*) induce PGC precursors (*open circles*) in the proximal epiblast. These move from anterior to posterior (A-P; *green arrow*). By E7.5, mesoderm has been generated (*red*) in the primitive streak and extraembryonic region (Xm). Descendants of the PGC precursors are allocated to either the extraembryonic mesoderm or PGC (*filled circles*) lineages. [Reproduced from Hogan (2001).]

Following establishment of the germ cell lineage, there is an extensive reprogramming of the genome, i.e. erasure of epigenetic marks and establishment of new marks (Surani 2001; Hajkova *et al.* 2002). Imprinting must be reprogrammed in the germ line, as a maternal allele in one generation may be a paternal allele in the next. PGCs do initially acquire genome wide *de novo* methylation; however, following entry into the gonadal ridge, there is rapid demethylation, simultaneously in male and females, prior to their sex-specific differentiation. The timing of erasure in humans is not known, but in mice it begins between E10.5 and E11.5, i.e. after arrival in the gonadal ridge (Lee *et al.* 2002).

1.10.2 PGC Migration

PGCs migrate from the allantois through the gut mesentery to the genital ridges of the developing gonad (Figure 1.7). This migration occurs at approximately E10.5 in the mouse and between weeks 5 and 8 of human gestation, and has been followed in the mouse using germ cells which express green fluorescent protein (GFP) (Molyneaux *et al.* 2001). Studies in mice have identified various molecules involved in the migratory process. The chemokine stromal-cell derived factor 1 (Sdf1), expressed in high levels in the gonad and genital ridge, is released from somatic cells and acts on the chemokine (C-X-C motif), receptor 4b (Cxcr4b), on the germ cell surface (Guardavaccaro *et al.* 2000; Stebler *et al.* 2004). The importance of the Sdf1-Cxcr4b signal was demonstrated in mice lacking the ligand or the receptor; fewer germ cells were able to reach the gonadal ridge and total PGC number was reduced. In addition, Sdf1 influenced the migration of PGCs expressing GFP. A second Sdf receptor, Cxcr7, has been identified as being required for migration in zebrafish: interestingly, it does not function as a signalling receptor, but sequesters Sdf1 thereby reducing levels in the extracellular environment (Boldajipour *et al.* 2008).

Additional molecules thought to be involved in migration include the c-Kit receptor tyrosine kinase, expressed by PGCs, and its ligand, stem cell factor (SCF)[7], expressed by somatic cells along the migratory route. An interaction between these molecules may support the migration and survival of the PGCs, as disruption leads to germ cell apoptosis and reduced numbers reaching the gonadal ridge (De Miguel *et al.* 2002). Mutations in either *Steel* (which codes for SCF) or *W* (which codes for c-Kit) interfere with PGC proliferation and migration (Mintz and Russell 1957; Donovan 1994), and in extreme mutants few PGCs, if any, reach the gonadal ridge (Buehr *et al.* 1993). However, the reduced number of germ cells do become distributed along the hindgut, which may be a passive mechanism. Knock-out/mutation of *β2 integrin* (Anderson *et al.* 1999), *Fgf8* (Sun *et al.* 1999) and the *germ cell deficient (gcd)* locus (Pellas *et al.* 1991) also interfere with germ cell migration indicating these gene products are also involved.

[7] Also known as steel factor (SF), mastocyte growth factor (MGF) or kit ligand (KL).

1.10.3 PGCs in the Gonad: Sex Determination

By E11.5-12.5 in the mouse, PGCs which have not reached the gonads undergo apoptosis (Stallock *et al.* 2003). The altered environment of the gonad induces several changes in the germ cells: they acquire a more rounded morphology, and the dead-box transcription factor *Deadbox 4 (Ddx4)* is expressed. *Ddx4* is the mouse orthologue of the *Drosophila Vasa* gene, also known as mouse *Vasa* homologue *(Mvh)*, which encodes an ATP-dependent RNA helicase essential for male germ cell proliferation and subsequent differentiation (Tanaka *et al.* 2000). After entering the genital ridges, the cells continue to proliferate for two days with a doubling time of approximately 16 hours. By E12.5, germ cells in female and male embryos enter a pre-meiotic stage and up-regulate meiotic genes such as *Synaptonemal complex protein 3 (Scp3)* (Di Carlo *et al.* 2000; Chuma and Nakatsuji 2001). At about E13.5, the germ cells in the female gonad proceed into prophase of the first meiotic division, progressing through leptonema, zygonema, pachynema and diplonema, and they enter a prolonged arrest stage termed dictyate around the time of birth (Speed 1982). They remain in this stage until just before ovulation, when they complete the first meiotic division, begin the second, and arrest again; meiosis is completed only at fertilisation. Germ cells enter meiotic prophase at about the same time even if outside the genital ridge, for example in the adrenal gland (Zamboni and Upadhyay 1983; McLaren 1995), or on a feeder layer *in vitro* (Chuma and Nakatsuji 2001); therefore, it appears that the default pathway for a germ cell is to develop as an oocyte, unless in the male genital ridge.

In the developing testis, germ cells down-regulate meiotic genes and enter mitotic arrest in G0/G1 as prospermatogonia between E12.5 and E14.5 (Western *et al.* 2008), resuming mitosis after birth (McLaren 1984), with meiosis delayed until well after birth (McLaren 1988). At 5 days postnatum in the mouse, primary spermatocytes can be seen, indicating some spermatogonia have entered meiosis. This is the equivalent stage to puberty in humans. Primary spermatocytes then complete both meiotic divisions promptly to generate spermatids, which mature further into spermatozoa (for a detailed description, see Chapter 4, Figure 4.1). Waves of meiosis continue throughout life, generating sperm from spermatogonial stem cells which reside at the periphery of the testis cords.

Exposure to retinoic acid and the function of the *Stimulated by retinoic acid gene 8 (Stra8)* cause female germ cells in the fetal ovary to enter meiosis (Baltus *et al.* 2006; Bowles *et al.* 2006; Koubova *et al.* 2006), whereas in males mitotic arrest is induced by

somatic cells, probably the Sertoli cells (Adams and McLaren 2002). Male specific expression of *Cyp26b1*, a P450 enzyme that degrades all-*trans* retinoic acid, inhibits meiosis in the fetal testis (Bowles *et al.* 2006; MacLean *et al.* 2007). Completion of meiosis and formation of functional gametes are influenced by the sex chromosome composition of the germ cell (McLaren 1981; Bradbury 1983; Burgoyne 1987; Taketo-Hosotani *et al.* 1989; Amleh *et al.* 2000; Park and Taketo 2003). Retinoic acid may also regulate meiotic progression in the pubertal testis (Bowles and Koopman 2007).

1.11 Human PGCs

Human PGC (hPGC) development is less well understood but is presumed to mirror that in mice. During the fifth week of human embryonic development, PGCs are apparent in the genital ridges and gut mesentery, and can be marked by alkaline phosphatase (AP) activity. The route of germ cell migration in the developing human embryo is depicted in Figure 1.9.

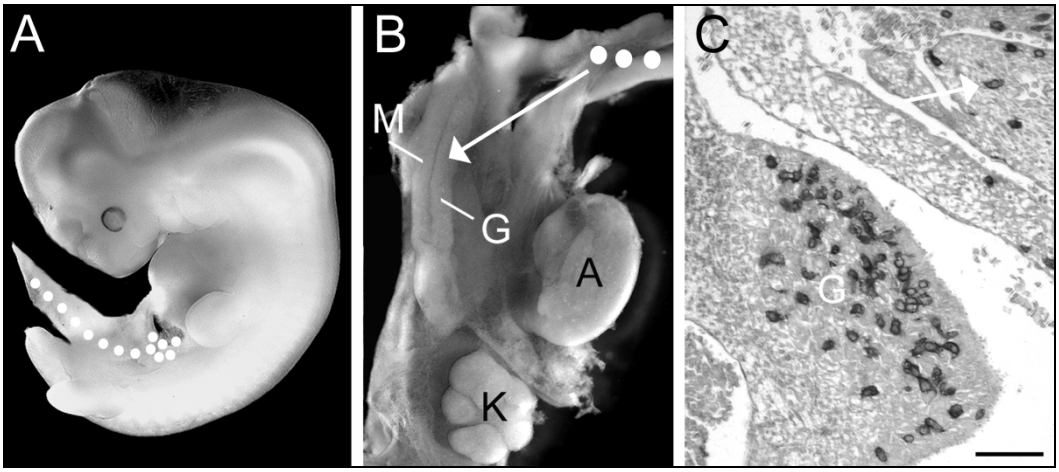


Figure 1.9 PGC migration in the developing human embryo

A. Path of PGC migration in the human embryo, from the allantois to the gonadal ridge. **B.** PGC migration through the gut mesentery within the dissected abdomen at 6 weeks post-conception (wpc). The gonadal ridge (G) has developed on the medial surface of the mesonephros (M) adjacent to the adrenal gland (A) and superior to the kidney (K). **C.** Human embryo section corresponding to panel B showing PGCs darkly stained for alkaline phosphatase activity in the gonad (G) and throughout the folds of the gut mesentery (white arrow). Size bar represents 250 μm. [Reproduced from Turnpenny *et al.* (2006).]

PGC number increases rapidly in the gonad: the number of oogonia per ovary increases from ~26,000 in week six to ~250,000 in week nine (Bendsen *et al.* 2006), and germ cells per testis increases from ~3000 in week six to ~30,000 in week nine (Bendsen *et al.* 2003). At around 41-44 days post-conception (dpc; Carnegie Stage [CS] 17-18) male PGCs begin to undergo mitotic arrest, associated with Sertoli cell differentiation and testicular cord formation, whereas female PGCs increase in number until approximately 12 weeks post-conception (wpc), when they undergo meiotic prophase (Gondos and Hobel 1971).

1.12 Derivation of Pluripotent ESCs from PGCs

1.12.1 Mouse EGCs

Early attempts at long-term mouse PGC (mPGC) culture had little success, even with feeder layers as had been used successfully for mESC culture. mPGCs (from 8.5-10.5 dpc) could only be grown in culture for three to five days with feeder layers (Donovan *et al.* 1986; Godin *et al.* 1990; De Felici and Dolci 1991). Certain agents were found to enhance PGC survival and/or proliferation, including LIF, which enhances survival (De Felici and Dolci 1991) and forskolin, which increases intracellular cyclic adenosine monophosphate (cAMP), thereby promoting proliferation (De Felici *et al.* 1993). In addition, a number of groups reported that SCF was essential for PGC survival, and that the PGC life-supporting effect of STO feeder layers[8] was partly due to the production of this growth factor (Dolci *et al.* 1991; Godin *et al.* 1991; Matsui *et al.* 1991). SCF was also found to promote PGC proliferation (Matsui *et al.* 1991). SCF and LIF promote PGC survival by suppressing apoptosis (Pesce *et al.* 1993), and SCF activates telomerase (Dolci *et al.* 2002). However, these *in vitro* cultures had a finite proliferative capacity (Donovan *et al.* 1986; De Felici and Dolci 1991; Dolci *et al.* 1991; Godin *et al.* 1991; Matsui *et al.* 1991), which correlated with the timing of their mitotic arrest (male) or the beginning of meiosis (female) *in vivo*.

In 1992, two groups found that the addition of FGF2 to PGC cultures already containing LIF and membrane-bound SCF enhanced the growth of PGCs and allowed their continued proliferation beyond the time when they normally stopped dividing *in vivo*

[8] STO is a mouse embryonic fibroblast cell line (S, SIM; T, 6-thioguanine resistant; O, ouabain resistant).

(Matsui *et al.* 1992; Resnick *et al.* 1992). The cells formed colonies of AP-, SSEA1-positive cells, that resembled ESCs and differentiated *in vitro* and *in vivo,* contributing to a variety of tissues in chimaeras, including the germ line (Labosky *et al.* 1994; Stewart *et al.* 1994). These cells, termed embryonic germ cells (EGCs), have since been derived from PGCs before and during migration (E8.5-9.5), and from PGCs in the genital ridge up to E13.5 (Matsui *et al.* 1992; Resnick *et al.* 1992; Labosky *et al.* 1994; Stewart *et al.* 1994; Tada *et al.* 1998; Durcova-Hills *et al.* 2001; Shim *et al.* 2008). It is presumed that after this point mitotic arrest in males and the entry into the first stage of meiosis in females prevents successful EGC derivation.

The major difference between ESCs and EGCs is that the PGCs from which EGCs are derived may have undergone the epigenetic changes that characterise the germ cell lineage, including demethylation and erasure of epigenetic marks. However, recent studies have indicated that the methylation pattern of imprinted genes in EGCs do not accurately reflect those of their parental PGCs (Durcova-Hills *et al.* 2001; Hajkova *et al.* 2002). During EGC derivation, *de novo* methylation occurs in the *Igf2* and *H19* genes in EGCs, with EGCs from the male gonad showing higher levels of methylation (Tada *et al.* 1998; Shim *et al.* 2008).

1.12.2 Human EGCs

Human EGCs (hEGCs) were first derived in 1998 by Shamblott *et al.*, and subsequently by additional groups (Turnpenny *et al.* 2003; Liu *et al.* 2004; Aflatoonian and Moore 2005; Pan *et al.* 2005). Material is obtained from first trimester termination, specifically human fetuses aged 5-9 wpc. PGCs are prepared for culture by mechanical disaggregation of the fetal gonad, with or without additional enzymatic digestion, and cells are plated onto a feeder layer of STO. Compared to mEGC derivation, the efficiency for generating hEGCs is low. hEGCs fulfill some criteria for pluripotency; they differentiate *in vitro* as EBs, containing derivatives of all three germ layers (Shamblott *et al.* 1998; Turnpenny *et al.* 2003; Aflatoonian and Moore 2005; Pan *et al.* 2005); however, no group has reported teratomas in immunocompromised mice following injection of hEGCs, attempted within our group (Turnpenny *et al.* 2006).

Despite the reprogramming which occurs in the germ line, monoallelic expression and methylation of imprinted genes has been observed in hEGC derivatives, and hEGCs derived from the gonadal ridge do not appear to have undergone epigenetic erasure

(Onyango *et al.* 2002). This indicates that imprinting may not be a significant barrier to hEGC transplantation. Encouragingly, hEGCs show therapeutic potential in transplantation therapy: hEGC-derived neural stem cells can successfully engraft, differentiate, and replace cells in the damaged neonatal mouse brain (Mueller *et al.* 2005), and hEGC-derived EB cells differentiate towards a musculoskeletal lineage *in vitro* (Kim *et al.* 2005). In addition, hEGC derivatives facilitate motor recovery of rats with diffuse motor neuron injury by enhancing host neuron survival and function (Kerr *et al.* 2003).

1.12.3 Mechanisms Underlying PGC-EGC Conversion

Relatively little is known regarding the mechanism behind the conversion of PGCs to EGCs. Knock-out of the tumour suppressor gene *phosphatase and tensin homologue (PTEN)* results in mice whose PGCs convert more readily to EGCs, although LIF, FGF2 and SCF are still required. This may increase signals for PI3K, a downstream molecule for signalling via LIF, FGF2 and SCF (Kimura *et al.* 2003). Further investigations demonstrated that Akt activation increases EGC formation in a similar manner (Kimura *et al.* 2008). Akt is a serine/threonine kinase, one of the downstream effectors of PI3K: this signalling pathway plays an important role in the regulation of ESC pluripotency [(Paling *et al.* 2004; Ivanova *et al.* 2006; Watanabe *et al.* 2006) and Section 1.8.3].

1.13 This Thesis

Compared to mouse and human ESCs, and mEGCs, hEGCs are notoriously difficult to derive and culture. Work delineating the mechanisms governing pluripotency, maintenance and differentiation in human stem cells has been conducted in hESCs. Continued research into hEGCs might provide further insights into these mechanisms, which may differ among different cell populations and/or between species. As well as having potential therapeutic benefit, research may provide insights into testicular germ cell tumours (TGCTs), which are of increasing frequency in the Western world. Studies may also provide useful information for the *in vitro* generation of gametes from human stem cells for future fertility treatments. For these reasons, the chapters of this thesis investigate:

- **Chapter 3: Expression of 'pluripotency regulators' within the human germ cell lineage**

hEGCs, while demonstrating pluripotency, fail to maintain this in long-term culture. This contrasts with mEGCs, and human and mouse ESCs. In addition, teratoma formation *in vivo* from hEGCs, a criterion for pluripotency, has not been described. Work within this chapter examines the expression of the key regulators of pluripotency, *OCT4*, *SOX2* and *NANOG*, and their proposed target genes, in hEGCs and their starting population, hPGCs, in comparison with the other pluripotent cell types.

- **Chapter 4: SOX2 expression in normal and neoplastic germ cell derivatives and its association with pluripotency**

Despite varying phenotypes, all TGCTs originate from a common precursor, the carcinoma *in situ* (CIS) cell, which is of germ cell antecedence. Previous studies have demonstrated that the transcription factor SOX2 is expressed by nonseminomas (which contain a pluripotent stem cell component) but is absent from seminomas. This chapter seeks to verify these findings and also extends analysis to examine SOX2 expression in CIS cells and during normal testicular development.

- **Chapter 5: Investigation of the role of the OCT4-SOX2-NANOG transcription factor complex in pluripotency**

The transcription factors OCT4, SOX2 and NANOG have been proposed to maintain pluripotency in human and mouse ESCs. Whilst expression of *OCT4* and *NANOG* is restricted to PGCs after gastrulation, *SOX2* is also expressed in embryonic and fetal tissues during development. In work described within this chapter, SOX2-positive cells are isolated from the human embryo/early fetus and culture methods optimised to grow the cells *in vitro* with retention of SOX2 expression. The expression of SOX2 target genes is examined, and attempts made to introduce OCT4 and NANOG, to determine whether establishing expression of the SOX2-OCT4-NANOG transcription factor complex in the cells induced pluripotency.

- **Chapter 6: Identification of candidate genes for germ cell proliferation**

This chapter describes efforts to identify factors which promote germ cell proliferation, by performing an expression microarray on RNA isolated from developing ovary and testes aged approximately eight weeks post-conception. At this stage of development, male germ cells have entered mitotic arrest whereas female germ cells are still proliferating. It is hypothesised that genes showing higher expression in the developing ovary may be involved in promoting germ cell proliferation. An initial RT-PCR screen is conducted to confirm differential gene expression, in order to draw up a short-list of likely candidate genes for further investigation.

CHAPTER 2: Methods

2.1 Preparation of Tissue Sections

2.1.1 Carnegie Staging of Human Embryos

The collection and use of human embryonic and fetal material was carried out following ethical approval from the Southampton & South West Hampshire Local Research Ethics Committee [Appendix 3 (i)-(iii)], under guidelines issued by the Polkinghorne committee (Polkinghorne 1989). Written consent for the use of embryos was obtained from women undergoing termination of pregnancy. Human embryos were collected following surgical termination of pregnancy and staged by stereomicroscopy according to the Carnegie classification [Table 2.1; O'Rahilly and Muller (1987); Bullen and Wilson (1997)]. Fetal material was also staged as weeks post-conception (wpc) by measuring foot length.

Carnegie stage (CS)	Human days post-ovulation	Equivalent mouse embryonic days (E)
8	18	7.5
9	20	7.5-8.5
10	22	8.5-9.0
11	24	9.0-9.5
12	26	9.5-10.25
13	28	10.25-10.5
14	32	10.5-11.0
15	33	11.0-11.5
16	37	11.5-12.0
17	41	12.0-12.5
18	44	12.5-13.0
19	48	13.0-13.5
20	50	13.5-14.0
21	52	13.5-14.0
22	54	13.5-14.0
23	56	13.5-14.0

Table 2.1 The Carnegie Classification - a precise staging of human gestation age
[Adapted from O'Rahilly and Muller (1987); Bullen and Wilson (1997).]

2.1.2 Tissue Fixation and Embedding

Immediately following dissection, human embryos and fetal material were placed in RNase-free bottles, containing 4% paraformaldehyde (PFA; Sigma Chemical Co., St. Louis, MO, US) in phosphate buffered saline (PBS; Sigma) overnight with gentle agitation. Samples were dehydrated by passing sequentially through 70%, 80%, 90% and 100% RNase-free ethanol (Fisher Scientific, Leicestershire, UK) for 2 hours each, at room

temperature with gentle agitation. The embryonic and fetal tissues were treated with chloroform (Fisher) for 16 hours to remove ethanol, followed by three changes of molten paraffin (2 hours each) at 72°C, the final change being under vacuum. Embryos and fetal tissue were embedded in paraffin (BDH, Dorset, UK), in the desired orientation and allowed to solidify. Embedded samples were stored at 4°C until required.

2.1.3 Slide Coating

Glass slides (22 × 60 mm; Fisher) were coated with 3-aminopropyltriethoxysilane (TESPA; Sigma). Batches of 24 glass slides were placed in polyacetal racks, and RNases inactivated by dipping in 0.1% diethyl pyrocarbonate (DEPC; Sigma) treated H_2O (DEPC-H_2O), followed by two washes in 0.1% DEPC in 96% ethanol for 5 minutes each. Slides were dried in a filtered air stream for 30 minutes. Slides were washed in 10% hydrochloric acid (HCl; Fisher) in 70% ethanol, DEPC-H_2O and 96% ethanol for 1 minute each, followed by drying in a filtered air stream for 1 hour. Coating was carried out by immersion for 45 seconds in 'dry' acetone (Fisher) containing 2% TESPA. The RNase-free coated slides were rinsed six times in 0.1% DEPC-H_2O, dried for 1 hour in a filtered air stream and then transferred to an oven overnight at 37°C. Coated slides were stored in RNase-free slide boxes at room temperature.

2.1.4 Tissue Sectioning and Slide Mounting

Paraffin blocks were mounted onto a dry microtome (Leica RM 2135, Leica Instruments, Wetzlar, Germany) in the required orientation for sectioning. Sections were cut at 5 μm intervals, as continuous ribbons, and placed onto RNase-free trays. Four serial sections were mounted onto RNase-free coated glass slides by floating on 0.1% DEPC-H_2O, heating to 45°C to expand the paraffin wax and aspirating the H_2O from underneath the embryo section. Slides were dried in an oven at 37°C overnight and then transferred to RNase-free slide boxes for storage at 4°C.

2.1.5 Haematoxylin & Eosin Staining

Every ninth section (40 μm intervals) cut from paraffin blocks was taken for histological staining. Paraffin was removed from the slides by immersion in xylene

(Fisher) for 5 minutes, followed by rehydration for 3 minutes each in 100% ethanol, 70% ethanol and H_2O. Slides were stained in Harris haematoxylin (ThermoShandon, PA, Pittsburgh, US) for 2-3 minutes, depending on the age of the solution, rinsed in H_2O and immersed in acid alcohol (95% ethanol, 1% HCl) and bluing solution (0.24 M $NaHCO_3$, 14 mM $MgSO_4$) for 10 seconds each. Counter-staining with eosin (ThermoShandon) for 30-40 seconds was followed by graded dehydration for 3 minutes in 50%, 70%, then 100% ethanol. Slides were placed in xylene for 5 minutes before mounting in Entellan (Merck Laboratories, Darmstadt, Germany).

2.2 Immunohistochemistry

A modified method of indirect immunohistochemistry (IHC) was used (Figure 2.1). Slides were dewaxed in xylene for 5 minutes, rehydrated in 100%, 70%, then 50% ethanol, followed by H_2O, for 3 minutes each, followed by washing in PBS. For the use of biotinylated HRP secondary antibody, sections were pretreated with 3% (v/v) hydrogen peroxide (Sigma) in PBS to quench endogenous peroxidase. Antigen unmasking was achieved by immersing slides in 10 mM sodium citrate buffer heated at 95°C, for 10-20 minutes, depending on the antibody. Sections were washed once more in PBS for 5 minutes. The primary antibody (Table 2.2) was diluted in buffer (PBS/0.1% Triton X-100) containing 3% serum (from the species in which the secondary antibody was raised; Vector Laboratories, Burlingame, CA, US), to reduce background and subsequent non-specific binding by secondary antibody. Sections were incubated with primary antibody in buffer/serum overnight at 4°C.

Sections were washed in PBS and biotin- or fluorescently-labelled secondary antibodies (Figure 2.1) applied in buffer (PBS/0.1% Triton X-100) for 2 hours at 4°C, at appropriate dilutions as listed in Table 2.3. For biotinylated secondary antibodies, further washing was followed by incubation for 1 hour at 4°C with the appropriate antibody (Table 2.3).

Dual immunofluorescence labelling was carried out sequentially (Figure 2.1), and sections were coverslip-mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). For bright-field immunohistochemistry, the colour reaction was developed with diaminobenzidine (DAB; Merck) containing 0.1% hydrogen peroxidase for 3 minutes, then counter-stained with

toluidine blue and coverslip-mounted using Entellan (Merck). Image analysis utilised a Zeiss Axiovert/Axiovision imaging system and Adobe Photoshop processing.

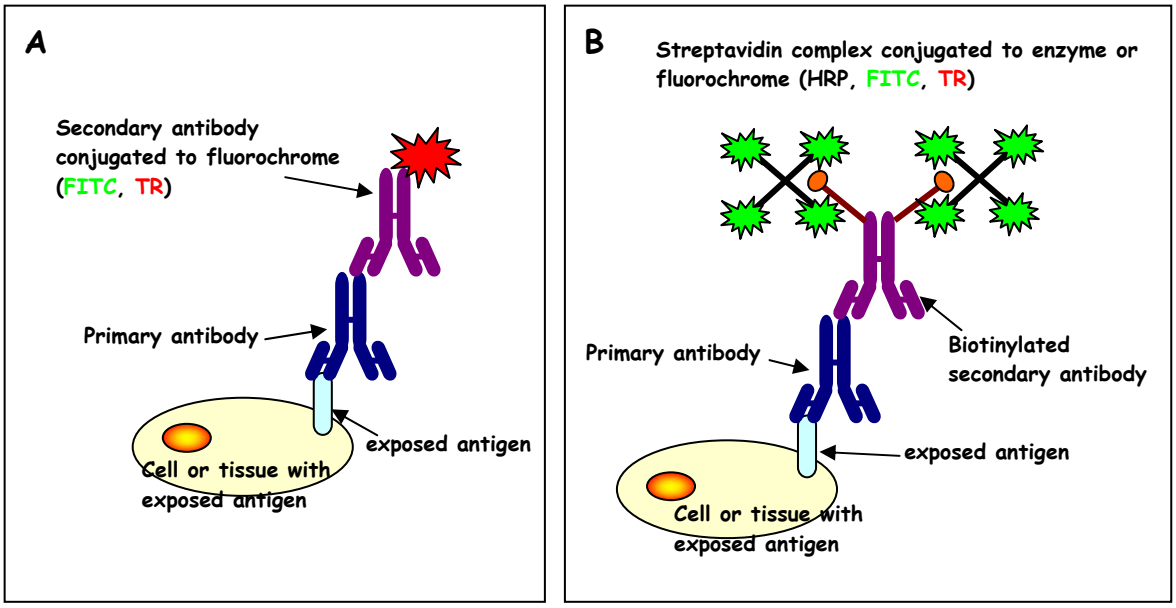


Figure 2.1 Indirect methods of immunohistochemistry

The antigen was detected using a primary antibody. The protein of interest was then visualised either using a secondary antibody conjugated to a fluorochrome (**A**), or using biotinylated secondary antibodies followed by streptavidin detection conjugated to a fluorochrome (e.g. FITC; Fluorescein Isothiocyanate; TR, Texas red), or horse radish peroxidase (HRP) which is detected enzymatically (**B**). **A** and **B** techniques were used sequentially to achieve dual expression profiles of two antigens in the same cell.

Primary antibody	Raised in	Dilution	Source
Monoclonal anti-β tubulin III	Mouse	1:500	Covance Research Products, Inc., Berkeley, CA, US
Monoclonal anti-CK19	Mouse	1:100	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
Monoclonal anti-EMA1	Mouse	1:20	Developmental Studies Hybridoma Bank (DSHB), Iowa University, IA, US
Monoclonal anti-GFAP	Mouse	1:500	Novocastra Laboratories Ltd.
Monoclonal anti-OCT4	Mouse	1:100	Santa Cruz Biotech. Inc., Santa Cruz, CA, US
Monoclonal anti-SSEA1	Mouse	1:20	DSHB
Monoclonal anti-SSEA3	Rat	1:5	DSHB
Monoclonal anti-SSEA4	Mouse	1:5	DSHB
Monoclonal anti-TDGF1	Mouse	1:50	R&D Systems, Abingdon, UK
Monoclonal anti-TRA-1-60	Mouse	1:5	Gift from Prof. Peter Andrews, University of Sheffield, UK
Monoclonal anti-TRA-1-81	Mouse	1:20	Gift from Prof. Peter Andrews, University of Sheffield, UK
Monoclonal anti-Vimentin	Mouse	1:50	DSHB
Polyclonal anti-CX43	Rabbit	1:200	Sigma Chemical Co., St. Louis, MO, US
Polyclonal anti-DPPA4	Sheep	1:500	R&D Systems
Polyclonal anti-FGF4	Rabbit	1:50	Abcam Ltd., Cambridge, UK
Polyclonal anti-Ki67	Rabbit	1:200	Novocastra Laboratories Ltd.
Polyclonal anti-Nanog	Goat	1:20	R&D Systems
Polyclonal anti-Nestin	Rabbit	1:50	Gift from Dr. Ron McKay, NIH, Bethesda, MD, US
Polyclonal anti-OCT4	Goat	1:150	Santa Cruz Biotech. Inc.
Polyclonal anti-PLAP	Rabbit	1:2000	Neomarkers, Fremont, CA, US
Polyclonal anti-SOX2	Rabbit	1:500	Chemicon International Inc., Temecula, CA, US

Table 2.2 Primary antibodies used in IHC experiments

Optimal antibody concentrations were determined by titration.

Secondary antibody	Dilution	Source
Biotinylated anti-rabbit	1:800	Vector Laboratories, Burlingame, CA, US
Biotinylated anti-goat	1:300	Vector Laboratories
Biotinylated anti-sheep	1:300	Vector Laboratories
Biotinylated anti-mouse	1:500	Vector Laboratories
Biotinylated anti-rat	1:100	Vector Laboratories
FITC anti-mouse	1:64	Sigma Chemical Co., St. Louis, MO, US
FITC anti-goat	1:64	Sigma
Texas red anti-rat	1:100	Vector Laboratories
Alexa Fluor 594 anti-rabbit	1:200	Molecular Probes, Inc, Invitrogen Life Technologies, Paisley, UK
Alexa Fluor 594 anti-mouse	1:200	Molecular Probes, Inc
Streptavidin horseradish peroxidase (SA-HRP)	1:200	Vector Laboratories
SA-FITC	1:150	Sigma
SA-Texas Red	1:200	Vector Laboratories

Table 2.3 Secondary antibodies used in IHC experiments

2.2.1 Germ Cell Tumour Stainings

The Regional Committee for Medical Research Ethics in Denmark approved the use of human tissue for this project. The tissue samples from adults with testicular neoplasms were obtained directly after orchidectomy and macroscopic pathological evaluation. Testicular samples were fixed overnight at 4^{o}C in formalin or PFA (both Sigma), and subsequently embedded in paraffin. A series of 20 overt testicular tumours were analysed by IHC, including six classical seminomas (mean age 32 years / range 28-37), nine nonseminomatous tumour components (mean age 29 years / range 24-35), four spermatocytic seminomas (mean age 66 years / range 42-87) and a testicular B-cell lymphoma (58 years). Eleven samples of testicular CIS were analysed (mean age 30 years / range 26-37). The seven normal fetal tissue samples [four testicular (14-39 wpc) and 21 ovarian 15-38 wpc) specimens] were obtained from spontaneous or induced abortions or autopsies of stillbirths mainly due to placental or maternal problems. The developmental age was calculated from the date of the last menstrual bleeding, supported by the foot size of the fetus. The six samples of infantile and prepubertal tissues (ranging from 2 months to 10 years) were obtained either from autopsies of infants who died suddenly of causes unrelated to the reproductive system or as testicular biopsies performed in boys with acute leukaemia for monitoring the spread of disease. Finally, normal adult testis was examined

using nine specimens of tissue removed because of TGCT, where there were normal preserved tubules with complete spermatogenesis (mean age 31 years / range 26-37).

IHC was carried out as per the protocol for bright-field IHC (Section 2.2), apart from the following amendment: the bound antibody was visualised using aminoethyl carbazole substrate (Zymed, San Francisco, CA, US). Sections were lightly counterstained with Mayer's haematoxylin to mark unstained cell nuclei.

The sections were examined under a light microscope (Zeiss, Oberkochen, Germany) and scored systematically by two investigators (RMP and SBS). The staining was assessed using an arbitrary, semi-quantitative score of the proportion of cells stained: +++: nearly all cells stained, ++: approximately half of the cells stained, +: a low percentage of cells stained, +/-: only single cells stained, -: no positive cells detected. The staining intensity was furthermore evaluated as strong, medium, weak, very weak or no staining.

2.3 Immunoblotting

2.3.1 Preparation of Cell Lysates

Tissues and cells were rinsed with PBS, and ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM sodium chloride, 0.5% Triton X-100; all Sigma), containing a set of protease inhibitors ('Complete'; Roche Applied Science, West Sussex, UK), was added for 30 minutes with gentle trituration. Lysates were stored at -80^{o}C. Protein was quantified by the Bio-Rad Protein Assay (Bio-Rad, Hertfordshire, UK), based on the Bradford dye-binding procedure (Bradford 1976). This utilises the colour change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein; the absorbance maximum shifts from 465 nm to 595 nm when protein binding occurs. A range of dilutions of a bovine serum albumin (BSA) standard was produced in water, and dilutions of the cell lysate were also made in water. 180 µl of the diluted standard or sample was added to 20 µl of the concentrated reagent in a 96-well plate (Bibby Sterilin Ltd., Stone, UK) in triplicate. The absorbances were read at 595 nm on a microplate reader (Bio-Rad Model 680). The readings for the standard dilutions were used to plot a standard curve to enable an estimation of the protein concentration of the extract. The protein extracts were then equalised.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Single dimension SDS-PAGE was carried out vertically using the Laemmli discontinuous buffer system, in a Mini-Protean 3 Cell (Bio-Rad), assembled according to the manufacturer's instructions. Stacking gels comprised 5% bisacrylamide, 125 mM Tris-HCl [pH 6.8], 0.1% sodium dodecyl sulphate (SDS), 0.1% ammonium persulphate (APS) and 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED). Resolving gels comprised 10% bisacrylamide, 375 mM Tris-HCl [pH 8.8], 0.1% SDS, 0.1% APS and 0.05% TEMED. The resolving gel was poured first, overlaid with isopropanol, and allowed to set. The isopropanol was poured off and any unpolymerised acrylamide removed by rinsing with distilled deionised H_2O (ddH_2O). The stacking gel was then poured onto the resolving gel and Teflon well combs inserted. The gel was allowed to set at room temperature.

15 µg of protein, prepared as described (Section 2.3.1), was combined with an equal volume of 2 × SDS gel-loading (Laemmli) buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue) and heated at 95^{o}C for 5 minutes. A prestained protein marker (Broad-range; New England Biolabs Inc., Beverly, MA, US) was also heated for 95^{o}C for 5 minutes before gel loading. The gel combs were removed and unpolymerised acrylamide removed from the wells by flushing with ddH_2O. Gels were mounted vertically in the electrophoresis apparatus and 1 × SDS-PAGE buffer (25 mM Tris-HCl [pH 8.3], 250 mM glycine, 0.1% SDS) added to the top and bottom reservoirs. Prepared samples were then loaded, with any unused wells being loaded with 1 × Laemmli buffer. A current of 55 mA was applied by connection to a Bio-Rad Power Pac 300 power supply and ran until proteins were sufficiently resolved as determined by the separation of the prestained marker.

2.3.3 Immunoblotting

Immunoblotting or Western blotting of proteins in SDS-PAGE gels was carried out by electrophoretic transfer onto nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, Piscataway, NJ, US). A piece of nitrocellulose was soaked in ddH_2O, then equilibrated in transfer buffer (48 mM Tris-HCl [pH 8.3], 39 mM glycine, 20% methanol). The gel was sandwiched between layers of Whatman 3MM paper and two porous pads, put in a Trans-Blot electrophoresis tank (Bio-Rad) filled with transfer buffer, and proteins transferred by applying a current of 250 mA for 2 hours.

2.3.4 Immunodetection

Following immunoblotting, success of protein transfer was confirmed by staining with a solution of Ponceau S (0.1% Ponceau S and 5% glacial acetic acid; Sigma) for 1 minute, followed by washes in ddH_2O until all red stain was removed. Blocking of non-specific binding sites was carried out by immersing the membrane in PBS containing 5% non-fat powdered milk and 0.1% Tween-20 (Sigma) for 2 hours at room temperature. The membrane was washed three times[9] in PBS containing 0.1% Tween-20, and then incubated with the selected primary antibody at a suitable dilution (determined by preliminary titration experiments) in blocking buffer overnight at 4°C. Membranes were washed three times in PBS containing 0.1% Tween-20, and then incubated with either peroxidase labelled anti-rabbit antibody (1:50,000; Amersham), anti-goat antibody (1:200,000; Sigma), or anti-sheep antibody (1:2000; Dako, Glostrup, Denmark) in blocking buffer for 1 hour at room temperature. β-actin was detected by 1 hour incubation with mouse anti-β-actin peroxidase conjugated antibody (1:50,000; Sigma) at room temperature. Membranes were washed three times in PBS / 0.1% Tween-20 and once in PBS, followed by detection.

2.3.5 Detection of Antibody-Immobilised Antigen Complexes

Location of antibody bound to antigen on nitrocellulose membranes was detected by luminescence, using either Standard or Advanced Enhanced Chemiluminescence (ECL) reagents (Amersham), depending on the quantity of target protein present. Equal volumes of the two chemical detection solutions were combined, sufficient to cover the membrane. The membranes were drained of excess wash buffer and laid protein side up on polythene wrap. The detection mixture was applied to the surface of the blots, ensuring complete coverage, for one (Standard) or five minutes (Advanced). The excess chemical was drained, and blots overlaid with polythene, placed in a film cassette, and under dark room conditions overlaid with photographic film (Kodak Scientific Imaging Film) with exposure for 30 seconds. Subsequent exposures were carried out for time periods (10 seconds to 30 minutes), according to the intensity of the signal observed on the first developed film.

[9] Each wash lasted 10 minutes and was carried out on a rotating platform at room temperature.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Primer Design

DNA sequences were obtained from the National Centre for Biotechnology Information website (NCBI; http://www.ncbi.nlm.nih.gov/) and downloaded into the Primer Select programme (DNASTAR Inc., Madison, WI, US). A sorted list of primers was generated based on the thermodynamic properties of the annealing reactions. Primer pairs were chosen according to product length, position within the target sequence, annealing temperature and the absence of hairpin loops. Where possible, primers that spanned introns were chosen so that any amplification from contaminating genomic DNA within the PCR reaction could be distinguished. Primers were ordered from Operon Biotechnologies, Inc. (Cologne, Germany).

2.4.2 Amplification of PCR Products

A typical PCR reaction contained 50-100 ng template DNA, along with DNA polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$; Promega Corporation, Madison, WI, US), 200 µM dNTPs (Roche), 0.2 µM of the relevant primer and 1 unit of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega). For GC rich template DNA, 1M betaine (Sigma) or 5% dimethyl sulphoxide (DMSO; Sigma) was often included. To optimise conditions where necessary, the annealing temperature and $MgCl_2$ concentration were varied by using a DNA polymerase reaction buffer without $MgCl_2$ (Promega) and adding varying concentrations of $MgCl_2$ (provided as a 25mM stock [Promega], concentration range of 0.5-3.5 mM used). Reactions were carried out on a MJ Research DNA Engine Tetrad. A typical PCR cycle began with denaturation at 94^{o}C for 3 minutes, followed by 30-40 cycles of: 1 minute at 94^{o}C (denaturation); 1 minute at 55-65^{o}C (annealing) and 1 minute at 72^{o}C (extension). The reaction was completed by a final extension step at 72^{o}C for 10 minutes. Products were identified by running on an agarose gel and recovered by gel extraction (see Section 2.5).

2.5 Agarose Gel Electrophoresis

DNA samples in the size range of 75 bp – 10 kb were analysed on 0.8 – 3% agarose gels. Agarose (Sigma) was dissolved in 1 × TAE buffer (40 mM Tris-acetate and 1 mM EDTA [pH 8.0]), and approximately 200 μg/ml of ethidium bromide (Roche) added to visualise reaction products under UV light. The gel was poured into casting apparatus containing a comb to mould wells for sample application. Once solidified, the gel was submerged in 1 × TAE running buffer in an electrophoresis tank (Bio-Rad Sub-Cell GT). DNA and 1 × Ficoll orange (0.25% orange G, 15% Ficoll and 0.5 M EDTA at [pH 7.0], all Sigma) were electrophoresed in parallel with 100 bp and 1 kb DNA step ladders (Promega), or in-house DNA ladders, at 80 V for 1-2 hours. The separated samples and markers were observed under UV light (High Performance UV Transilluminator, UVP, Cambridge, UK) and photographed using Doc-It software (UVP).

2.5.1 Gel Extraction

DNA was separated by gel electrophoresis and purified by QIAquick gel extraction (QIAGEN, West Sussex, UK). This entailed excising selected product bands from agarose gels with a scalpel, dissolving the agarose in Buffer QG (containing guanidine thiocyanate) at 50^{o}C for 10 minutes before mixing with isopropanol. This mixture was applied to a QIAquick column and the DNA bound to the silica-gel membrane by centrifugation at 12,000 g. Residual agarose was eliminated by adding further Buffer QG and centrifuging at 12,000 g, before washing with Buffer PE (tris-hydroxy amino-methane-buffered 80% ethanol). DNA was eluted in ddH_2O.

2.6 DNA Cloning: General Principles

2.6.1 Preparation of Insert DNA

Generally, insert DNA was amplified using primers incorporating the recognition sequences for restriction endonucleases (see Section 2.7.4 and 2.7.5), allowing subsequent cloning into appropriate vectors [Appendix 2(i)-(ii)]. Following amplification, products were gel purified, digested with the relevant restriction enzyme, and re-purified via gel extraction. The resultant DNA was cloned into the vector of choice.

2.6.2 Preparation of Vectors

The vector was digested with the appropriate restriction enzymes. To avoid self-annealing, vectors were dephosphorylated by adding 1 µl of calf intestinal phosphatase (CIP; New England Biolabs) directly into the digestion reaction, and incubating at 37°C for 15 minutes. The vector DNA was then purified by gel electrophoresis and extraction.

2.6.3 Ligation of DNA Inserts into Vectors

Ligations were carried out in a 10 µl volume containing 1 × DNA ligase buffer (50 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 5% polyethylene glycol), dephosphorylated vector, digested insert DNA, and 1 unit of T4 DNA ligase (Promega). Vector:insert ratios were estimated for efficient ligation using the following calculation:

$$\text{ng insert} = \frac{\text{ng vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{desired ratio of } \frac{\text{insert}}{\text{vector}}$$

For most ligations <100 ng of vector DNA was sufficient, with vector:insert ratios optimised at 1:1 or 1:3. Ligations were carried out at room temperature overnight.

2.7 Transformation of Plasmids into Bacterial Cells

2.7.1 'Heat-Shock' Transformation

50 µl of chemically competent cells (JM109; Promega) were thawed on ice, mixed with 1-5 µl of recombinant DNA and incubated on ice for 10 minutes. The DNA cell mix was heat shocked by incubation in a H_2O bath at 42°C for 45 seconds. Cells were immediately chilled on ice for a further 2 minutes, rescued with 950 µl of warmed SOC media (SOB[10] media [0.5% yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$] with 20 mM glucose) and incubated at 37°C for 1 hour with

[10] SOB stands for Super Optimal Broth. SOC had the 'B' in SOB changed to 'C' , for catobolite repression, reflective of the added glucose.

shaking. The transformed cells were plated out onto LB[11] agar containing 50 µg/ml of carbenicillin (Sigma), and grown overnight at 37°C.

2.7.2 Growth of Bacterial Cultures and Plasmid Preparation

Individual colonies from LB/carbenicillin agar plates were picked with a sterile loop and inoculated into an appropriate volume of LB medium[11] containing 50 µg/ml carbenicillin (Sigma). To prepare smaller amounts of plasmid DNA, 3 ml cultures were incubated overnight at 37°C / 225 rpm. Cells were harvested by centrifugation at 2000 g for 10 minutes. High-copy recombinant plasmids were purified from pelleted cells by the alkaline lysis method, employing the QIAGEN QIAprep Miniprep kit. Following resuspension in 250 µl of Buffer P1 (50 mM Tris-Cl, 10 mM EDTA, 100 µg/ml RNase A [pH 8.0]), cells were lysed by the addition of 250 µl of Buffer P2 (200 mM NaOH, 1% SDS)[12] and gentle mixing. The lysis reaction was limited to 5 minutes[13], before neutralisation by gentle mixing with 350 µl of Buffer P3 (3.0 M potassium acetate [pH 5.5])[14]. The precipitate was compacted by centrifuging for 10 minutes at 12,000 g and the supernatant applied to a QIAprep silica-gel membrane column placed in a 2 ml collection tube. Following centrifugation for 1 minute at 12,000 g, the flow-through was discarded. The membrane-bound plasmid DNA was then washed by applying 0.75 ml of ethanol-containing Buffer PE and centrifuging for 1 minute at 12,000 g. After discarding the flow-through, residual ethanol was removed by a repeat centrifugation. The column was then transferred to a clean microcentrifuge tube and the purified plasmid DNA eluted in 50 µl ddH_2O by centrifuging again at 12,000 g for 1 minute.

To prepare larger amounts of plasmid DNA for transfection into mammalian cells, 5 ml starter cultures were grown for 8 hours at 37°C / 225 rpm. The starter cultures were then used to inoculate larger overnight cultures (100 ml of LB/carbenicillin) at a dilution of 1:100. Cells were harvested by centrifugation at 6000 g for 15 minutes at 4°C. The alkaline lysis method was again used to purify high-copy recombinant plasmids from pelleted cells,

[11] LB stands for Lysogeny broth, also known as Luria Bertani medium or Luria broth. LB medium comprises 0.5 % yeast extract; 1% tryptone and 171mM NaCl. LB agar is LB medium plus 1.5% agar.
[12] Contains SDS,which disrupts the cell membrane, releasing the cell contents, and NaOH for the denaturation of the exposed DNA and proteins.
[13] Prevents release of chromosomal DNA, but permits optimum release of plasmid DNA while minimising denaturation.
[14] This high-salt buffer precipitates denatured proteins, chromosomal DNA, cellular debris and SDS, while the plasmid DNA renatures and remains in solution. The high-salt conditions also enable selective adsorption of plasmid DNA to the membrane of the QIAprep column.

employing the QIAGEN Plasmid Maxi Kit. Following resuspension in 10 ml Buffer P1, cells were lysed by adding 10 ml Buffer P2, mixed thoroughly, and incubated at room temperature for 5 minutes, before neutralisation by the addition of 10 ml of chilled Buffer P3. The lysate was poured into the barrel of a QIAfilter cartridge, and incubated at room temperature for 10 minutes. The cell lysate was then filtered into a QIAGEN-tip previously equilibrated with Buffer QBT (750 mM NaCl, 50 mM MOPS [pH 7.0], 15% isopropanol and 0.15% Triton X-100); as the lysate gravitates through the tip, DNA binds to the QIAGEN resin. The QIAGEN-tip was washed twice with Buffer QC (1.0 M NaCl, 50 mM MOPS [pH 7.0], 15% isopropanol), and DNA eluted with 15 ml Buffer QF (1.25 M NaCl, 50 mM Tris-Cl [pH 8.5] and 15% isopropanol). DNA was precipitated by adding 0.7 volumes (10.5 ml) room temperature isopropanol, and centrifuged at 15,000 g for 30 minutes at 4°C. The DNA pellet was washed with 5 ml room temperature 70% ethanol, and centrifuged at 15,000 g for 10 minutes, followed by drying and redissolving in ddH_2O.

2.7.3 DNA Quality and Concentration

Plasmid DNA was analysed on an agarose gel. The separated samples and markers were observed under UV and photographed. Pure plasmid DNA was found as two separate bands, one for relaxed (nicked) DNA and the other for supercoiled DNA, which migrates more rapidly due to its compact form. DNA was quantified by measuring the A260 using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, US).

1 A_{260} Unit of double stranded DNA ~50 µg/ml

The quality of plasmid DNA was estimated by the ratio between A260:A280, to ensure the solution was relatively free from proteins. For a pure DNA solution the A260:A280 ratio should be at least 1.8, with the A260:280 ratio at ≥1.8 indicative of DNA of sufficient purity.

2.7.4 Expression Vector Constructs

The coding sequences for the human (h) *OCT4*, *NANOG*, and *SOX2* genes were amplified by PCR[15] and cloned into the expression vector pcDNA 3.1 Zeo(+) [see Appendix 2(i), Invitrogen] which drives expression from a CMV promoter. The primers in Table 2.4 were used to amplify insert DNA for cloning into the pcDNA 3.1 Zeo(+) vector. All reverse primers contained an *EcoR*I recognition sequence, the OCT4 and SOX2 forward primers contained a *BamH*I site, and the NANOG forward primer a *Bgl*II site.

	Forward primer	**Reverse primer**
h*NANOG*	5' GAAGATCTTTTTTCCTCCTCTTCCTCTAT 3'	5' GGAATTCATGGGAGGAGGGGAGAGGAAGGAT 3'
h*OCT4*	5' CGGGATCCGCTTGGGGCGCCTTCCTTCC 3'	5' GGAATTCCCCTCCCCCTGTCCCCCATTCCTA 3'
h*SOX2*	5' GGGATCCCGCCCGCCTCCCCTCCTCCTCTC 3'	5' GGAATTCTGCACCCCTCCCATTTCCCTCGTT 3'

Table 2.4 Primers used for generating expression vector constructs
Restriction sites are underlined.

2.7.5 Short Interfering RNA Constructs

Short interfering RNA (siRNA) constructs were cloned into pSUPER.gfp/neo [see Appendix 2(ii), Oligoengine, Seattle, WA, US], a mammalian expression vector which directs intracellular synthesis of siRNAs 19 nucleotides in length, and which also contains a neomycin resistance gene and the gene for green fluorescent protein (GFP). A 19 nucleotide region of the h*SOX2* gene was selected using the Wistar Institute siRNA Selector (from http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAdescrip.htm). A BLAST search was performed on the highest scoring sequences, and one selected with fewer than 15 nucleotides matching with any other human gene. Complementary forward and reverse oligonucleotides were designed containing the 19 nucleotide regions and restriction enzyme recognition sequences at either end. These were annealed, digested and ligated into the *Bgl*II and *Hind*III sites of the vector. The oligonucleotides used are shown in Table 2.5 (siRNA 1). In addition, a second 19 nucleotide region (Table 2.5, siRNA 2) was chosen to which siRNA has been directed and shown to reduce the level of *SOX2* transcripts by 50% (Chew *et al.* 2005). This sequence also shows less than 15 base pair

[15] For *SOX2,* genomic DNA could be used as template, as the gene contains no introns. However, for the intron-containing genes *OCT4* and *NANOG,* cDNA clones were used, obtained from the I.M.A.G.E. Consortium (Geneservice Ltd., Cambridge, UK)

match with other human genes. This was cloned into the *Xho*I and *BgI*I sites of the plasmid. A control sequence directed against the luciferase gene was also used.

	Forward primer	**Reverse primer**
h*SOX2* siRNA1	5'GATCCCCACCAGCGCATGGACAGTTATTCAAG AGATAACTGTCCATGCGCTGGTTTTTTGGAAA 3'	5'AGCTTTTCCAAAAAACCAGCGCATGGACAGTTAT CTCTTGAATAACTGTCCATGCGCTGGTGGG 3'
h*SOX2* siRNA2	5'GATCCCCGAAGGAGCACCCGGATTATTTCAAG AGAATAATCCGGGTGCTCCTTCTTTTTC 3'	5'TCGCGAAAAAGAAGGAGCACCCGGATTATTCTC TTGAAATAATCCGGGTGCTCCTTCGGG 3'

Table 2.5 Primers used for generating siRNA constructs

19 nucleotide regions complementary to the h*SOX2* gene are underlined. Restriction enzyme sequences flank these regions (*Bgl*II and *Hind*III for siRNA 1, *Bgl*II and *Xho*I for siRNA 2).

2.8 DNA Sequencing

All plasmid sequences were verified by sequencing, using the Beckman Coulter CEQ 8000 Genetic Analysis System (Human Genetics Division, University of Southampton). This is a dye terminator cycle sequencing process which involves a PCR reaction containing the DNA, primer and DTCS Quick Start Master Mix, followed by ethanol precipitation. The sample is resuspended in loading solution, run on the CEQ Machine and analysed using CEQ Analysis Software.

2.9 RNA Extraction and Analysis

2.9.1 Total RNA Extraction

All RNA work was carried out using baked glassware (180°C for 4 hours) and DEPC-treated reagents, to ensure work was carried out in RNase-free conditions. Total RNA was extracted using Tri reagent (Sigma). 1 ml of reagent was added to tissue or cells and pipetted up and down to dissociate the material. This was left at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added and the solution mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The sample was then centrifuged at 10,000 rpm for 15 minutes at 4°C. This separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). The RNA containing phase was removed and transferred to a fresh tube, and the RNA

precipitated by adding 500 µl of isopropanol, with incubation at room temperature for 10 minutes, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C; the RNA forms a precipitate on the side and bottom of the tube. The supernatant was removed, the pellet washed with 75% ethanol, centrifuged at 7,000 rpm for 5 minutes at 4°C, air-dried and resuspended in DEPC-H_2O for storage at -80°C.

2.9.2 RNA Quality and Concentration

RNA was quantified by measuring the A260 using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

1 A_{260} Unit of single stranded RNA ~40 µg/ml

The quality of the RNA was estimated by the ratio between A260:A280, to ensure the solution was relatively free from proteins. An A260:A280 ratio of 1.8 is indicative of highly purified RNA.

2.9.3 First Strand cDNA Synthesis

This procedure converts 1-5 µg of total RNA into first strand cDNA using an oligo(dT) primer, which hybridises to the 3' poly(A) tail sequences found in most eukaryotic mRNAs (Figure 2.2). An RNA/primer mixture was prepared in a 10 µl volume containing 1-5 µg of total RNA, 0.5 mM dNTP, 0.5 µg oligo(dT) primer, and DEPC-H_2O. The RNA mixture was denatured at 65°C for 5 minutes. During this step a reaction mixture (maximum volume 7 µl) was prepared containing 1 × first strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, and 4 U RNase inhibitor (Promega). The reaction mixture was added to the denatured RNA mixture, followed by an annealing step at 42°C for 2 minutes. 200 U of Superscript III Reverse Transcriptase (RT; Invitrogen) was added to the solution and cDNA synthesised at 42°C for 50 minutes. The reaction was terminated at 70°C for 15 minutes and stored at -20°C.

As a control for genomic contamination in subsequent PCR reactions, 'minus RT' controls were prepared, containing DEPC-H_2O in place of Superscript III Reverse Transcriptase enzyme.

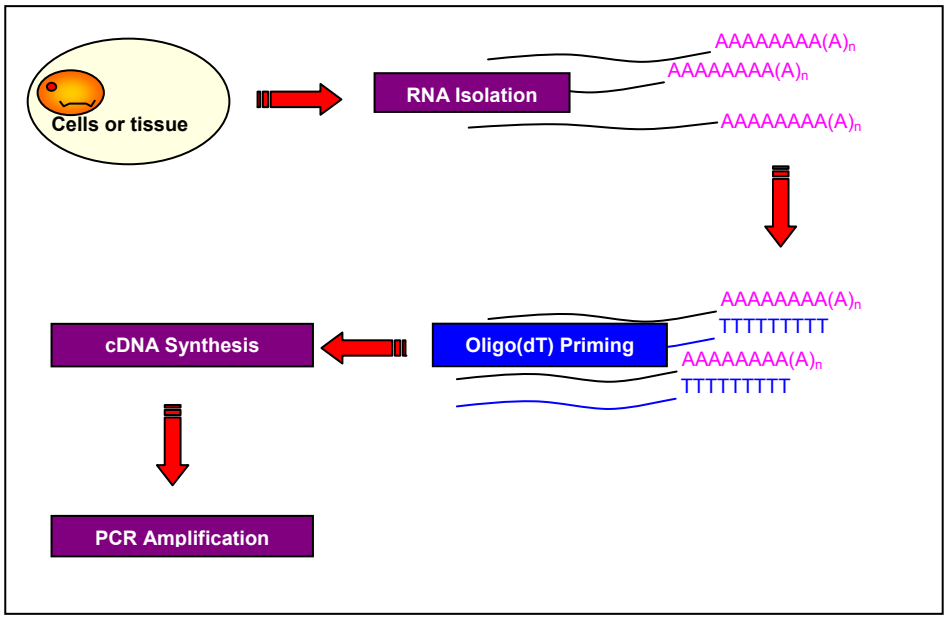


Figure 2.2 RT-PCR

Total RNA was extracted from tissue or cells using Tri reagent. First strand cDNA was synthesised using an oligo(dT) primer, which hybridises to 3' poly(A) tails found in the RNA. This cDNA can then be used to carry out conventional PCRs to identify expression of genes of interest.

2.10 Expression Microarray

2.10.1 RNA Extraction and Clean-up

RNA was extracted from male and female gonads as described (Section 2.9.1) and purified using the RNeasy MinElute Cleanup Kit (QIAGEN). The sample was adjusted to a volume of 100 µl with RNase-free water, and 350 µl Buffer RLT (containing 25-50% guanidinium thiocyanate and 143 mM β-mercaptoethanol)[16] added. 250 µl 96-100% ethanol[17] was added, an the solution applied to an RNease MinElute Spin Column in a 2 ml collection tube, and centrifuged for 15 seconds at 8000 g, and the flow-through discarded. The spin column was transferred into a new 2 ml collection tube, and 500 µl Buffer RPE (wash buffer containing ethanol) added, followed by centrifugation at 8000 g for 15 seconds. Further washing was performed by adding 500 µl 80% ethanol, and centrifuged for 8000 g for 2 minutes, followed by a further centrifugation at 5 minutes at

[16] β-mercaptoethanol irreversibly denatures RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality. Guanidinium isothiocyanate (GITC) is a strong but temporary denaturing agent, therefore the action of both these agents is to inactivate RNases.
[17] Ethanol is added to provide appropriate binding conditions.

full speed to remove residual ethanol. RNA was eluted in RNase-free water by centrifugation for 1 minute at maximum speed.

2.10.2 RNA Quality and Concentration

The quality and concentration of the RNA were analysed on an Agilent 2100 Bioanalyzer.

2.10.3 Expression Microarray

The expression microarray was conducted using the Amersham CodeLink Uniset Human 20K I Bioarray, which targets 21,000 transcripts using 30-mer probes. The expression microarray assay process is summarised in Figure 2.3. This work was performed by Dr. Feng Lin (Cancer Sciences Division, University of Southampton). Results were analysed using Amersham CodeLink Expression Analysis v4.1 and GeneSifter software.

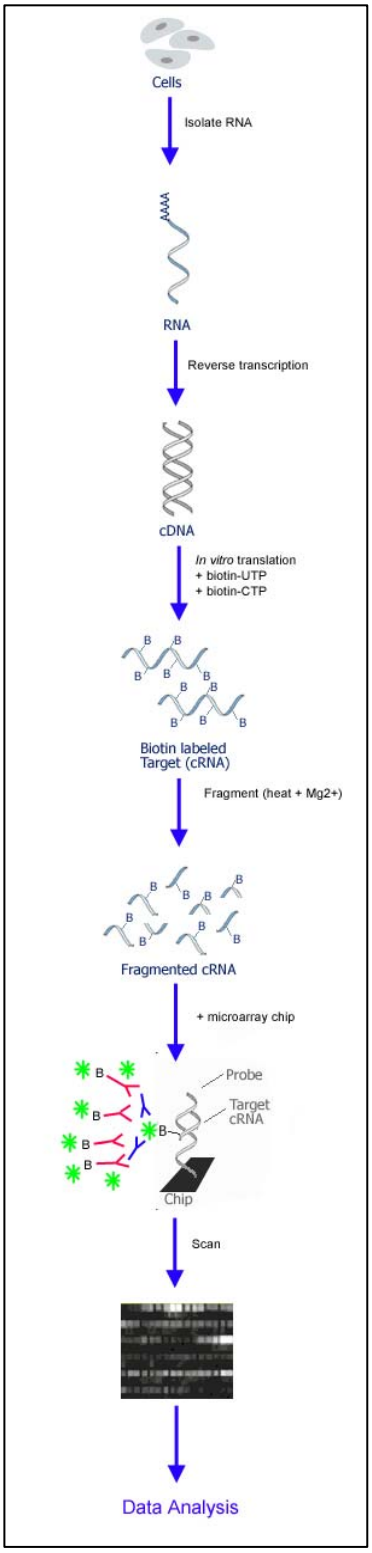


Figure 2.3 Summary of expression microarray procedure

Double stranded cDNA is synthesised from the RNA sample. This cDNA serves as a template in an *in vitro* transcription reaction that produces amplified amounts of biotinylated labelled antisense (copy) mRNA (cRNA). The cRNA is fragmented reducing it to 25-200 bp fragments and hybridised to the gene chip. The chip is then stained with streptavidin-phycoerythrin which binds to the biotin, scanned with a confocal laser and the fluorescent signal from phycoerythrin recorded.

2.11 Cell Culture and Transfections

2.11.1 Culture Conditions and Cell Maintenance

2.11.1(i) Human Embryonal and Pancreatic Carcinoma Cell Lines

The hECC line NTERA2 clone D1 (NT2) and the human pancreatic carcinoma cell line PANC1 were obtained from European Collection of Cell Cultures (ECCAC numbers 01071221 and 87092802 respectively). The hECC lines TERA1 and 2102Ep were a gift from Professor Peter Andrews (University of Sheffield). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; PAA Laboratories, Somerset, UK), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (all PAA Laboratories) in 5% CO_2/95% humidity at 37°C in a Heto-Holten Cell house incubator. NT2 cells were subcultured using sterile glass beads (VWR International Ltd., Poole, UK). Other cell lines were subcultured using sufficient Trypsin/EDTA (0.05%/0.02% in D-PBS; PAA Laboratories) to cover the bottom of the culture vessel and placed at 37°C until adherent cells detached (2-5 minutes). Trypsin was inactivated by adding horse serum (PAA Laboratories) and the contents transferred to a 50 ml tube. Further medium was added and the cells centrifuged at 1,000 rpm for 4 minutes. The pellet was resuspended in fresh culture medium and diluted to the appropriate density (~1:3 dilution for routine passaging) in fresh 75 cm^2 culture flasks.

2.11.1(ii) Human Embryonic Stem Cell Lines

The hESC lines HUES1 and HUES7 were obtained under MTA from Harvard University (Cowan *et al.* 2004) and maintained in Knockout (KO)-DMEM containing 1% penicillin/streptomycin, 1% Gluta-MAX, 0.1 mM non-essential amino acids, 10% KO-Serum Replacement (KO-SR; all Invitrogen), 10 ng/ml human recombinant fibroblast growth factor 2 (FGF2; Peprotech Ltd, London, UK) and 0.1 mM β-mercaptoethanol (Sigma) in 5% CO_2/95% humidity at 37°C. hESCs were cultured on mitotically-inactivated primary mouse embryonic fibroblasts (pMEFs; C5BL6 background; a gift from Dr. Neil Smyth, University of Southampton) and passaged using 1 mg/ml type IV collagenase (GIBCO, Invitrogen) with mechanical disaggregation using a cell scraper (Greiner Bio-One, Stonehouse, UK).

2.11.1(iii) Human Embryonic Germ Cells

- **Embryo Collection, Gonadal Dissection, and Disaggregation**

With local research ethics committee approval [Appendix 3 (i)-(iii)] and written informed consent, following Polkinghorne guidelines (Polkinghorne 1989), human fetuses at 7-9 wpc were collected at surgical termination of pregnancy. In total, 25 gonadal cell cultures were initiated. Dissection was carried out using stereomicroscopy, and gonads were washed in Hanks' Balanced Salt Solution (HBSS; PAA Laboratories). Gonads were mechanically disaggregated using hypodermic needles (0.4 × 40 mm; Sterican, Braun, Melsungen, Germany) and trituration, and then plated. Puncturing the intact organ is equally effective as enzymatic dissociation in generating hEGC cultures (Turnpenny *et al.* 2006), and avoids protracted washing and resuspension, along with any possible (although theoretical) damage caused to cell surface markers and receptors caused by proteolytic enzymes.

- **Cell Culture**

Mouse STO fibroblasts (American Type Culture Collection CRL-1503) were mitotically inactivated by exposure to 50 Gy of γ-radiation and plated in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (all PAA Laboratories). Dissociated PGCs were plated onto this feeder layer in KO-DMEM (Invitrogen), containing either 15% KO-SR (Invitrogen) or ESC-tested FCS (PAA Laboratories), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (both Sigma), 0.1 mM nonessential amino acids (Invitrogen), and antibiotics as above. To promote their survival, proliferation, and maintenance in the undifferentiated state, PGCs/EGCs were cultured in the presence of 10 μM forskolin (Sigma), 4 ng/ml human recombinant FGF2 (Peprotech) and 1,000 U/ml human recombinant leukaemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA, US). During the first 14 days, cultures were sacrificed or sampled for characterisation (see below). Cells were passaged using Trypsin/EDTA (0.05%/0.02% in D-PBS; PAA Laboratories) onto fresh feeder layers, and samples were taken for additional characterisation. All cultures were maintained in 5% CO_2/95% humidity at 37°C.

- **Characterisation of PGCs and EGCs**

Cells were fixed in 4% PFA in PBS (both Sigma), and dehydrated via 2 minute sequential steps in 50%, 70% and 100% ethanol. Alkaline phosphatase (AP) activity was detected using the colour substrates nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP; both Roche). 4.5 µl of each chemical was added per ml of detection buffer (100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl), the solution added to the fixed cells and left in the dark for 2 hours at room temperature. Cells were washed and mounted with glass cover slips using Aquamount (VWR).

2.11.2 Freezing and Thawing Cells

Cells were trypsinised and pelleted as described. The cell pellet was resuspended at a density of ~6 x 10^6 cells/ml in media containing 50% FCS, 40% DMEM (both PAA Laboratories) and 10% DMSO (Sigma) and aliquoted into cryotubes (Greiner Bio-one). Cells were frozen at a rate of 1°C/minute to -80°C in a Nalgene Cryo 1^{o}C freezing container and then transferred to storage in liquid nitrogen.

Frozen cells were thawed rapidly at 37°C, diluted with prewarmed medium and centrifuged at 1,000 rpm for 4 minutes. The cell pellet was resuspended in medium and transferred to a culture vessel.

2.11.3 Transient Transfections

OCT4 and NANOG expression vectors (3 µg total DNA) were transfected into primary cells (stomach epithelial cells and neuroprogenitors) by nucleofection (Amaxa GmbH, Cologne, Germany). Cells were pelleted by centrifugation, resuspended in 100 µl Nucleofector Solution V, DNA added, put into a cuvette and nucleofected using the X-05 program[18] on The Nucleofector (Amaxa). Media was added, and cells were plated directly into 8-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, US), with media being changed 24 hours later, and the cells being fixed with 4% PFA after 48 hours.

[18] A selection of programs and Nucleofector solutions were tested with the pmaxGFP vector (GFP expression vector [3.49 kb] provided in the Nucleofector kit). The X-05 program was found to transfect the most primary cells (neuroprogenitors).

Cells were either plated on pMEFs in hESC medium (Section 2.11.1[ii]); or directly onto glass in NT2 conditioned medium[19], to mimic ESC-like growth conditions.

2.11.4 G418 Titration ('Kill Curve Analysis')

Cells successfully transfected with pSUPER.neo/gfp can be selected using the antibiotic G418 (PAA Laboratories), as the plasmid contains a neomycin resistance gene[20]. The optimal concentration of G418 for the selection of stable NT2 clones was determined by titration. NT2 cells were plated at ~80% confluency onto 6-well plates (Iwaki Glass Co. Ltd., Tokyo, Japan) and incubated with varying concentrations of G418 (0, 25, 50, 100, 150, 200, 300, 400, 500 μg/ml) for two weeks. The concentration of selection agent chosen was the least amount which caused total cell death in this period: 200 μg/ml G418.

2.11.5 Generation of Stable Transfectants using Calcium Phosphate-Mediated Transfection

24 hours prior to transfection, NT2 cells were trypsinised and plated at 8×10^5 cells per 100 mm culture plate. Three hours prior to transfection, the medium was removed from the cells and replaced with fresh growth medium. Cells were transfected with DNA using the ProFection Mammalian Transfection System (Promega). 15 μg of DNA (pSUPER.neo/gfp) was added to 62 μl 2M $CaCl_2$ and sterile, deionised water added to 500 μl. This solution was added dropwise to 500 μl of 2 × HEPES-Buffered Saline (HBS), and incubated at room temperature for 30 minutes[21]. The solution was vortexed and added to cells; 5 hours later the culture medium was replaced with fresh growth medium to minimise cell toxicity. After 24 hours, 200 μg/ml G418 was added, and 48 hours later the cells were subcultured 1:2 and maintained in medium containing 200 μg/ml G418. Media was changed every 3 days, and approximately 2 weeks later, green colonies were visible; these were trypsinised using cloning cylinders and transferred to multiwell plates for further propagation in selective growth medium.

[19] Collected after 24 hours and filter-sterilised.
[20] The gene for aminoglycoside 3'-phosphotransferase.
[21] The solution appears cloudy due to the formation of a fine calcium phosphate-DNA coprecipitate.

CHAPTER 3: Expression of SOX2 within the Human Germ Cell Lineage

3.1 ABSTRACT

OCT4, NANOG and SOX2 act cooperatively to maintain pluripotency in cells of the inner cell mass (ICM) of human and mouse blastocysts, and in the derived embryonic stem cells (ESCs). Knock-out/down-regulation of any one of these transcription factors causes loss of pluripotency. Pluripotent mouse embryonic germ cells (mEGCs), and their founder cells, mouse primordial germ cells (mPGCs), also express Oct4, Nanog and Sox2. Thus, a similar expression profile could be anticipated for human PGCs (hPGCs) and hEGCs. However, by RT-PCR, immunoblotting and immunohistochemistry, hPGCs were found to express OCT4 and NANOG, but not SOX2, with no evidence of redundancy within the Group B family of human *SOX* genes. Although lacking SOX2, proliferative human germ cells can still be identified *in situ* during early development and can be cultured *in vitro*. Upon conversion to the hEGC state, which fulfills some but not all criteria for pluripotency, cells remain SOX2-negative, indicating *SOX2* expression is not required for conversion to EGC. Surprisingly, with the exception of *FGF4*, many stem cell-restricted SOX2 target genes remained detected within the human SOX2-negative germ cell lineage. The hPGC is the first primary cell-type described to express *OCT4* and *NANOG* but not *SOX2,* exemplifying a striking difference in gene expression between human and mouse. The data also provide a new reference point for studies attempting to turn human stem cells into gametes by normal developmental pathways, for the treatment of infertility.

3.2 INTRODUCTION

3.2.1 Mouse ESCs and EGCs

Original experiments in mice have demonstrated that ESCs and EGCs are, unequivocally, two types of pluripotent stem cell. The former are derived from the ICM of the blastocyst taken into laboratory culture; the latter are presumed to represent an analogous *in vitro* conversion of the PGC, the diploid precursor of the male and female gametes. The criteria which mark the derivation of EGCs from PGCs are a suspension of meiosis and a state of rapid self-renewing proliferation. Mouse EGCs (mEGCs) were first derived in 1992 independently by two groups (Matsui *et al.* 1992; Resnick *et al.* 1992). Derivation is most successful from earlier stages of development, including from PGCs still migrating (McLaren and Durcova-Hills 2001). Derivation efficiency is high: around 95% of cultures initiated generate EGC lines (Shamblott *et al.* 2004). In many respects, mEGCs resemble mESCs, derived eleven years earlier. Both satisfy all criteria for pluripotency *in vitro* and *in vivo*. Both their precursor cells (i.e. cells of the ICM/PGCs) and their derived cell types (ESCs/EGCs) are diploid and express the pluripotency related genes *Oct4*, *Nanog* and *Esg1* (Western *et al.* 2005), and share AP activity (Ginsburg *et al.* 1990) and SSEA1 expression (Solter and Knowles 1978), along with that of other markers (Table 3.1).

Both mESCs and mEGCs grow rapidly and indefinitely *in vitro* while retaining pluripotency and tumourigenic capability (Matsui *et al.* 1992; Stewart *et al.* 1994). They both show comparable cell cycle phase distribution (Rohwedel *et al.* 1996), and cell cycle-related gene transcripts and proteomic signatures are indistinguishable based on global gene expression alone (Kurosaki *et al.* 2007; Sorrentino *et al.* 2007); although detailed comparison identified some differences (Sharova *et al.* 2007). In addition, minor dissimilarities in differentiation capabilities have been noted: in the presence of retinoic acid, EGCs show lower expression of muscle- and cardiac-related genes and a higher expression of gonad-related genes (Sharova *et al.* 2007). EGCs differentiate more efficiently to neuronal cells and less efficiently to cardiac and skeletal muscle cells than ESCs (Rohwedel *et al.* 1996), and fail to differentiate in co-culture with lung tissue (Durcova-Hills *et al.* 2003). Another difference between the cells is the DNA methylation patterns of some imprinted genes. During migration and maturation of PGCs, the somatic status of imprinted genes is progressively erased (Yamazaki *et al.* 2003), and EGCs can

retain some of these differences, for example reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova *et al.* 2002).

Gene	Species	ESCs	Early GCs	Late GCs	ICM
Oct4 (Pesce and Scholer 2001)	M	+	+	+	+
Nanog (Chambers *et al.* 2003)	M	+	+	+	+
Stella[22] (Saitou *et al.* 2002)	M	+	+	+	+
Fragilis (Saitou *et al.* 2002)	M	+	+	+	+
c-Kit (Horie *et al.* 1991)	M	+	+	+	-
Dazl (Pan *et al.* 2008)	H	+	+	+	-
Ddx4 (Toyooka *et al.* 2003)	M	-	-	+	-
Akp2[23] (Chiquoine 1954)	M	+	+	+	+
Rex1[24] (Rogers *et al.* 1991)	H	+	-	+	+

Table 3.1 Comparison of marker expression between embryonic stem cells (ESCs), early and late germ cells (GCs), and the inner cell mass (ICM)

+ denotes expression at that developmental stage; – denotes no expression. Abbreviations; Dazl, deleted in azoospermia-like; Ddx4, Dead (Asp-Glu-Ala-Asp) box polypeptide 4; Akp2, alkaline phosphatase 2, liver; Rex1, reduced expression 1; H, human; M, mouse. [Adapted from Zwaka and Thomson (2005).]

The similarities between ESCs and EGCs have led to the hypothesis that ESCs are derived from PGC precursors that originated from the ICM (Zwaka and Thomson 2005). This theory is further supported by data which demonstrate that the SCF-KIT signal transduction pathway is required for ESC differentiation *in vitro*: there is no pre-implantation defect in mice lacking either KIT or SCF function, indicating this pathway is not essential for ICM differentiation *in vivo*. Therefore, ESCs *in vitro* may not accurately reflect their *in vivo* counterpart, the ICM (Chambers and Smith 2004), and may have more resemblance to PGCs where the SCF-KIT pathway plays an important role in survival and differentiation (Godin *et al.* 1991; Matsui 1998; Kissel *et al.* 2000).

[22] Also known as Dppa3 (Developmental pluripotency associated 3).
[23] Also known as Tnap (Tissue non-specific alkaline phosphatase).
[24] Also known as Zfp42 (Zinc finger protein 42).

3.2.2 Human ESCs and EGCs

Human EGC (hEGC) derivation has been less frequently reported, by only a handful of groups (Shamblott *et al.* 1998; Turnpenny *et al.* 2003; Liu *et al.* 2004; Aflatoonian and Moore 2005; Pan *et al.* 2005). This is due, in part, to the limited availability of human embryonic/fetal material. However, it also reflects the difficulties encountered with hEGC culture: the cells are difficult to derive, in our experience only approximately one-eighth (≥14%) of cultures demonstrate 'EG-like' properties, and they are difficult to maintain over prolonged passaging (Turnpenny *et al.* 2003). No groups have maintained hEGCs indefinitely in culture; our experience is that loss occurs after 10-12 passages, and is exacerbated by freeze-thaw routines (Turnpenny *et al.* 2003; Turnpenny *et al.* 2006). No lines have been banked in public depositories, and the cells have not been clonally expanded thus far. The only report describing continuous culture of hEGCs for over a year (Park *et al.* 2004) was subsequently retracted. It is possible that the ease of mEGC derivation is related to the high susceptibility of the 129/sv strain to spontaneously developing teratocarcinomas[25], due to a recessive mutation in the *ter* gene (Noguchi and Noguchi 1985). Human TGCTs do not have this tendency towards embryonal carcinomas: half of human TGCTs are seminomas, which lack a stem cell component and are difficult to culture *in vitro*: only one human seminoma cell line has been grown, likely facilitated by an activating mutation in the *BRAF* oncogene (de Jong *et al.* 2008).

Germ cells could potentially be of therapeutic benefit, if pure populations could be isolated. In addition, further research might provide insight into cancer cell biology. TGCTs, which originate from a precursor cell, the carcinoma *in situ* cell, are of germ cell antecedence (Skakkebaek 1972), and have increased in incidence over the last several decades; they now constitute the most prevalent malignant cancer among young male adults in Northern and Western Europe (Richiardi *et al.*, 2004).

In addition, hEGCs may provide a comparator with which to study hESC pluripotency, offering the opportunity to define important factors, markers and signalling pathways. They could provide an additional tool with which to study aspects of human development. Relatively little is known regarding human development from 14 days (the time limit for human embryo culture under the Human Fertilisation and Embryology Act)

[25] Teratocarcinomas are comprised of teratoma and embryonal carcinoma; the stem cell component is contained within the embryonal carcinoma compartment.

to the receipt of material from social (or voluntary) termination of pregnancy at approximately 7-12 weeks of gestation, and hEGCs provide another pluripotent cell type with which to mimic these events, and provide a comparator to hESCs.

Information gleaned from studying the human germ line will provide a reference point for studies aiming to replicate normal development for the *in vitro* generation of gametes from human stem cell populations for future fertility treatment (Nagano 2007). Germ cell induction from mESCs has been reported by a number of groups (Hubner *et al.* 2003; Toyooka *et al.* 2003; Geijsen *et al.* 2004), with one reporting the successful production of offspring (Nayernia *et al.* 2006). Furthermore, hESCs can differentiate to cells expressing PGC and meiotic markers (Clark *et al.* 2004; Aflatoonian and Moore 2006; Clark and Reijo Pera 2006). However, germ cell induction from ESCs is currently a rare event. In addition, it is not clear whether the *in vitro* process follows the same pathway as that of normal germ cell development *in vivo*.

Work described within this chapter sought to further characterise aspects of the human germ cell lineage, specifically comparing hEGCs to PGCs, the ICM, ESCs and ECCs. The difficulties in initiating hEGC cultures and maintaining them in the long-term led to the postulation that they may lack some factor present in mEGCs, or they may fail to induce expression of a factor which would maintain their pluripotency.

3.3 SPECIFIC AIMS

3.3.1 To characterise hEGCs in comparison to PGCs, ESCs and ECCs.

OCT4, SOX2 and NANOG are proposed to maintain pluripotency in human and mouse ESCs (Boyer *et al.* 2005; Loh *et al.* 2006). The expression of these transcription factors was examined in hEGCs and their precursors, hPGCs, and in hESCs, by RT-PCR, immunohistochemistry and immunoblotting, and also compared to the mouse germ cell lineage.

3.3.2 To characterise the expression of transcription factors central to maintaining pluripotency in pluripotent and nullipotent hECC lines.

This involved determining whether pluripotency/nullipotency of hECC lines correlated with expression of key transcription factors involved in maintaining pluripotency.

3.3.3 To analyse the expression of OCT4, NANOG and SOX2 target genes in the various pluripotent stem cells and their starting cell types.

OCT4, SOX2 and NANOG are proposed to co-occupy over 350 gene promoters in hESCs, activating transcription from those which maintain pluripotency and repressing those which promote differentiation (Boyer *et al.* 2005). OCT4 and SOX2 are known to act cooperatively in up-regulating *FGF4* (Ambrosetti *et al.* 1997), *UTF1* (Nishimoto *et al.* 1999) and *FBX15* (Tokuzawa *et al.* 2003) transcription. Expression of these genes, and a further 13 genes which are expressed in ESCs when their respective promoter regions are bound by OCT4, NANOG and SOX2 (Boyer *et al.* 2005), were analysed by RT-PCR in human fetal gonads, hESCs and hECCs.

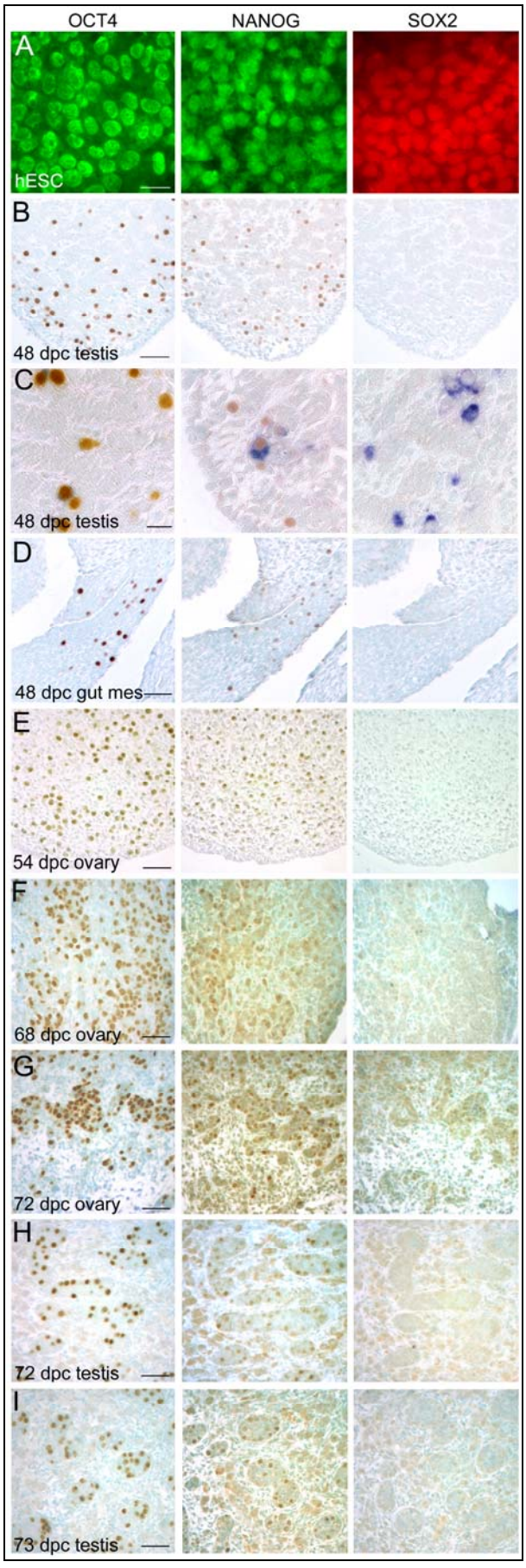
3.4 RESULTS

3.4.1 Primordial Germ Cell Expression of *SOX2* Differs between Human and Mouse

The human fetal gonad is apparent as a distinct structure from 32 dpc with sex determination marked by the expression of *SRY* and *SOX9* at ~41-44 dpc. Thereafter, sex cords comprised of Sertoli cells and germ cells become increasingly apparent within the testis during late embryonic (up to 56 dpc) and early fetal (thereafter) development (Hanley *et al.* 2000). OCT4, NANOG and SOX2 are known as three critical transcription factors in hESCs (Boyer *et al.* 2005) and were expressed accordingly (Figure 3.1 A). Nuclear OCT4 and NANOG proteins were clearly detected within germ cells in the gonad of the embryonic ovary and testis ranging from Carnegie stages 19-23 (48-56 dpc), and in the early fetus (56-73 dpc; Figure 3.1 B, D-H). In sharp contrast, SOX2 was not detected within the human female or male gonad either prior to sex determination or later during the first trimester (48-73 dpc; Figure 3.1 B, D-H). The expression of some genes, such as *Ddx4*, is altered upon the arrival of the PGC to the gonadal ridge in mice (Tanaka *et al.* 2000). Therefore, PGCs were also studied within the gut mesentery at 48 dpc, a location consistent with their migration from yolk sac wall to gonadal ridge. Nuclear OCT4 and NANOG were again robustly detected; however, SOX2 was absent (Figure 3.1 C).

Figure 3.1 OCT4, NANOG and SOX2 expression in hESCs and late embryonic/early fetal ovary and testis

A. hESCs (HUES1); **B.** 48 dpc testis; **C.** Immunohistochemistry [IHC] combined with AP staining; **D.** 48 dpc gut mesentery; **E.** 54 dpc ovary; **F.** 68 dpc ovary; **G.** 72 dpc ovary; **H.** 72 dpc testis; **I.** 73 dpc testis. Abbreviation: gut mes, gut mesentery. Size bars represent 50 μm (A,B,D-I) and 20 μm (C).



[For legend see facing page]

Sox2 transcripts have been demonstrated in the gonadal ridge of mouse embryos (Western *et al.* 2005). Consistent with this finding, nuclear Sox2 protein was present in PGCs within the mouse embryonic testis and ovary (E13.5 and E12.5 respectively; Figure 3.2 A, B). As the PGC is the precursor to the EGC, the ICM is the starting cell-type for ESC derivation. Nuclear Sox2 was also clearly detected within the ICM of mouse blastocysts (Figure 3.2 D) (Avilion *et al.* 2003). In human blastocysts, SOX2 detection was more diffuse but included nuclear localisation within the ICM (Figure 3.2 C). These data, demonstrating an inter-species difference in PGC SOX2/Sox2 expression, were further validated by immunoblotting of protein isolated from fetal gonads (Figure 3.2 E). SOX2 was present as a 35 kDa band in protein isolates from mouse gonads at E14 but not from the corresponding human organs. OCT4 detection is shown as a positive control for the presence of PGCs.

3.4.2 SOX2-Negative Germ Cells are Proliferative *in situ*

The SOX2-negative human germ cells included ones that were proliferative *in situ*, as demonstrated by dual immunoreactivity for OCT4 and the proliferative marker MK167, marked by the Ki67 antibody[26] (Figure 3.3). There were more of these double-stained cells in the embryonic ovary (Figure 3.3 A) than in the embryonic testis (Figure 3.3 B), consistent with male PGCs entering mitotic arrest following testicular cord formation.

[26] Ki-67 is a commercially available monoclonal antibody that reacts with a nuclear antigen expressed in proliferating cells but not in quiescent cells. Expression of this antigen occurs preferentially during late G1, S, G2, and M phases of the cell cycle, while in cells in G0 phase the antigen cannot be detected.

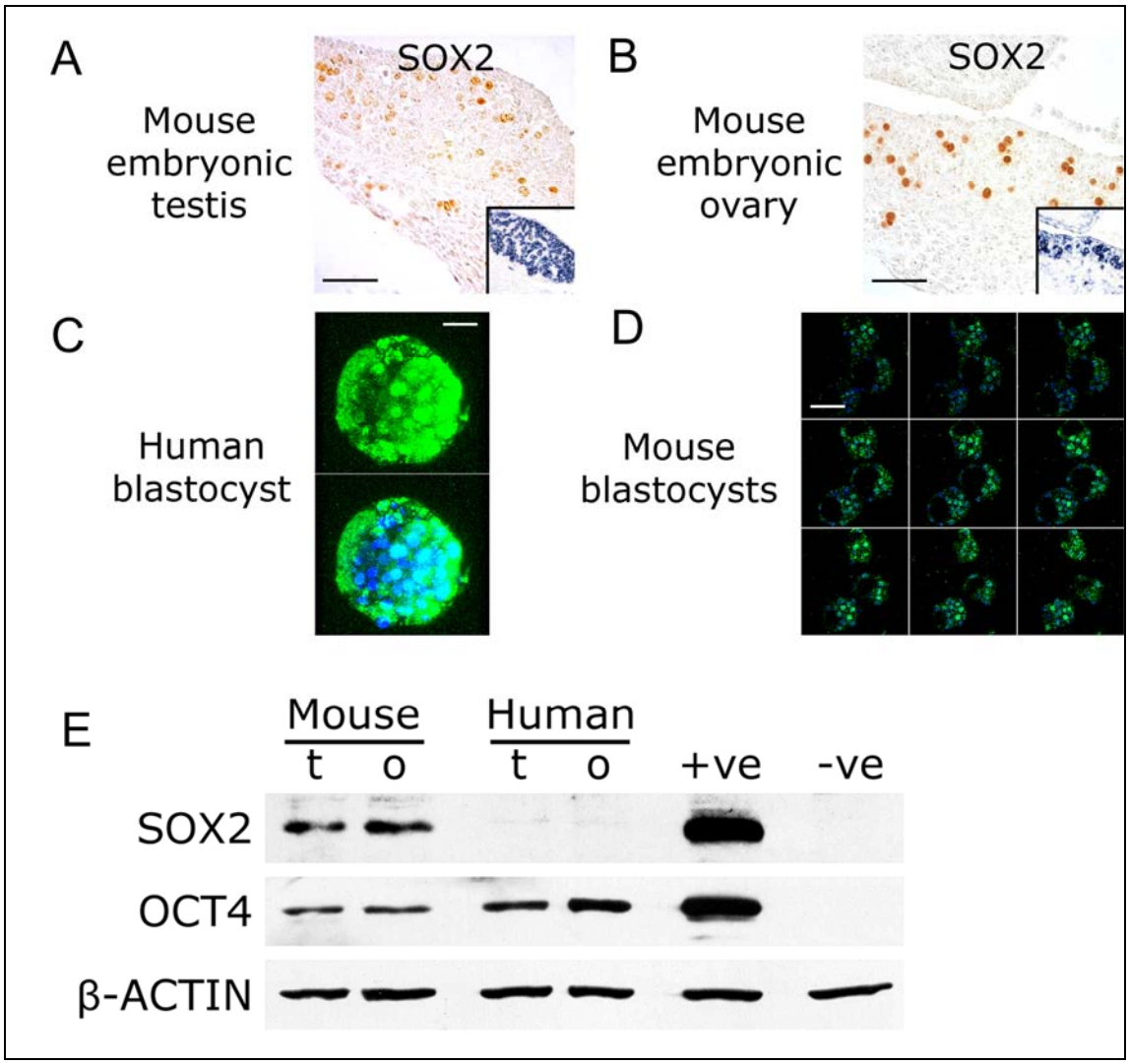


Figure 3.2 SOX2 expression in human and mouse embryonic gonads and blastocysts

Immunocyto/histochemistry for SOX2 in mouse embryonic testis (**A**; E13.5), ovary (**B**; E12.5; insets show IHC combined with AP staining), and human (**C**) and mouse (**D**) blastocysts. Size bars represent 50 μm. **E.** Immunoblotting for the detection of SOX2, OCT4 and β-actin in mouse and human embryonic gonads. Positive control, NTERA-2 D1 hECCs; negative control, PANC1 cells. Ovarian and testicular samples were prepared from at least three human fetuses. [Parts C and D Dr. Judith Eckert, Developmental Origins of Health and Disease Division, University of Southampton.]

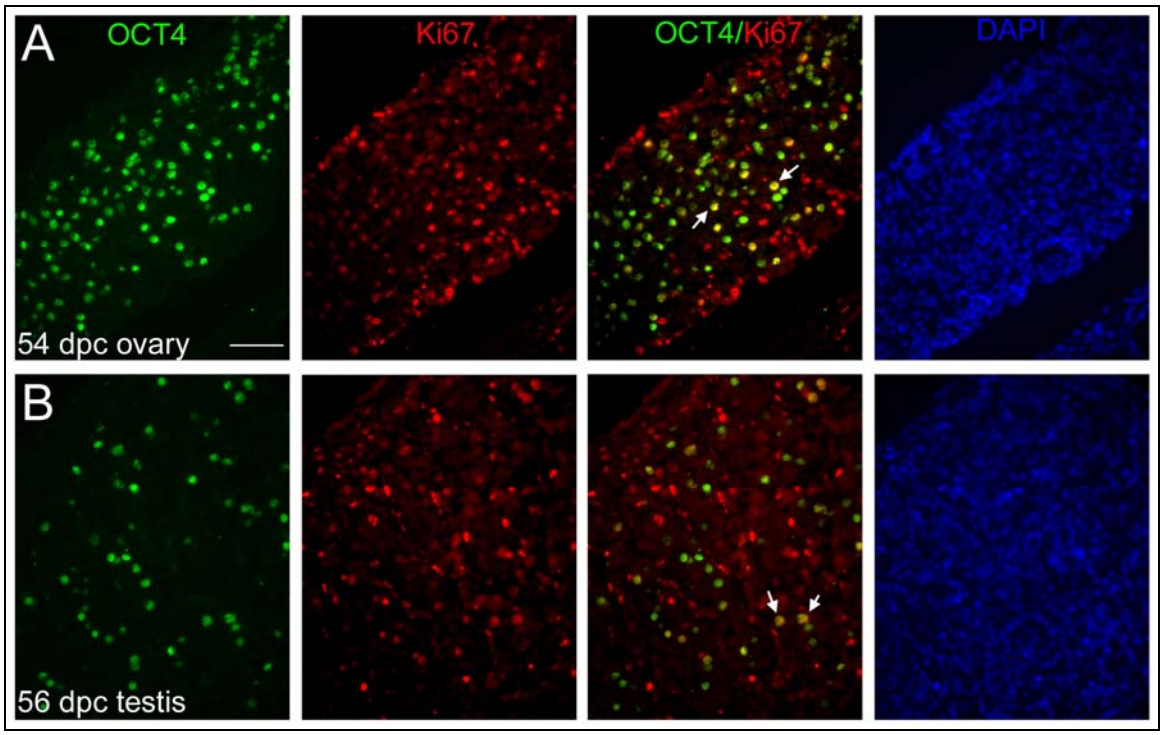


Figure 3.3 PGCs are proliferative *in situ* during early human development
Dual IHC for OCT4 and the proliferation marker MK167, identified using the Ki67 antibody, in human embryonic ovary (**A**) and testis (**B**). Arrows point to examples of PGCs that are dual stained. From left to right, individual band pass images are shown for FITC (OCT4), Texas Red (Ki67), dual band pass image (OCT4 and Ki67) and DAPI. Size bar represents 100 μm.

3.4.3 Redundancy is Unlikely to Compensate for Absence of SOX2 in Human Germ Cells

One potential explanation for this species difference is redundancy for SOX2 within Group B of the *SOX* gene family, which comprises *SOX1*, *SOX2*, *SOX3* (the SOXB1 group), and *SOX14* and *SOX21* (SOXB2). This group shows significant homology both between and within the B1 and B2 groups (Figure 3.4).

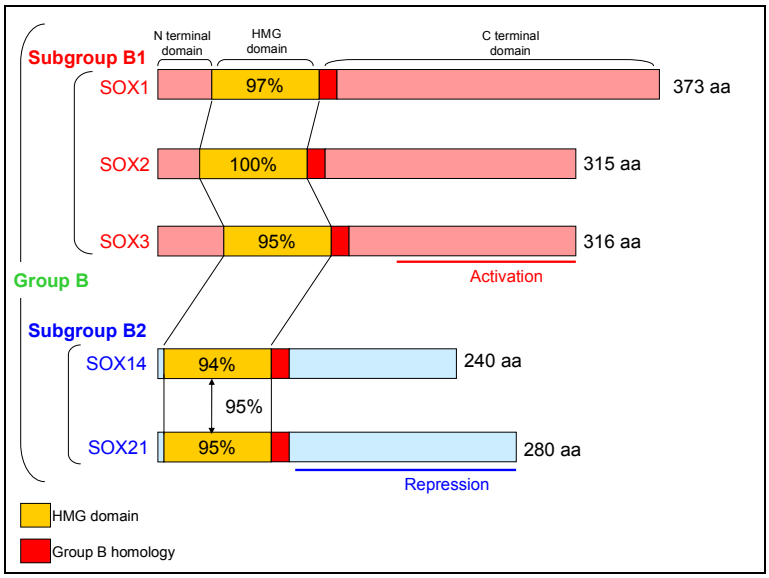


Figure 3.4 Comparison of the domain organisation of chick SOXB proteins
Conserved subdomains within the B1 and B2 subgroups are indicated. Between subgroups B1 and B2, homology is found within the Group B homology domain, depicted in red, in addition to the HMG domain. [Adapted from Kamachi *et al.* (2000).]

By RT-PCR for 35 cycles (Figure 3.5), *SOX2*, *SOX3*, *SOX14* and *SOX21* transcripts were detected robustly in both hESCs (HUES1) and hECCs (NTERA-2 D1). The weak detection of *SOX1* in hESCs may represent low level expression *per se* or, feasibly, minor spontaneous ectodermal differentiation as is commonly encountered in hESC culture. However, all transcripts were absent in four samples of human testes collected during the late embryonic and early fetal periods (51-59 dpc). Only *SOX14*, encoding a putative repressor of SOX2 function (Uchikawa *et al.* 1999), was clearly detected within the ovary after amplification for 35 cycles. In four ovarian samples ranging from 51 to 61 dpc, expression of *SOX1* and *SOX21* was weakly discernable in some samples. The failure to detect *SOX2* transcripts within these testicular and ovarian samples following extensive PCR cycles corroborates the protein data from immunohistochemistry and immunoblotting (Figure 3.1 and Figure 3.2).

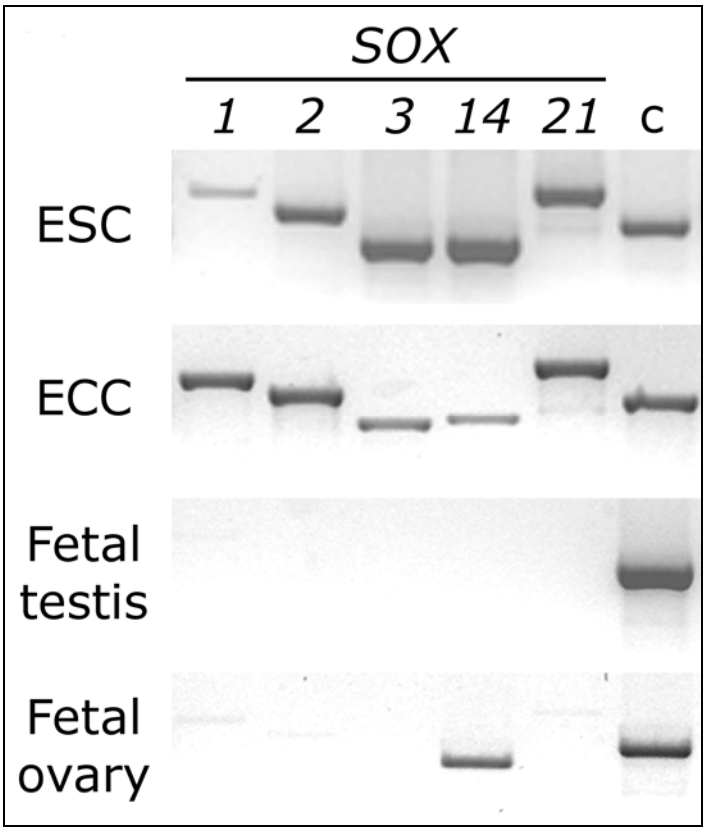


Figure 3.5 The potential for SOX2 redundancy in the human germ cell lineage
RT-PCR panel is shown for members of the Group B *SOX* gene family in hESCs (HUES1), hECCs (NTERA-2 D1 cells), and one of four samples each of human fetal testis and fetal ovary at 8 wpc, following PCR for 35 cycles. Abbreviations: c, control - the housekeeping gene *HPRT (hypoxanthine-guanine phosphoribosyl-transferase)*; ECC, embryonal carcinoma cell; ESC, embryonic stem cell. For primer sequences see Appendix 1.

3.4.4 SOX2 in Human Embryonal Carcinoma Cell Lines

The ECC represents malignant transformation of the PGC. SOX2 expression was examined in different ECC lines. In contrast to NTERA-2 D1 cells, once taken into culture some ECs have yielded nullipotent cell lines that are no longer capable of differentiation to derivatives of all three germ layers, for example TERA-1 and 2102Ep cells (Damjanov 1990; Harrison *et al.* 2007). However SOX2 expression did not correlate with pluripotency versus nullipotency: it was clearly expressed equivalently in both pluripotent and nullipotent hECCs localising indistinguishably to the nucleus in all lines tested (Figure 3.6 A-C), and confirmed by immunoblotting (Figure 3.6 D).

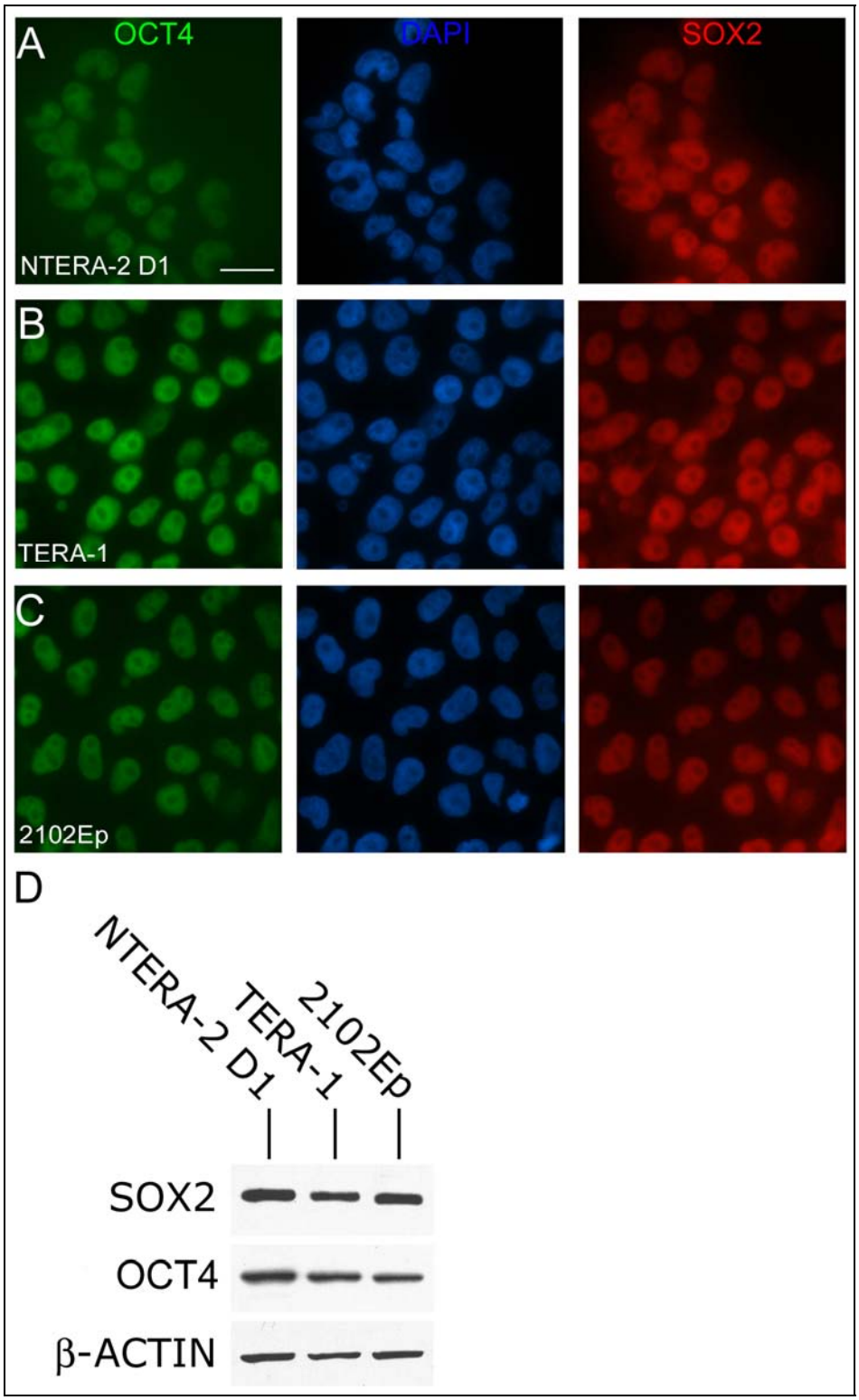


Figure 3.6 OCT4 and SOX2 expression in hECCs

A-C: Immunocytochemistry for OCT4 and SOX2 in hECC lines fixed after culture on fibronectin-coated glass slide. **A.** pluripotent hECC line, NTERA-2 D1 and nullipotent hECC lines **B.** TERA-1 and **C.** 2102Ep. Individual band pass images are shown for FITC (left: OCT4), DAPI, and Texas Red (right: SOX2). Size bar represents 40 μm. **D.** Immunoblotting for the detection for SOX2, OCT4 and β-actin in NTERA-2 D1, TERA-1 and 2102Ep hECC lines.

3.4.5 Human Germ Cells Persisting in Culture *in vitro* Remain SOX2-Negative

Human germ cell cultures have been previously assigned as 'poorly proliferative' ('PP') or 'vigorously proliferative' ('VP'), according to their growth characteristics, with the latter taken as indicative of conversion to the EGC state (Turnpenny *et al.* 2003). Akin to ESCs, hEGCs demonstrate expression of the nuclear transcription factor OCT4 and AP activity (Turnpenny *et al.* 2006). However, SOX2, required for the maintenance of pluripotent ESCs (Avilion *et al.* 2003), was not expressed in the human germ line *in situ*. In order to test whether SOX2 expression was induced in culture, numerous human gonad-derived cultures were established and samples of both PP and VP/EGC cultures were analysed. OCT4/*OCT4* and AP activity served as positive controls for the presence of the germ cell lineage. Neither SOX2 protein nor *SOX2* transcripts were detected by immunoblotting or RT-PCR, respectively, in either early, PP or VP/EGC cultures (Figure 3.7).

3.4.6 SOX2 is Dispensable for the Expression of Genes Previously Identified to Require OCT-SOX Interaction or Cooperative OCT4-SOX2-NANOG Function

Given the absence of SOX2 in human germ cells, yet the presence of both OCT4 and NANOG, a selection of genes which are recognised as targets of these critical transcription factors were investigated. OCT4 and SOX2 are known to act cooperatively in up-regulating *FGF4* (Ambrosetti *et al.* 1997), *UTF1* (Nishimoto *et al.* 1999) and *FBX15* (Tokuzawa *et al.* 2003). However, despite the absence of SOX2, *UTF1* and *FBX15* transcripts were detected in the human fetal ovary and testis. In contrast, *FGF4* was absent (Figure 3.8 A). A further set of 13 genes were analysed, which are expressed in ESCs when the respective promoter regions are bound by OCT4, NANOG and SOX2 (Boyer *et al.* 2005). All 13 stem cell-restricted transcripts were detected in fetal gonads, albeit more weakly for *DKK1*, *SET*, *ZIC3* and *STAT3* in the human fetal ovary (Figure 3.8 B).

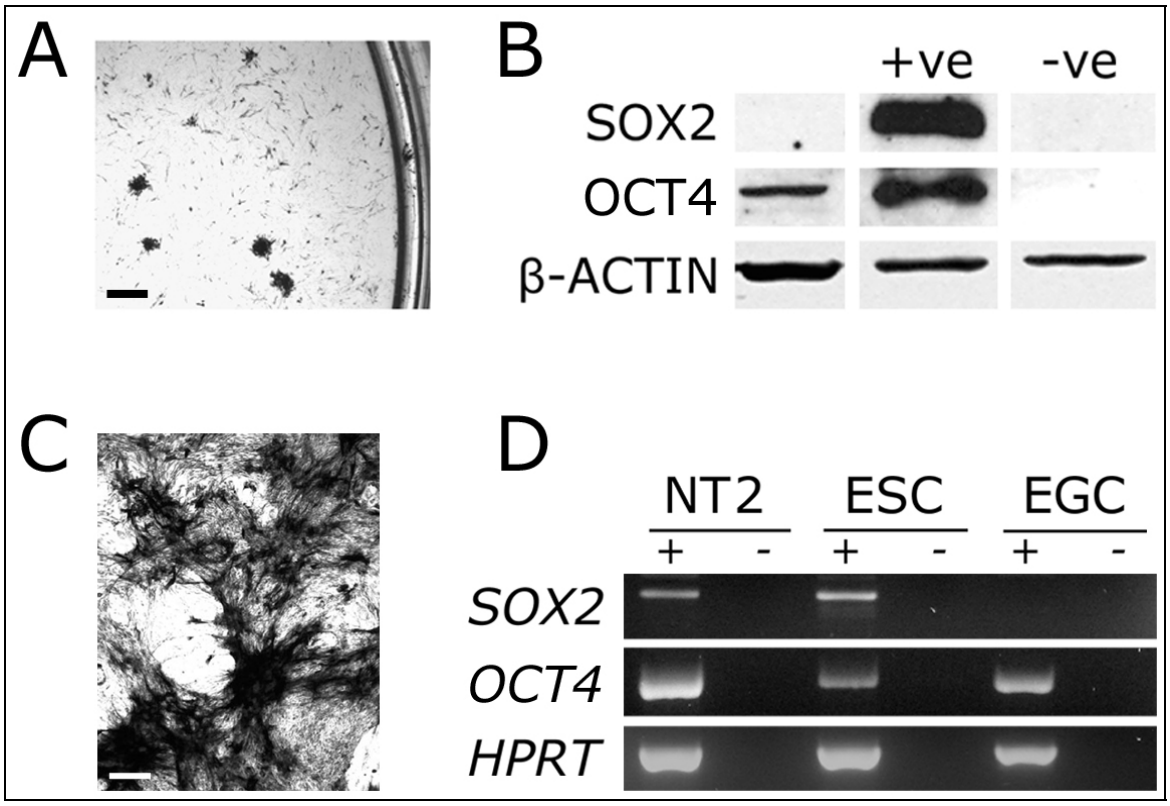


Figure 3.7 SOX2 is not detected in human germ cell cultures

A. Image of germ cell-derived culture showing colonies after one week that were positive for AP activity. Size bar represents 2 mm. **B.** Immunoblotting for the detection of SOX2, OCT4 and β-actin. Left column, germ cell culture; positive control, NTERA-2 D1 cells; negative control, PANC1 cells. Four OCT4-positive/AP-positive cultures were analysed after one week and SOX2 was not detected in any of them. **C.** Image of a passaged germ cell culture fulfilling VP/EGC criteria. Size bar represents 250 μm. **D.** RT (+) of mRNA isolated from the EGC culture shown in C after 3.5 weeks and PCR for 32 cycles. NTERA-2 D1 cells (NT2) and ESCs are shown as positive controls. Omission of RT (-) is shown as negative control. The same results were obtained from another EGC culture. [Parts C and D Dr. Lee Turnpenny, Human Genetics Division, University of Southampton.] For primer sequences see Appendix 1.

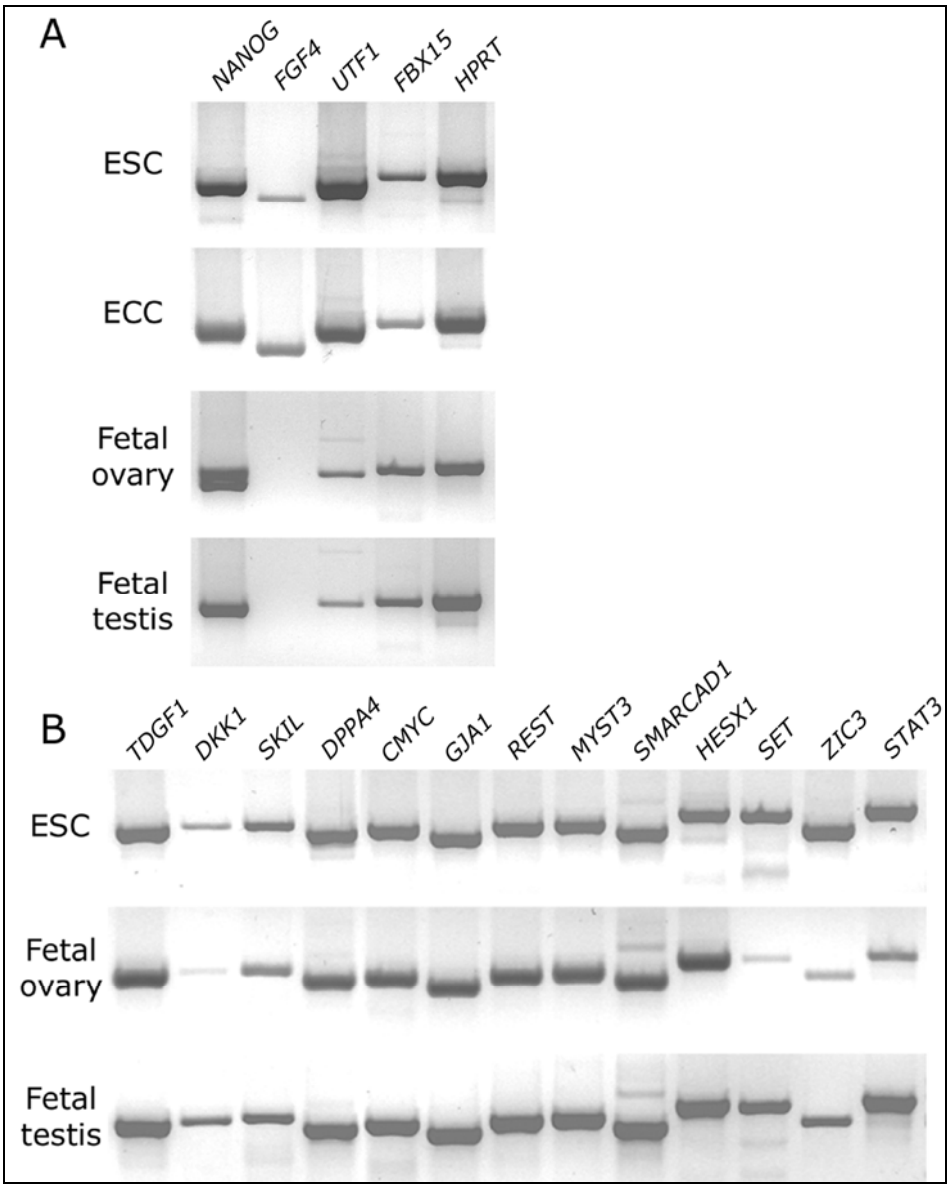


Figure 3.8 RT-PCR analysis of genes regulated by SOX-OCT enhancers and those identified by bioinformatic analysis as targets of OCT4, NANOG and SOX2, following PCR for 35 cycles

A. Genes previously recognised as targets of OCT4 and SOX2 (Ambrosetti *et al.* 1997; Nishimoto *et al.* 1999; Tokuzawa *et al.* 2003) and **B.** genes previously described to be expressed in human ESCs when OCT4, NANOG and SOX2 are bound to the respective promoter regions (Boyer *et al.* 2005). *NANOG* and *HPRT* are shown in A as controls. Abbreviations[27]. For primer sequences see Appendix 1.

[27] FGF4, fibroblast growth factor 4; UTF1, undifferentiated embryonic cell transcription factor 1; FBX15, F-box only protein 15; HPRT, hypoxanthine-guanine phosphoribosyl-transferase; TDGF1, teratocarcinoma-derived growth factor 1; DKK1, Dickkopf Xenopus, homolog of 1; SKIL, SKI-like; DPPA4, developmental pluripotency associated gene 4; CMYC, myelocytomatosis oncogene; GJA1, gap junction protein, alpha 1; REST, RE-1 silencing transcription factor; MYST3, MYST histone acetyltransferase; SMARCAD1, SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1; HESX1, homeobox gene expressed in ESCs; SET, suppressor of variegation, enhancer of zeste and Trithorax; ZIC3, zinc finger protein of cerebellum; STAT3, signal transducer and activator of transcription 3; ECC, embryonal carcinoma cell; ESC, embryonic stem cell.

3.5 DISCUSSION

Studies have demonstrated that pluripotent stem cells share gene and marker expression, growth characteristics and tumourigenic capability. hEGCs share some of these characteristics: these cells express the transcription factors OCT4 and NANOG, the surface markers SSEA4, TRA1-60 and TRA1-81, AP activity, and differentiate *in vitro* (Shamblott *et al.* 1998; Turnpenny *et al.* 2003; Liu *et al.* 2004; Aflatoonian and Moore 2005; Pan *et al.* 2005). However, hEGCs have not been demonstrated, thus far, to generate teratomas upon engraftment or injection into nude mice (Turnpenny *et al.* 2006), a criterion for pluripotency. The cells do not remain undifferentiated long-term *in vitro*, and clonal expansion has not been shown.

This study sought to determine whether hEGCs lacked a critical factor(s), or whether attempts to derive hEGCs fail to induce and/or retain the expression of a key gene(s) required for the maintenance of self-renewal and pluripotency.

3.5.1 The Human Germ Cell Lineage Does Not Express SOX2

Data herein demonstrate that hPGCs within the embryonic/early fetal (48-73 dpc) testis and ovary do not express *SOX2*, a gene identified as fundamental to stem cell pluripotency (Avilion *et al.* 2003; Boyer *et al.* 2006). In addition, hPGCs and hEGCs in culture also fail to express *SOX2*. This contrasts with the germ cell lineage in the mouse: mPGCs (data herein) and mEGCs (Western *et al.* 2005; Sharova *et al.* 2007) express *SOX2*. This difference in gene expression is contrary to predictions from previous studies (Western *et al.* 2005), or previously presumed conservation (Clark 2007). The absence of SOX2 in the early human germ cell lineage was confirmed as a lack of both transcripts and protein. In contrast, SOX2 was present in the human ICM, ESCs, and various ECCs. Levels of Sox2 and Oct4 are known to alter in cells derived from different stages of mouse development; mouse germline stem cells, which are cultured spermatogonial stem cells of highly restricted developmental potential, express abundant transcripts, but limited Sox2 and Oct4 protein (Imamura *et al.* 2006). However, mPGCs clearly express Sox2 (Western *et al.* 2005 and data herein). These data make the human early germ cell lineage unique in expressing *OCT4* and *NANOG*, yet lacking SOX2, both *in vivo* and in *in vitro* culture.

The regulation of *SOX2* in germ cells must differ from that in ESCs (and presumably the epiblast) where an upstream enhancer is bound by OCT4 and a further downstream composite element by both OCT4 and SOX2 (Catena *et al.* 2004; Chew *et al.*

2005). The high inter-species sequence conservation of these elements and the wider *SOX2* locus suggest that alternative factors, such as epigenetic mechanisms, may be responsible for divergent *SOX2* expression (Katoh and Katoh 2005; Zhan *et al.* 2005). Although the earliest germ cell lineage is inaccessible in humans (i.e. prior to those cells in the gut mesentery at 6-7 weeks), the failure to detect SOX2 within the earliest specimens (48 dpc) implies that SOX2 must be extinguished during germ cell specification from cells of the SOX2-positive epiblast or soon afterwards. As reports unfold of hESCs differentiated towards functional germ cells (Clark *et al.* 2004; Aflatoonian and Moore 2006), it will be interesting to observe whether SOX2 disappears in mimicry of normal human development.

Redundancy amongst Group B *SOX* family members would limit functional consequences of absent SOX2 for the hPGC, however, this appears unlikely, as only *SOX14* was detected clearly in fetal ovary samples and only after relatively high numbers of PCR cycles. Furthermore, to date, this transcription factor has been recognised to counteract, rather than substitute for the role of SOX2 (Uchikawa *et al.* 1999).

3.5.2 SOX2: Necessary but not Sufficient for Pluripotency

The collective data infer that, alongside OCT4 and NANOG, SOX2 is necessary but not sufficient for pluripotency. The epiblast from *Sox2*-null mice fails to progress *in vivo* and *Sox2*-null blastocyst outgrowths fail to generate ESCs *in vitro* (Avilion *et al.* 2003); however, whereas mPGCs give rise to robust pluripotent EGC lines, the Sox2-positive PGCs themselves are nullipotent (Donovan and de Miguel 2003). Similarly, although previous data on nullipotent hECC lines were largely limited to analysis of transcripts (Sperger *et al.* 2003), this study reveals relatively equivalent quantities of correctly localised nuclear SOX2 protein regardless of an ability for broad differentiation.

The few groups to record hEGC derivation to date have all demonstrated differentiation *in vitro* to cells that represent the three germ layers. However, none has reported teratoma formation *in vivo*; although this was attempted within our group (Turnpenny *et al.* 2006). Stable transfection of h*SOX2* cDNA into cultured human germ cells has also been attempted; however, no teratomas formed upon engraftment of these cells into immunocompromised mice, and reliable long-term SOX2-positive cultures were not generated (Dr. Marie Best, Human Genetics Division, University of Southampton; pers. comm.). Aside from the graft environment being foreign, human germ cell

proliferation would be expected as, distinct from the mouse epiblast (Avilion *et al.* 2003), SOX2 is dispensable for hPGC and EGC proliferation *in vitro*. Furthermore, germ cells were proliferative *in situ* during development. Discovery of ways to induce *SOX2* expression in cultured human germ cells will be important to facilitate further work in this area. Indeed, with the initiation of neoplastic transformation of human germ cells proposed to occur *in utero* (Rajpert-De Meyts 2006), the absence of SOX2 in proliferative hPGCs may explain the predilection for testicular seminomas in humans compared to mice. Further experiments to investigate the role of SOX2 in pluripotent cells included attempts to generate long-term clones of NTERA-2 cells with reduced *SOX2* expression using two SOX2 siRNA vectors; however, such clones could not be established, despite clones being obtained with the control vector (containing siRNA directed against the luciferase gene). This may indicate a fundamental requirement for SOX2 in pluripotent cells.

3.5.3 Expression of OCT4-SOX2-NANOG Target Genes in Human PGCs

Identifying the consequences of missing SOX2 offers comparative insight into its role when present in other cell types. Where co-expressed, SOX2 is considered to act cooperatively with OCT4 and NANOG in regulating critical cohorts of target genes. From bioinformatic studies, their collective association with gene promoters is proposed to induce the expression of genes that determine self-renewal, whilst repressing those associated with differentiation (Boyer *et al.* 2005). Data herein suggest that SOX2 is dispensable in the former function as in its absence all 13 of the arbitrarily selected 'ESC-restricted' target genes from Boyer and colleagues, as well as *UTF1* and *FBX15*, were expressed in the fetal ovary and testis. Indeed, most recently, evidence has been published that the major role for Sox2 in mESCs is in maintaining *Oct4* expression (Masui *et al.* 2007). This implies that the regulation of both *OCT4* and *SOX2* is different in human germ cells; *SOX2* on account of its absence, and *OCT4* due its independence from SOX2 protein. In contrast, the data do support a pivotal role for SOX2 in promoting *FGF4* expression (Yuan *et al.* 1995; Ambrosetti *et al.* 1997); *FGF4* transcripts were undetected in the SOX2-negative germ cell lineage. The difference between *FBX15* (present) and *FGF4* (absent) is interesting as both have been shown hypermethylated and correspondingly absent in mouse germline stem cells (Imamura *et al.* 2006).

These findings suggest that exogenous FGF addition to the media might be able to compensate for the lack of SOX2 in cultured human germ cells. Although FGF2 has been

added to human germ cell cultures by all groups reporting derivation, definitive evidence for the roles of any FGF family member in human equivalents is lacking (Turnpenny *et al.* 2006).

Finally, it has been discussed whether, on the basis of gene expression profiles, the closest equivalent of the hESC is an early germ cell and, indeed, whether ESC derivation arises from an early germ cell phenotype (Zwaka and Thomson 2005). On the basis that the earliest human germ cells detected are SOX2-negative, and remain so – including in culture – this theory appears unlikely.

3.5.4 Summary

Studies described in this chapter analysed the expression of OCT4, NANOG and SOX2 during early human germ cell development in comparison with that in hESCs, various ECCs, EGCs and mPGCs. The data highlight a stark human-mouse species difference, confounding previous predictions of human development (Western *et al.* 2005) and demonstrating the limitations of inter-species extrapolation. Surprisingly, despite the absence of SOX2, many of the stem cell-restricted target genes reported to require OCT4 and SOX2 (Ambrosetti *et al.* 1997; Nishimoto *et al.* 1999; Tokuzawa *et al.* 2003) or OCT4, NANOG and SOX2 (Boyer *et al.* 2005) remained expressed in the human fetal gonad. Furthermore, in contrast to the epiblast of *Sox2*-null mice (Avilion *et al.* 2003), SOX2-negative germ cells were able to grow in culture with retained expression of pluripotent markers. These data highlight SOX2 as an important transcription factor for further investigation in attempts to understand the relationship between hPGCs, GCTs and the derivation, self-renewal and pluripotency of hEGCs. The information is also instructive to attempts at generating human gametes from stem cell sources for ambitious fertility treatments.

Taken together, the data demonstrate a striking inter-species difference in the transcription factor determinants of pluripotency and a lack of stringency regarding their collective presence to elicit characteristic gene expression ‘signatures’.

CHAPTER 4: SOX2 Expression in Normal and Neoplastic Germ Cell Derivatives and its Association with Pluripotency

4.1 ABSTRACT

The transcriptional regulators of pluripotency, OCT4, NANOG and SOX2, are highly expressed in embryonal carcinoma (EC). In contrast to OCT4 and NANOG, SOX2 has not been demonstrated in seminoma and in carcinoma *in situ* (CIS), a common precursor of these testicular germ cell tumours (TGCTs). This study sought to clarify this unusual pattern and examined SOX2 expression at the mRNA and protein level in CIS and a panel of TGCTs, and compared this to the developmental ontogeny of SOX2 in normal testes.

In contrast to earlier studies, SOX2 expression was found in CIS and normal fetal, neonatal and adult testes. Cytoplasmic immunoreactivity was detected in fetal prespermatogonia and nuclear staining in a subset of adult spermatogonia and spermatocytes. Nuclear SOX2 protein was abundant in EC, absent in seminoma, but detected in a small subset of CIS cells as well as in a subset of Sertoli cells. However, SOX2 mRNA was detected in most CIS cells and in focal areas of seminomas. SOX2 was also detected in spermatocytic seminoma, a tumour not derived from CIS.

Changes in subcellular protein localisation during germ cell maturation are likely related to differing regulation of SOX2 during development and in adult spermatogenesis. The presence of *SOX2* mRNA in seminoma, despite the absence of protein, suggests post-transcriptional regulation linked to the germ cell phenotype. The expression of SOX2 protein in a subset of CIS cells may be linked to re-programming towards a pluripotent phenotype.

4.2 INTRODUCTION

4.2.1 Human Testicular Germ Cell Development: An Overview

The migration of germ cells into the developing gonad has already been described (Section 1.10.2). However, the subsequent development of germ cells into gonocytes and eventually functional spermatozoa, has not. In the developing testis gonocytes begin to migrate towards the periphery of the cords during the 14th week of development. Once in close contact with the basal lamina, they are referred to as prespermatogonia. By the 20th week, prespermatogonial germ cells make up the majority of germ cells in the fetal testis (Gondos and Hobel 1971). During infancy the testis is not quiescent (Chemes 2001): at around 2-4½ months, a transient increase in the production of testicular hormones occurs, known as the 'mini-puberty' (Forest *et al.* 1973), which coincides with the final stage of differentiation of gonocytes into infantile spermatogonia (Hadziselimovic *et al.* 1986). The newborn period is characterised by a higher rate of germ cell proliferation and a lower rate of germ cell apoptosis than in the rest of prepuberty (Berensztein *et al.* 2002). During infancy, there are abortive attempts at spermatogenic maturation, ending with the degeneration of newly developed spermatocytes, similar to the first waves of incomplete spermatogenesis that occur during puberty. These initial waves of incomplete spermatogenesis mean that 2-4 years pass between the onset of puberty and the acquisition of fertility, equivalent to 20-30 spermatogenic waves (Rey *et al.* 1993). Fertility is acquired when spermatogonia undergo meiotic divisions and successfully mature to form spermatozoa. Figure 4.1 depicts the stages of spermatogenesis in the adult human testis.

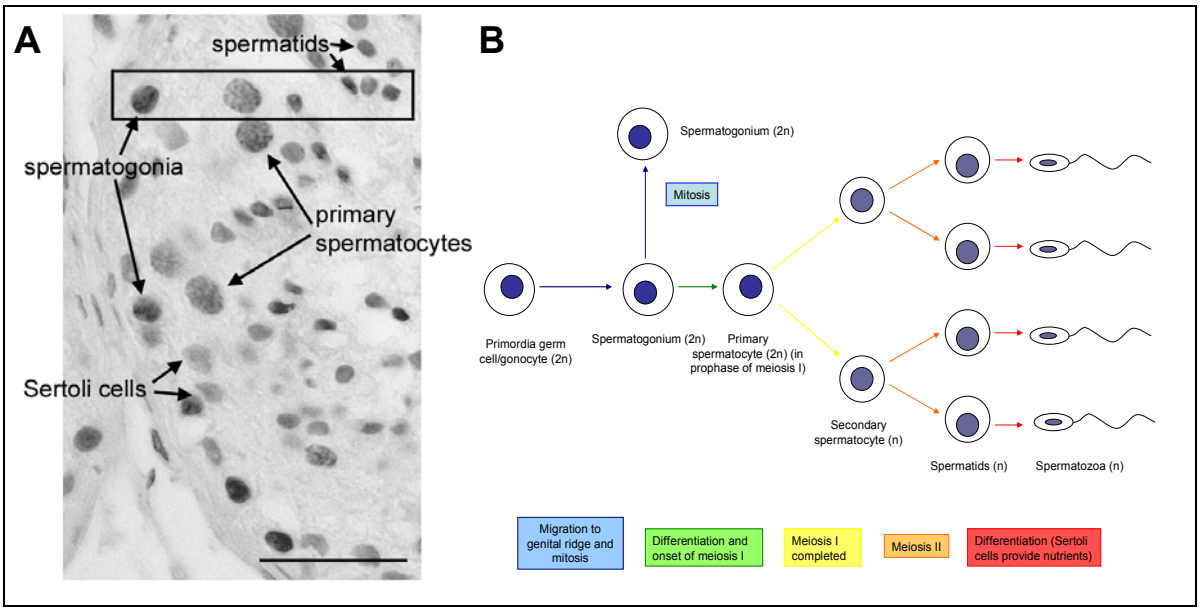


Figure 4.1 Spermatogenesis in the adult human testis

A. Bright-field image of a transverse section of a seminiferous tubule within normal adult testis; black box surrounds spermatogonium in contact with basal lamina, primary spermatocyte and spermatids. Size bar represents 20 µm. **B.** Summary of spermatogenesis. Diploid primordial germ cells migrate to the genital ridge and undergo mitosis forming spermatogonia. These self-renew as stem cells via mitosis or undergo two stages of meiosis to form haploid spermatids, which then differentiate (spermiogenesis) to form sperm. This process takes about 72 days in man.

4.2.2 Heterogeneous Subpopulations of Germ Cells Exist During Development

In mice, PGCs within the testis cords differentiate into a single population of fetal germ cells, usually referred to as 'gonocytes', that actively proliferate until about 15.5 dpc, and then remain arrested in G1 until day 1.5 postpartum, when they resume mitotic activity (Vergouwen *et al.* 1991; Nagano *et al.* 2000). Two days before they resume reproliferation, gonocytes relocate to the periphery of the cord, make contact with the basement membrane, and thereafter undergo further differentiation.

In contrast, germ cell maturation in humans is a gradual process, with a heterogeneous population of germ cells existing at any one time. In the second trimester all germ cells in the testis express DAZL (Ruggiu *et al.* 2000), VASA (Castrillon *et al.* 2000) and CHKD (Bartkova *et al.* 2001). However, other proteins are only expressed by subpopulations of these cells. Gaskell *et al.* (2004) identified three populations of germ cells: gonocytes (OCT4^{pos}/c-KIT^{pos}/MAGE-A4^{neg}), intermediate germ cells (OCT4$^{low/neg}$/c-

KIT^{neg}/MAGE-A4^{neg}), and prespermatogonia (OCT4^{neg}/c-KIT^{neg}/MAGE-A4^{pos}). In the first trimester, most germ cells have a gonocyte phenotype; however, from 18 weeks of gestation, prespermatogonia are the most abundant cell type. Pauls *et al.* (2006) described fetal male germ cells as being comprised of two groups with distinct immunohistochemical phenotypes: germ cells predominantly found before week 25 of gestation co-express the proteins OCT4, c-KIT, M2A and AP2γ. After week 25, most germ cells have lost their pluripotent potential and acquire a spermatogonial phenotype defined by expression of MAGE-A4. Kerr *et al.* (2008) demonstrated that PGCs initially express SSEA1, SSEA4, OCT4, NANOG, AP, and c-KIT but during early differentiation prior to mitotic arrest they gradually loose OCT4, AP and NANOG expression while retaining that of c-KIT and VASA.

Pluripotent stem cells have been isolated from neonatal mouse testis; termed multipotent germline stem cells (Kanatsu-Shinohara *et al.* 2004). These ES-like cells are phenotypically similar to ESCs/EGCs except in their genomic imprinting pattern. A subset of spermatogonial stem cells in adult mouse testes retain the embryonic phenotype of PGCs and gonocytes; these spermatogonial stem cells [termed multipotent adult germline stem cells (Nayernia 2007)] have been isolated and exhibit properties similar to ESCs; they are pluripotent and express Oct4, Sox2 and Nanog (Guan *et al.* 2006; Seandel *et al.* 2007). No cells retaining such an embryonic phenotype are present in adult human testes; germ cells expressing NANOG and OCT4 (together with c-KIT and AP2γ) are rapidly down-regulated from the second half of pregnancy until 2-4½ months of postnatal age (Rajpert-de Meyts *et al.*, 2004; Hoei-Hansen *et al.*, 2005). However downstream factors identified in ESCs, UTF1 and REX1 (Niwa, 2001; Nishimoto *et al.*, 2005), continue to be expressed throughout the male development in spermatogonia and are still present in the adult testes (Kristensen *et al.* 2008). Therefore a partial embryonic phenotype of the germ cells is still evident in the mature human testes.

4.2.3 TGCTs: A Classification

TGCTs that occur in young adults are subdivided into two histologically distinct groups; seminomas (also called classical seminoma to distinguish from spermatocytic seminoma) and nonseminomatous GCTs, each constituting approximately 50%. Whereas the seminoma is generally a uniform tumour and resembles early fetal germ cells, the nonseminomas are very heterogeneous and encompass embryonal carcinoma (EC), choriocarcinoma (CC), teratomas (TER), yolk sac tumour (YST), and can also contain elements of seminoma (SEM) (Ulbright *et al.*, 1999). These tumours mimic different stages of embryogenesis; ECs are pluripotent and undifferentiated like the ICM, TERs contain the differentiated somatic cells found in the embryo, and CCs and YSTs show extraembryonic differentiation (see Table 1.2 for a summary).

The carcinoma *in situ* (CIS) cell, also known as intratubular germ cell neoplasia (ITGCN) or testicular intraepithelial neoplasia (TIN), was first identified in 1972 and is the preinvasive stage of TGCTs of adolescents and young adults (Skakkebaek 1972). Spermatocytic seminomas in elderly men and infantile germ cell tumours are additional types of TGCT which do not arise from CIS cells (Muller *et al.* 1987). Both of these tumour types are rare and have remained at a steady low incidence level (Visfeldt *et al.* 1994). The opposite is true for the TGCTs in young adults, which have increased in incidence over the last several decades and now constitute the most prevalent malignant cancer among young male adults in Northern and Western Europe (Richiardi *et al.* 2004).

4.2.4 CIS Cells Express both Fetal and Pluripotent Markers

CIS cells have many morphological similarities with PGCs and early gonocytes (Nielsen *et al.*, 1974; Albrechtsen *et al.*, 1982; Skakkebaek *et al.*, 1987). They share expression of immunohistochemical markers such as placental-like alkaline phosphatase (PLAP) and the stem cell factor receptor c-KIT (Giwercman *et al.* 1991; Rajpert-De Meyts and Skakkebaek 1994; Jorgensen *et al.* 1995). These markers are commonly used in the clinical setting to help identify CIS in surgical biopsies. A fetal origin is also supported by the identification of CIS cells in prepubertal patients who later developed TGCTs, indicating that the cells had originated prior to puberty (Muller *et al.* 1984). More recently, the expression profiles of CIS cells studied by microarrays (Almstrup *et al.* 2004; Skotheim *et al.* 2006) provided a series of candidate marker genes, such as *TFAP2C*

(Transcription factor activator protein-2C; AP2γ) (Hoei-Hansen *et al.* 2004), which has shown promising results as a possible marker of CIS cells in semen samples (Hoei-Hansen *et al.* 2007).

Studies in mouse and human ESCs indicate that the transcription factors OCT4, SOX2 and NANOG are crucial in maintaining pluripotency, and do so by acting cooperatively (Boyer *et al.* 2005; Loh *et al.* 2006). The three factors are co-expressed simultaneously in the pluripotent cells in the embryo and homozygous knock-out of any of them in the mouse causes failure of the epiblast to form (Nichols *et al.* 1998; Avilion *et al.* 2003; Mitsui *et al.* 2003). Nuclear OCT4 and NANOG proteins have also been identified in fetal PGCs, gonocytes, seminoma, gonadoblastoma and in overt TGCTs derived from CIS except for fully differentiated TERs (Avilion *et al.* 2003; Gidekel *et al.* 2003; Looijenga *et al.* 2003; Honecker *et al.* 2004; Jones *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hart *et al.* 2005; Western *et al.* 2005; Kerr *et al.* 2008; Perrett *et al.* 2008).

Genome-wide expression profiling has revealed that the genes expressed by CIS cells and hESCs may overlap by as much as 50% (Almstrup *et al.* 2004). In particular, *OCT4* and *NANOG* were amongst the highest expressed genes in testes containing CIS compared to normal adult testes. Indeed, the expression of these transcription factors and other fetal germ cell markers by CIS cells can be viewed as support for the origin of CIS cells from gonocytes that failed to mature to spermatogonia (Rajpert-De Meyts 2006).

In comparison, the expression profile of *SOX2* is less clear. The gene was not among those significantly up-regulated in testes with CIS (Almstrup *et al.* 2004) instead, increased expression of *SOX17* has been reported (Almstrup *et al.* 2007). In contrast, nuclear SOX2 has been described in nonseminomatous TGCTs but not in CIS (Korkola *et al.* 2006) and is absent in seminomas (Santagata *et al.* 2007). It has also been reported negative in germ cells during later development but positive in Sertoli cells surrounding CIS (de Jong *et al.* 2008). Although SOX2 is present in mPGCs as both transcripts and protein (Western *et al.* 2005), the gene is not expressed in hPGCs (Perrett *et al.* 2008). Thus, compared to studies in mouse, *SOX2* expression is divergent from that of *OCT4* and *NANOG* in the untransformed human germ cell lineage. However, all three transcription factors appear present in EC, which is considered a pluripotent TGCT type (Skotheim *et al.* 2005).

From these studies it is evident that molecular understanding of the phenotypic differences amongst TGCT types and their relationship to CIS is incomplete. This study

sought to extend these previous studies and clarify the expression of SOX2, OCT4 and NANOG by systematic analyses of over thirty samples of TGCTs and CIS, and a broader range of TGCT types than previously reported.

4.3 SPECIFIC AIMS

4.3.1 To characterise the spatiotemporal expression profile of SOX2 during human testicular development from the second and third trimester until adulthood.

Immunohistochemistry (IHC) was used to determine the spatiotemporal expression profile of SOX2 in samples of fetal (mid-gestation: 14, 23, 29 and 39 wpc); postnatal (2, 3 and 4 months after birth) and prepubertal testis (1, 2.5, 8 and 10 years); and fetal ovarian (mid-gestation: 15, 25 and 38 wpc) samples held at the Department of Growth and Reproduction, Copenhagen University Hospital, Denmark.

4.3.2 To characterise the expression profile of SOX2 in various types of TGCTs, and their precursors, CIS cells.

A substantial selection of carcinoma samples were analysed using IHC for SOX2 (along with OCT4, NANOG and PLAP), utilising the collection held at the Department of Growth and Reproduction, Copenhagen University Hospital, Denmark. Eleven samples of CIS (comprising CIS-only samples and also those adjacent to seminoma, nonseminoma and mixed tumours); nine nonseminomatous tumour components (comprising four EC, three mixed tumours and two teratomas), and six samples of seminoma (three seminoma-only and three mixed) were examined. *In situ* hybridisation (ISH) was used to validate *SOX2* expression in three samples each of seminoma and nonseminoma (EC), two samples of CIS and three of normal testicular tissue.

4.3.3 To characterise SOX2 expression in testicular tumours not derived from CIS.

IHC was used to determine whether SOX2 was expressed in spermatocytic seminoma (four samples) and testicular B-cell lymphoma (one sample).

4.4 RESULTS

The results obtained for SOX2 IHC and ISH in the normal and neoplastic specimens are summarised in Table 4.1.

4.4.1 Testicular SOX2 Expression During Mid- to Late-Gestation and After Birth

SOX2, in stark contrast to OCT4 and NANOG, is absent from PGCs/gonocytes in specimens from the first trimester of human development (Perrett *et al.* 2008 and Chapter 3). Here, by IHC, SOX2 was not detected in a sample of fetal testis at 14 wpc (Figure 4.2 A). The majority of germ cells at this stage express OCT4 and NANOG (Honecker *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hoei-Hansen *et al.* 2005). By 23 wpc, expression of OCT4 and NANOG is only observed in approximately 0-2% of cells (Honecker *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hoei-Hansen *et al.* 2005). At this stage, immunoreactivity against SOX2 was apparent, localised to the cytoplasm of a few germ cells (arrowheads in Figure 4.2 B). Counts of ten fields of vision demonstrated 56% of tubules contained at least one SOX2-positive germ cell in testis of this age. Fewer germ cells demonstrated immunoreactivity at 29 and 39 wpc (approximately 50% of tubules contained at least one SOX2-positive germ cell), when staining was predominantly perinuclear, although some cytoplasmic staining was still visible (arrowheads in Figure 4.2 C for 29 wpc).

Postnatally, at 2 and 4 months of age (Figure 4.2 D and E respectively), some weak staining was observed in presumed prespermatogonia, which was frequently cytoplasmic (arrowhead in Figure 4.2 E) but occasionally included nuclear localisation (arrowhead in Figure 4.2 D), again with approximately 50% of tubules containing a SOX2-positive germ cell. At these stages, some staining was also observed in undifferentiated Sertoli cells. Weak cytoplasmic staining for SOX2 within germ cells was observed in testis 12 months of age (arrowheads on Figure 4.2 F), with only 8% of tubules containing a positive cell. Weak nuclear staining in some cells at the base of the tubules was observed in a testis specimen of 2.5 years of age (Figure 4.2 G; marked with arrowheads); 27% of tubules contained a positive cell.

Tissue and cell type	Age	N	IHC		N	ISH	
			Staining	Intensity and location		Staining	Intensity and location
Fetal and prepubertal testis							
Gonocytes	14 wpc	1	-	None	ND		
Prespermatogonia	23 wpc	1	+/-	Weak cytoplasmic	ND		
Prespermatogonia	29 wpc	1	+/-	Weak cytoplasmic/perinuclear	ND		
Prespermatogonia	39 wpc	1	+/-	Weak cytoplasmic/perinuclear	ND		
Infantile spermatogonia	2 m	1	+/-	Weak cytoplasmic/perinuclear	ND		
Infantile spermatogonia	3 m	1	+/-	Weak cytoplasmic/perinuclear	ND		
Infantile spermatogonia	4 m	1	+/-	Weak cytoplasmic/perinuclear	ND		
Infantile spermatogonia	1 y	1	+/- (very occasional)	Weak cytoplasmic	ND		
Infantile spermatogonia	2.5 y	1	+/- (very occasional)	Weak nuclear	ND		
Prepubertal spermatogonia	8 y	1	-	None	ND		
Prepubertal spermatogonia	10 y	1	-	None	ND		
Ovary							
Oogonia	15 wpc	1	-	None	ND		
Oocytes	25 wpc	1	+/-	Weak cytoplasmic	ND		
Oocytes	38 wpc	1	+/-	Weak cytoplasmic	ND		
Adult testis (including normal tubules in vicinity of CIS)							
Spermatogonia	Adult	9	++	Medium nuclear	3	+	Medium cytoplasmic
Sertoli, undifferentiated in CIS tubules	Adult	9	++	Medium nuclear	2	++	Weak cytoplasmic
Spermatocytes	Adult	9	+/-	Weak nuclear	3	++	Weak cytoplasmic
Spermatids	Adult	9	-	None	3	+	Weak cytoplasmic
Testicular neoplasms							
CIS	Adult	11	++	Weak nuclear	2	++	Strong/weak cytoplasmic
Seminoma	Adult	6	-	None	3	++	Medium cytoplasmic
Lymphocytes (infiltrating seminomas)	Adult	4	+/-	Weak	-	ND	Lymphocytes
Embryonal carcinoma	Adult	5	+++	Strong nuclear; overlaps with OCT4 and NANOG positive areas	3	+++	Strong cytoplasmic
Teratoma	Adult	4	++	Strong nuclear	-	ND	
Spermatocytic seminoma	66 y (42-87)	4	+/-	Weak cytoplasmic	-	ND	
Testicular B-cell lymphoma	58 y	1	-	None	-	ND	

Table 4.1 Summary of the SOX2 expression data by IHC and ISH during normal human testicular development, in fetal ovaries, and TGCTs

The proportion of cells stained was assessed using a semi-quantitative score: +++, nearly all cells stained; ++, approximately half of the cells stained; +, a low percentage of cells stained; +/-, only single cells stained; -, no positive cells detected. The staining intensity was furthermore evaluated as strong, medium, weak, very weak or none, along with cellular location (nuclear, perinuclear or cytoplasmic). All fetal, infantile and prepuburtal sections were from formalin-fixed archival tissues not suitable for ISH. Abbreviations: wpc, weeks post-conception; m, months; y, years; N, number; ND, not done.

SOX2 staining was not apparent in older testicular specimens prior to puberty (8-10 years of age; Figure 4.2 H-I respectively). Within the fetal ovary, SOX2 was not detected in oogonia at 15 wpc (Figure 4.2 J), whereas some weak expression was observed in the cytoplasm of oocytes, identified by their large nuclei, at 25 wpc (arrowhead in Figure 4.2 K), and more pronounced expression at 38 wpc (Figure 4.2 L). These results are summarised in Table 4.1.

4.4.2 SOX2 Expression in Adult Testes

Nuclear SOX2 protein was clearly detected in a subset of spermatogonial cells at the base of the normal adult testicular tubules (SPG on Figure 4.3 A-C). Expression within spermatogonia was heterogeneous, with some appearing strongly positive, some moderately so, and some negative. It was less robustly detected in some primary spermatocytes (SPC on Figure 4.3 A-C) and was not apparent in spermatids (SPT on Figure 4.3 A, C). SOX2 protein was not identified in adult Sertoli cells from normal tubules. A similar profile was observed by ISH, except that the strongest reaction was observed in spermatocytes (Figure 4.3 D, summarised in Table 4.1). In keeping with its detection in normal testis, *SOX2* transcripts were detected weakly by RT-PCR in a sample of whole normal testis (Figure 4.4).

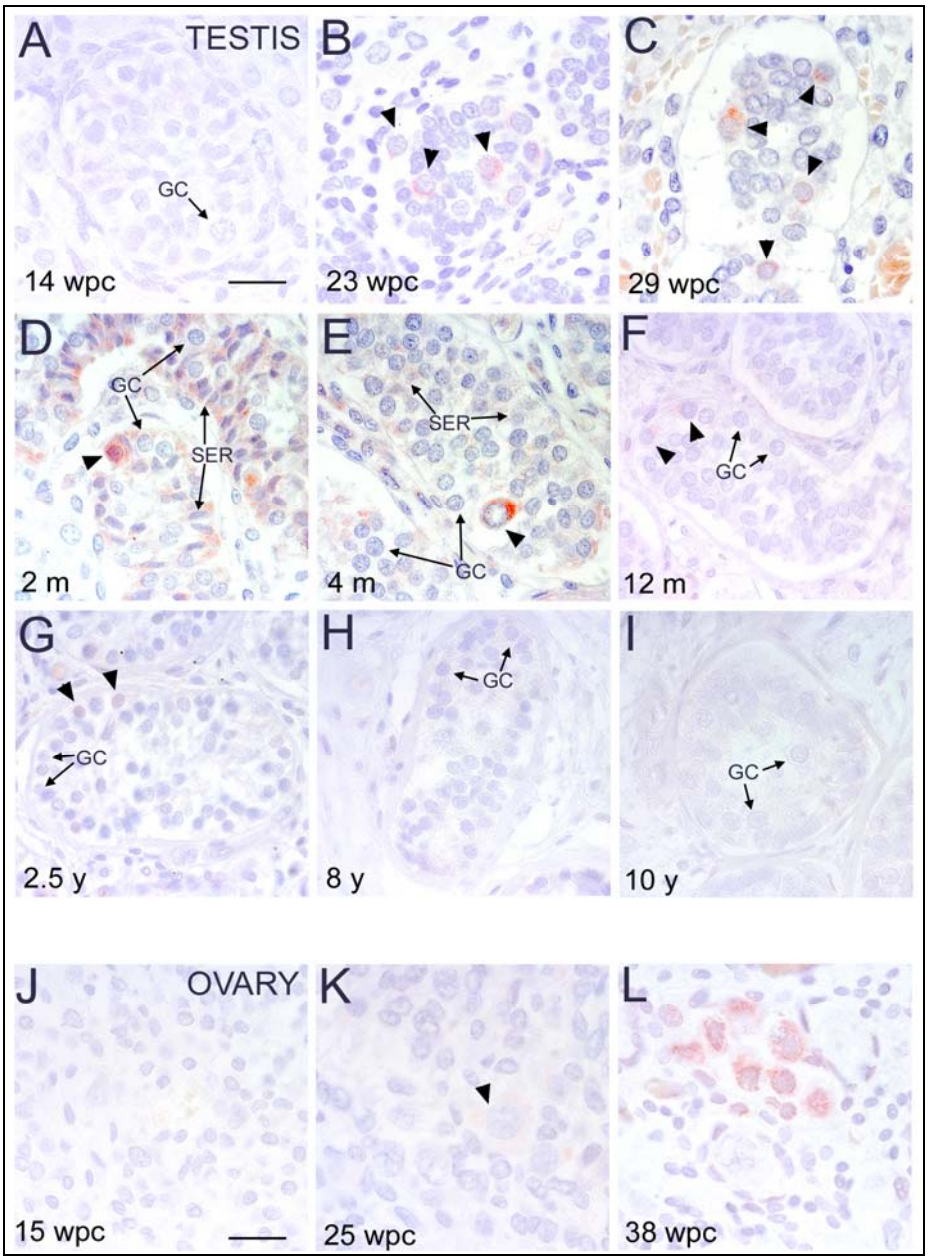


Figure 4.2 Localisation of SOX2 protein during human testicular development

Bright-field images of human testicular and ovarian sections counter-stained with Mayer's haematoxylin following IHC for SOX2. Fetal testis **A.** 14 wpc (arrow points to negative germ cell); **B.** 23 wpc; **C.** 29 wpc (arrowheads in B and C point to positive germ cells). SOX2 expression in postnatal testis aged **D.** 2 months (arrowhead points to positive germ cell, arrows to negative germ cells and positive Sertoli cells); **E.** 4 months (arrowhead points to positive germ cell, arrows to negative germ cells and weak staining in Sertoli cells); **F.** 12 months (arrowhead points to positive germ cells, arrows to negative germ cells); **G.** 2.5 years (arrowhead points to positive germ cells, arrows to negative germ cells, note nuclear staining); **H.** 8 years; and **I.** 10 years (arrows to negative germ cells). Ovarian expression at **J.** 15 wpc; **K.** 25 wpc (arrow depicts cytoplasmic staining) and **L.** 38 wpc. Abbreviations: GC, germ cell; m, month; SER, Sertoli cell; wpc, weeks post-conception; y, years. Size bars represent 20 μm.

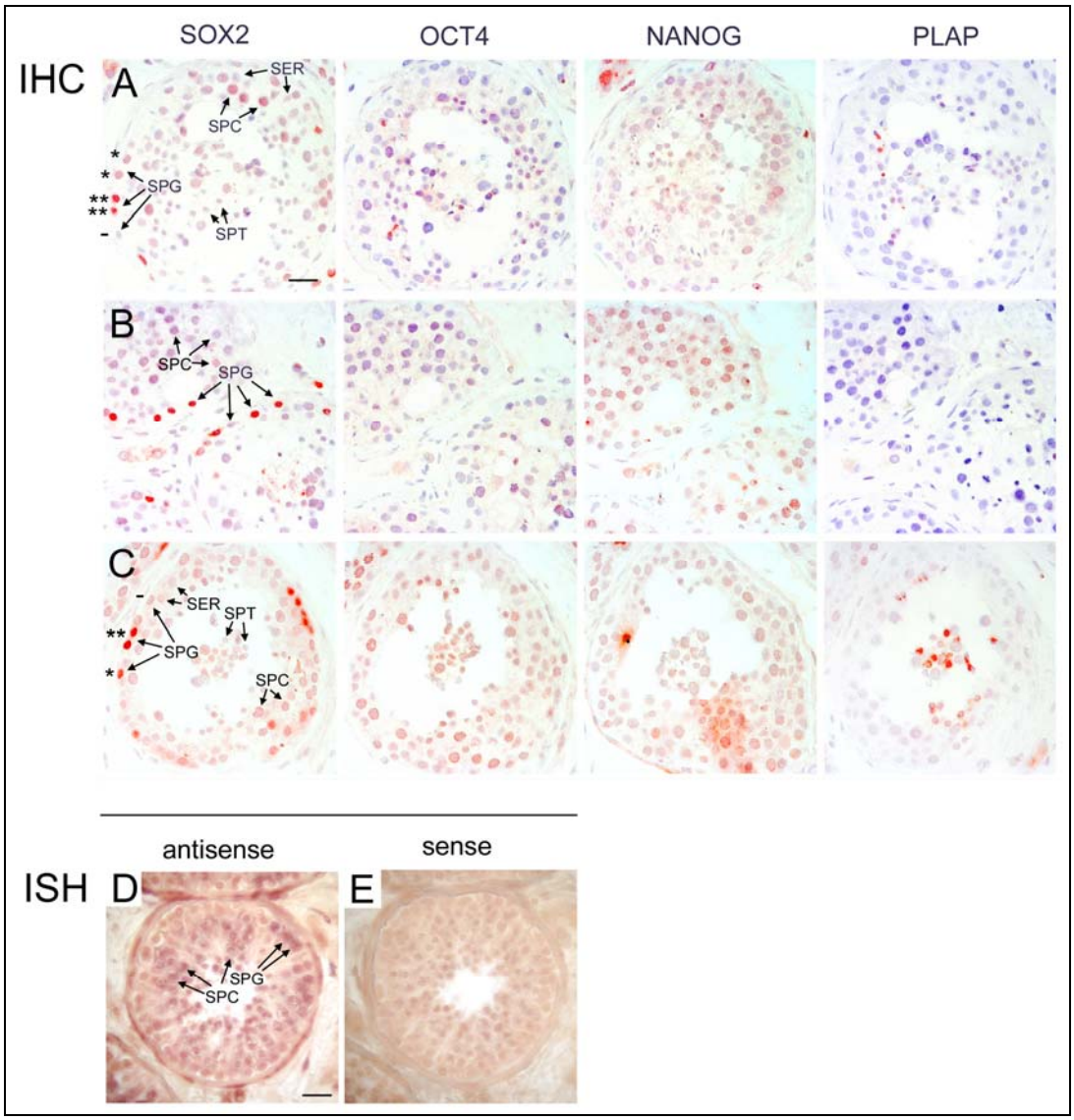


Figure 4.3 Localisation of SOX2, OCT4 and NANOG protein and *SOX2* mRNA in normal adult testes

Bright-field images of human adult testicular sections counter-stained with Mayer's haematoxylin. **A-C.** Immunohistochemistry (IHC) panels are shown for SOX2, OCT4 and NANOG in normal adult testis. Serial (6 µm) sections are shown from three different patients. PLAP-negativity demonstrates the absence of CIS cells (normal tissue was obtained from CIS-containing testes). Arrows indicate Sertoli cells (SER), spermatids (SPT), spermatocytes (SPC) and spermatogonia (SPG). ** signify spermatogonia showing strong SOX2 expression, * moderate, - none. **D-E.** mRNA *in situ* hybridisation (ISH) with *SOX2* antisense (**D**) and sense (**E**) riboprobes. Serial (6 µm) sections of the same tubule are shown. Size bars represent 20 µm.

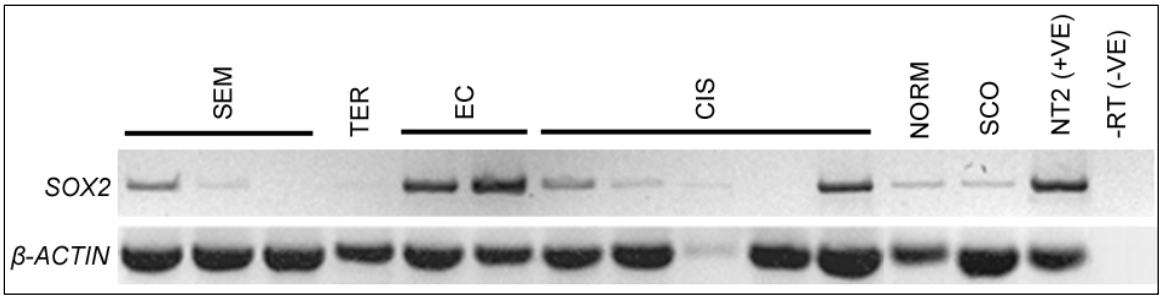


Figure 4.4 Expression of *SOX2* in normal and neoplastic human testes
RT-PCR panel is shown for *SOX2* and the housekeeping gene *β-ACTIN* in a range of TGCT types following PCR for 35 cycles. Abbreviations: SEM, seminoma; TER, teratoma; EC, embryonal carcinoma; CIS, carcinoma *in situ*; NORM, normal testis; SCO, Sertoli cell-only testis; NT2, NTERA2 cells (positive control) and –RT, negative control containing RNA but not reverse transcriptase (RT) (shown in this case for EC, but performed for all RNA samples with no *SOX2* detection). For primer sequences see Appendix 1.

4.4.3 SOX2 Expression in CIS

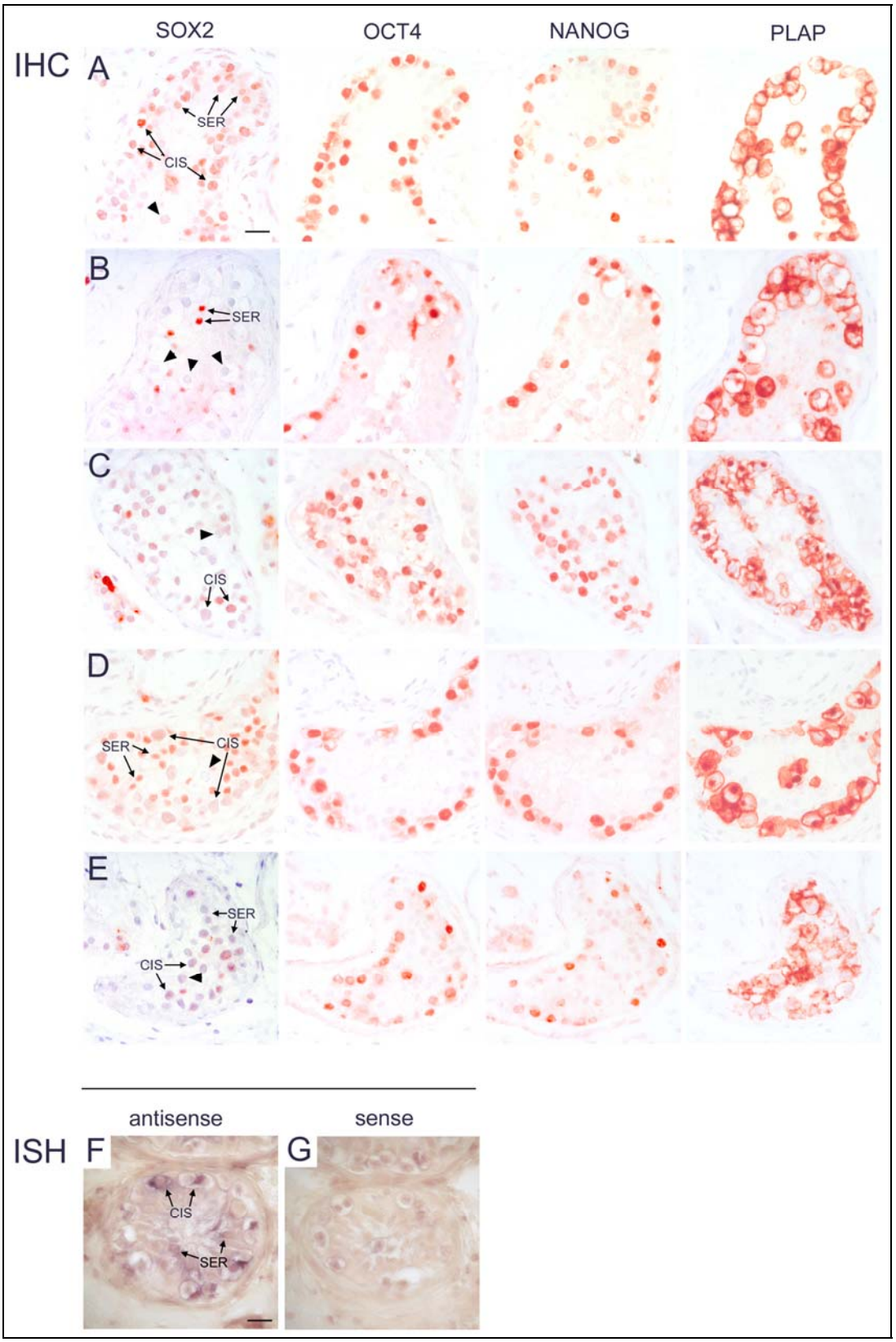
PLAP staining was used in order to distinguish CIS-containing tubules (PLAP-positive) from normal tubules (PLAP-negative). By staining serial sections, it could be concluded that whilst CIS cells were OCT4- and NANOG-positive to a similar degree, regardless of phenotypic proximity to a specific type of TGCT, SOX2 expression in CIS cells was very heterogeneous. Only a few CIS cells exhibited positive nuclear staining (arrows on Figure 4.5 A, C-E), with some CIS cells being negative (arrowheads on Figure 4.5 A-E). There was no difference in the relative expression between CIS cells adjacent to seminomas and those adjacent to nonseminomas. Comparable findings were observed at the RNA level; ISH showed a relatively strong signal in CIS (Figure 4.5 F). RT-PCR detected *SOX2* weakly to moderately in four out of five CIS samples, presumably reflecting the variable numbers of CIS cells and normal germ cells present (Figure 4.4). Transcripts were very weakly expressed in a Sertoli cell-only sample (Figure 4.4); also called germ cell aplasia, a condition of the testes in which only Sertoli cells line the seminiferous tubules.

In addition, nuclear SOX2 expression was observed in some Sertoli cells within CIS tubules (Figure 4.5 A-B, D), but not in others (Figure 4.5 E). *SOX2* expression was also detected in Sertoli cells by ISH (arrows on Figure 4.5 F), but not at the protein level

(Figure 4.3 A, C), although some staining was apparent during fetal testicular development (Figure 4.2 D-E).

Figure 4.5 Localisation of SOX2, OCT4 and NANOG protein and *SOX2* mRNA in carcinoma *in situ*

Bright-field images of carcinoma *in situ* sections counter-stained with Mayer's haematoxylin. **A-E.** IHC panels are shown for SOX2, OCT4 and NANOG in carcinoma *in situ*. Serial (6 µm) sections are shown from five different patients. Arrows point to SOX2-positive CIS and Sertoli cells; arrowheads to SOX2 negative CIS cells. PLAP positivity demonstrates the presence of CIS cells. **F-G.** mRNA *in situ* hybridisation with *SOX2* antisense (**F**) and sense (**G**) riboprobes. Serial (6 µm) sections of the same tubule are shown. Abbreviations: CIS: carcinoma *in situ*, IHC: immunohistochemistry, ISH: *in situ* hybridisation, SER: Sertoli cell. Size bars represent 20 µm.


SOX2
OCT4
NANOG
PLAP
IHC
A
SER
CIS
B
SER
C
CIS
D
SER
CIS
E
SER
CIS
antisense
sense
ISH
F
CIS
SER
G

4.4.4 SOX2 Expression in TGCTs Derived From CIS

4.4.4(i) Nonseminomas

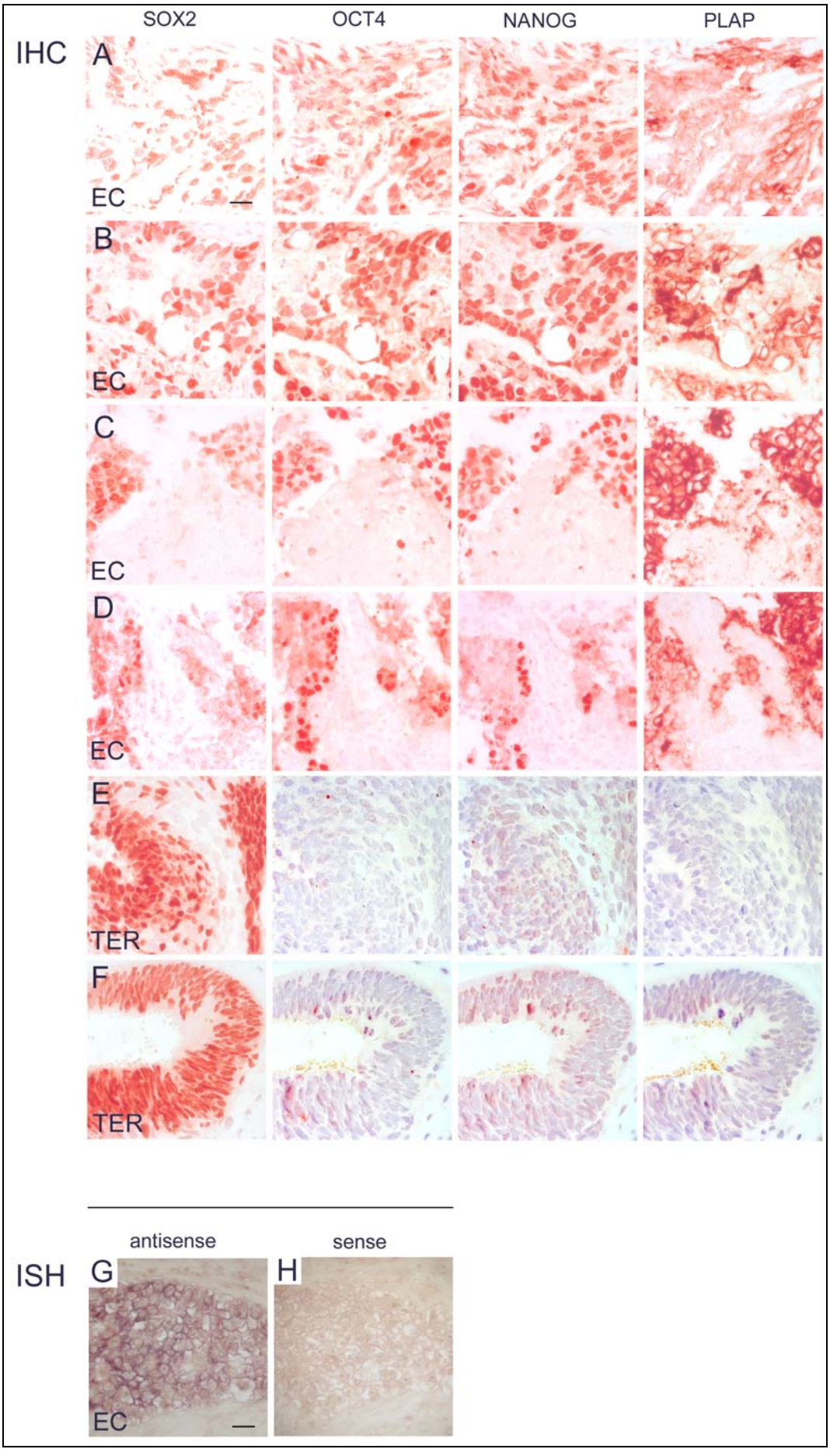
SOX2 protein co-localised with OCT4 and NANOG to the same areas of ECs. All three proteins showed clear nuclear localisation (Figure 4.6 A-D). Strong expression of *SOX2* was detected by both RT-PCR (Figure 4.4) and ISH (Figure 4.6 G) in EC. In addition, SOX2 was expressed within some differentiated areas of teratomas (Figure 4.6 E-F), particularly in papillary structures, which were OCT4- and NANOG-negative; data that are consistent with known SOX2 expression in neural and gut epithelial cells (Ishii *et al.* 1998; Wood and Episkopou 1999; Sakamoto *et al.* 2000; Graham *et al.* 2003). A very faint band could be detected by RT-PCR in teratoma (Figure 4.4), although protein detection was very strong. SOX2 expression was stronger in EC/teratoma than in CIS or normal adult testis; both at the mRNA and protein level.

4.4.4(ii) Seminomas

In contrast to nonseminomas, SOX2 protein was not detected by IHC in OCT4-positive/NANOG-positive seminomas (Figure 4.7 A-D), consistent with previous reports (Korkola *et al.* 2006; Santagata *et al.* 2007). However, by ISH, *SOX2* expression was detected in all samples, with focal or heterogeneous staining (Figure 4.7 E). In keeping with this finding, of three seminoma samples tested by RT-PCR, weak expression was detected in two samples (Figure 4.4). This discrepancy between mRNA and protein expression could be due to the presence of low level untranslated mRNA.

Figure 4.6 Localisation of SOX2, OCT4 and NANOG protein and *SOX2* mRNA in nonseminoma

Bright-field images of nonseminoma sections counter-stained with Mayer's haematoxylin. **A-F.** IHC panels are shown for SOX2, OCT4, NANOG and PLAP in embryonal carcinoma (**A-D**) and SOX2 positive areas of teratoma (**E-F**). Serial (6 µm) sections are shown from six different patients. **G-H.** mRNA *in situ* hybridisation with *SOX2* antisense (**G**) and sense (**H**) riboprobes. Serial (6 µm) sections of the same tubule are shown. Abbreviations: EC: embryonal carcinoma, IHC: immunohistochemistry, ISH: *in situ* hybridisation, TER: teratoma. Size bars represent 20 µm.


SOX2
OCT4
NANOG
PLAP
IHC
A
EC
B
EC
C
EC
D
EC
E
TER
F
TER
antisense
sense
ISH
G
EC
H

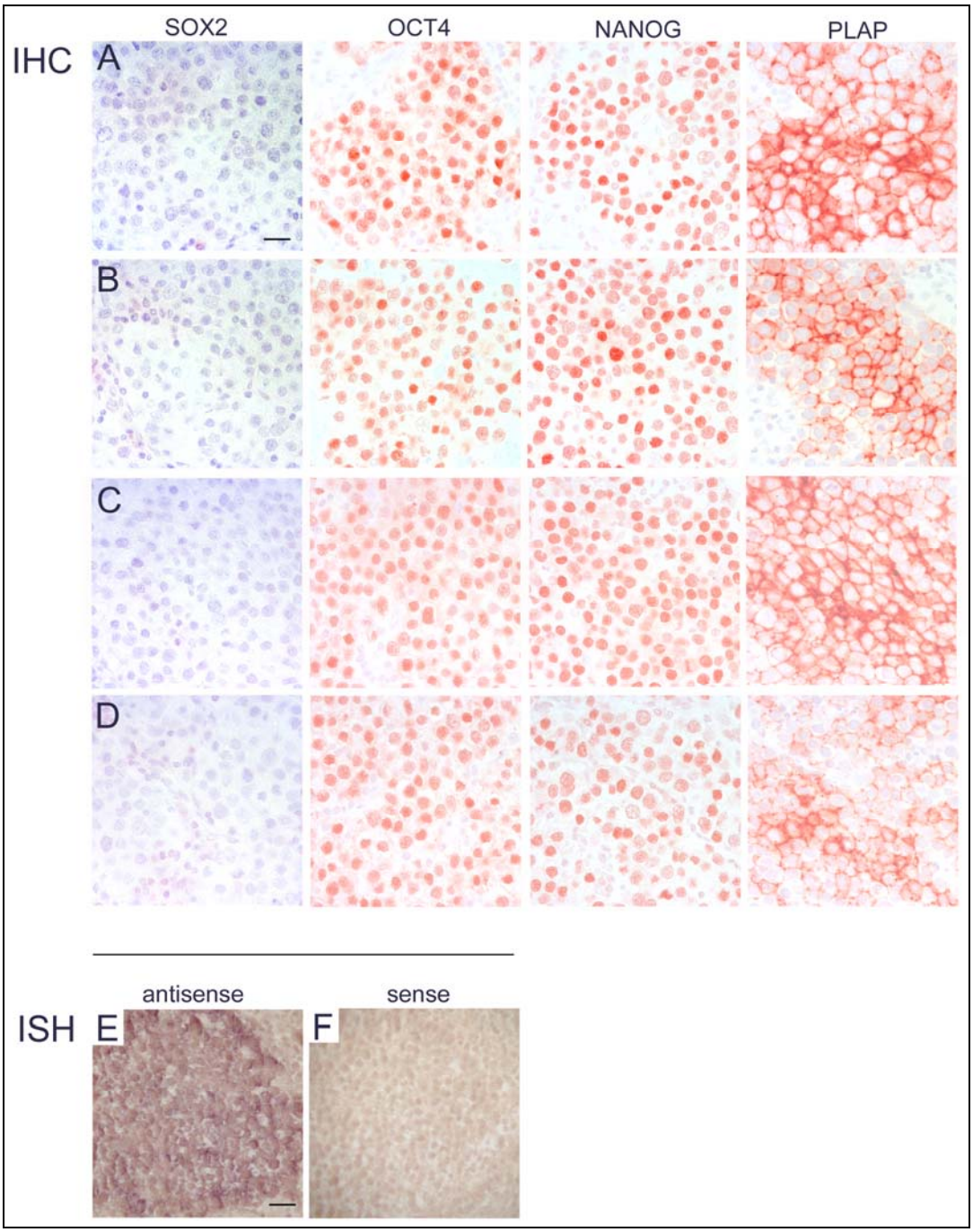


Figure 4.7 Localisation of SOX2, OCT4 and NANOG protein and *SOX2* mRNA in seminoma

Bright-field images of seminoma sections counter-stained with Mayer's haematoxylin. **A-D.** IHC panels are shown for SOX2, OCT4, NANOG and PLAP. Serial (6 µm) sections are shown from four different patients. **E-F.** mRNA *in situ* hybridisation (ISH) with *SOX2* antisense (**E**) and sense (**F**) probes. Serial (6 µm) sections of the same tubule are shown. Size bars represent 20 µm.

4.4.5 SOX2 Expression in TGCTs Not Derived From CIS

4.4.5(i) Spermatocytic Seminoma

Of four samples of spermatocytic seminoma tested, weak cytoplasmic staining was detected in two (arrows in Figure 4.8 A and B), albeit infrequently. A third sample showed nuclear staining in stromal cells (arrows in Figure 4.8 C), and a fourth was negative (Figure 4.8 D).

4.4.5(ii) Testicular B-Cell Lymphoma

Testicular B-cell lymphoma was negative for SOX2 (Figure 4.8 E).

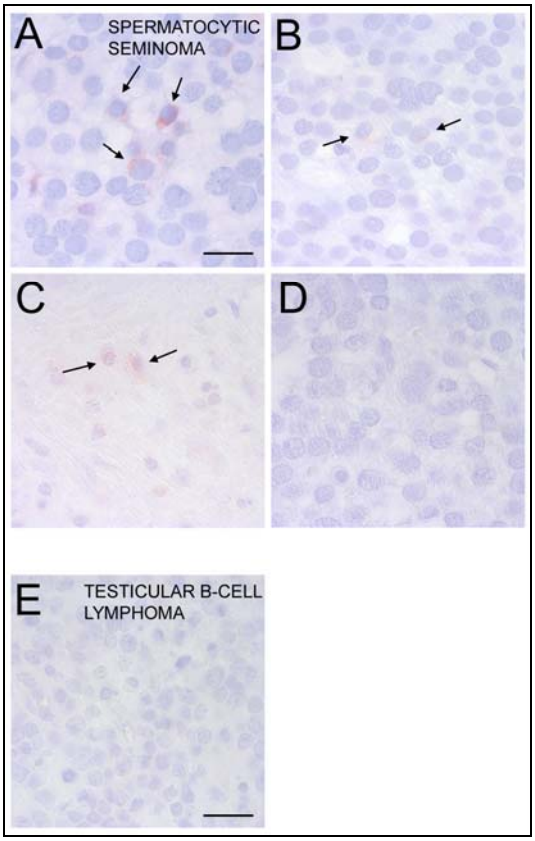


Figure 4.8 Localisation of SOX2 protein in spermatocytic seminoma and testicular B-cell lymphoma

Bright-field images of testicular carcinoma sections counter-stained with Mayer's haematoxylin. **A-D.** IHC staining for SOX2 in spermatocytic seminoma from four different patients. Arrows indicate cytoplasmic staining (A-B) and nuclear staining (C). **E.** IHC staining for SOX2 in testicular B-cell lymphoma. Size bars represent 20 μm.

4.5 DISCUSSION

4.5.1 Temporal Expression Profile of SOX2 During Human Testicular Development

This study, coupled with Chapter 3, provides a comprehensive analysis of SOX2 expression during testicular development. The temporal expression profile of SOX2 is depicted in Figure 4.9: nuclear localisation indicated by a solid line and cytoplasmic by a dashed line. The earliest germ cells detectable in the indifferent gonad (around 5 wpc) are positive for OCT4 and NANOG (Honecker *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hoei-Hansen *et al.* 2005). All germ cells express these transcription factors until both number stained and staining intensity begins to decline at around 13 wpc. At 17 wpc, around 50% are positive for OCT4. At 18 wpc, expression declines more rapidly, with 5-10% positive at week 20. Subsequently only single cells are positive. Expression of NANOG mirrors that of OCT4 but declines slightly later, being expressed in all germ cells at 17 wpc, then declining rapidly, becoming expressed by only a subset of gonocytes until 3-4 months (Honecker *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hoei-Hansen *et al.* 2005).

No SOX2 staining was detected in samples up to 14 wpc (Perrett *et al.* 2008 and Chapter 3); however, positive cells were detected in 23, 29 and 39 wpc testes. Germ cells in 2, 3, 4 and 12 month postnatal testis were also positive, with SOX2 localised to the cytoplasm in all; however, the number of germ cells positive in 12 month old testis was lower. Numbers increased again in 2.5 year old testis but expression was nuclear rather than cytoplasmic. The percentage of germ cells positive for SOX2 is an estimate at all stages (at 50% on the graph). Total germ cell number was unknown; however, it appeared, by identifying cells with large nuclei as being germ cells, that 50% or less were positive for SOX2 at each age. At 8 years there was no SOX2 expression; the unavailability of samples between 2.5 and 8 years means it is unclear at what stage during testicular maturation SOX2 expression declines. Nuclear expression was detected in adult spermatogonia and spermatocytes; although it is unclear at what stage expression is initiated, it is possibly pubertal. Analysis of more specimens would help to clarify the exact expression profile of SOX2 during testicular development.

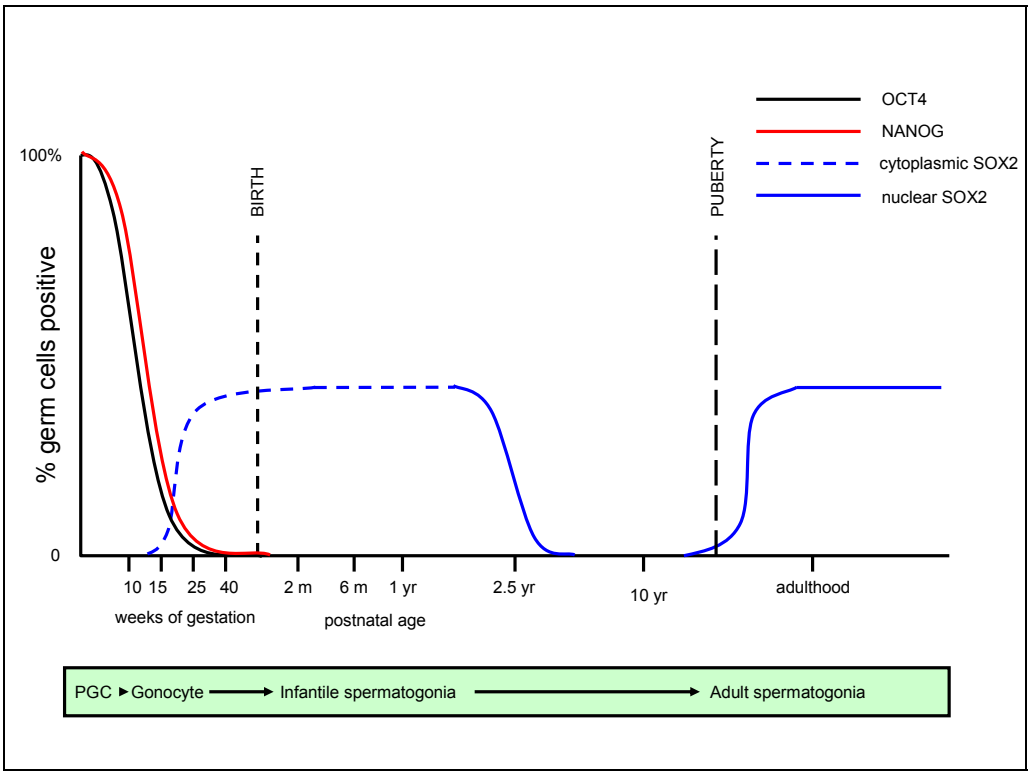


Figure 4.9 Schematic overview of the temporal expression profile of OCT4, NANOG and SOX2 in germ cells during human testicular development

The image shows approximately smoothed curves based on the combined results of several studies (Gaskell *et al.* 2004; Honecker *et al.* 2004; Rajpert-De Meyts *et al.* 2004; Hoei-Hansen *et al.* 2005; Anderson *et al.* 2007; Perrett *et al.* 2008) and this work. Nuclear SOX2 expression is indicated by a solid line, cytoplasmic expression by a dashed line.

4.5.2 SOX2 Expression in Infantile and Adult Spermatogonia

Cytoplasmic (along with perinuclear/nuclear) expression of SOX2 was visible in infantile spermatogonia in both fetal (23-39 wpc) and infantile (2-12 months) testes. Expression at 2.5 years was nuclear. Cytoplasmic detection of SOX2 has been reported previously: unlike OCT4 and NANOG which are exclusively nuclear in the ICM, SOX2 is localised in both the nuclei and cytoplasm in pre-implantation embryos from the human, but is solely nuclear in the mouse (Perrett *et al.* 2008 and Chapter 3). Other SOX proteins are regulated by subcellular localisation: for example, the lack of Sox9 nuclear translocation induces a sex-reversed phenotype in male gonads (Gasca *et al.* 2002); and, interestingly, continuous nucleocytoplasmic shuttling appears to be essential to the transactivation function of Sox10 (Rehberg *et al.* 2002). SOX2 has recently been shown to contain two nuclear localisation signals (NLSs; depicted for hSOX2 in Figure 4.10), which

it uses to shuttle between the nucleus and cytoplasm in mESCs (Li *et al.* 2007). These NLSs are contained within the HMG domain and are well conserved amongst other SOX proteins. In addition, the nuclear export signal (NES) essential for Sox9 subcellular localisation (Gasca *et al.* 2002), and required for Sox10 shuttling and subsequent transactivation (Rehberg *et al.* 2002), is also conserved within the SOX2 HMG domain (Figure 4.10), the only difference being the presence of isoleucine in place of leucine at the first residue (both hydrophobic). The existence of these NLSs and a NES within the HMG domain of SOX2 is further evidence for its cytoplasmic/perinuclear/nuclear location during testicular development.

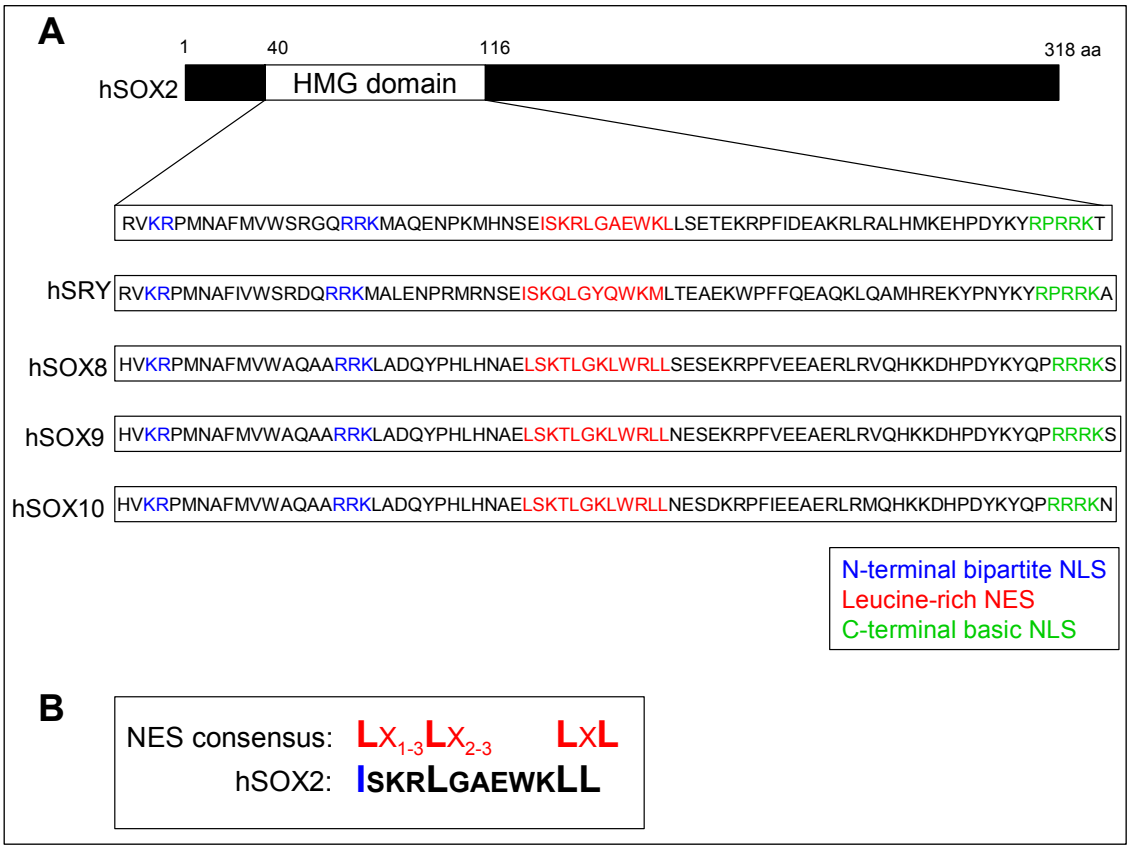


Figure 4.10 Conservation of nuclear localisation signals (NLS) and a nuclear export signal (NES) in human SOX proteins

A. The 318 amino acid (aa) SOX2 protein contains two NLSs positioned at either end of the HMG domain, originally identified for SRY and SOX9 (Sudbeck and Scherer 1997), but conserved in many if not all the SOX proteins. The HMG domain also contains a leucine-rich NES sequence (detailed in **B**), similar, but not identical to that conserved within the SOXE subgroup and required for Sox9 and Sox10 translocation (Gasca *et al.* 2002; Rehberg *et al.* 2002).

Akin to its putative role in maintaining adult neural stem cells (Rex *et al.* 1997; Graham *et al.* 2003), the identification of SOX2 in selected adult spermatogonial cells may be marking their stem cell population. This is supported by the recent discovery of UTF1 protein in spermatogonia (Kristensen *et al.* 2008). UTF1 is a transcription factor linked to pluripotency in ESCs (van den Boom *et al.* 2007), but apparently involved in proliferation of spermatogonia in the adult (Kristensen *et al.* 2008). The expression of these presumed pluripotency factors in mature spermatogonia suggests a different biological role, as indicated in the case of SOX2 by a shift from a cytoplasmic to nuclear location during testis development. This finding warrants further studies, also in view of the fact that *SOX2* transcripts were more abundant in primary spermatocytes than in spermatogonia. It is possible that ISH staining may detect the presence of several types of single stranded RNA, which germ cells may use to selectively silence transcripts that are no longer needed after stem spermatogonia commit to enter meiosis (Novotny *et al.* 2007).

In humans, it is proposed that there are two species of spermatogonia in the adult testes [Figure 4.11 C (Clermont 1966; Clermont 1966)], similar to the monkey (Figure 4.11 B). This contrasts the seven types of A spermatogonia described in the mouse [Figure 4.11 A (de Rooij 1998; Dettin *et al.* 2003)]. A_{dark} spermatogonia are testicular stem cells and function as regenerative reserve, they show low proliferative activity during normal spermatogenic activity, but are active during pubertal expansion (Simorangkir *et al.* 2005). A_{pale} spermatogonia are self-renewing progenitors which proliferate continuously during each spermatogenic cycle. A_{pale} spermatogonia generate B spermatogonia, which then generate spermatocytes (depicted in Figure 4.11).

It is unclear in which population of spermatogonia SOX2 is expressed. If expressed by A_{dark} spermatogonia, SOX2 may be functioning to maintain the testicular stem cell population, whereas if expressed by A_{pale} spermatogonia, it may be maintaining spermatogonia in an undifferentiated progenitor state, so called 'transit-amplifying cells'. When SOX2 is expressed in the absence of the pluripotency-maintaining transcription factors OCT4 and NANOG in neural stem cells, it maintains a state of proneural commitment and the capacity for self-renewal, its activity being down-regulated during terminal division (Graham *et al.* 2003). Forced expression of SOX2 inhibits neuronal differentiation preserving a naïve progenitor state. However, the requirement for Sox2 in the conversion of somatic cells to induced pluripotent stem cells (iPS cells), first reported

by Takahashi and Yamanaka (2006), suggests it more likely has a stem cell-specific role than simply a transit-amplifying role.

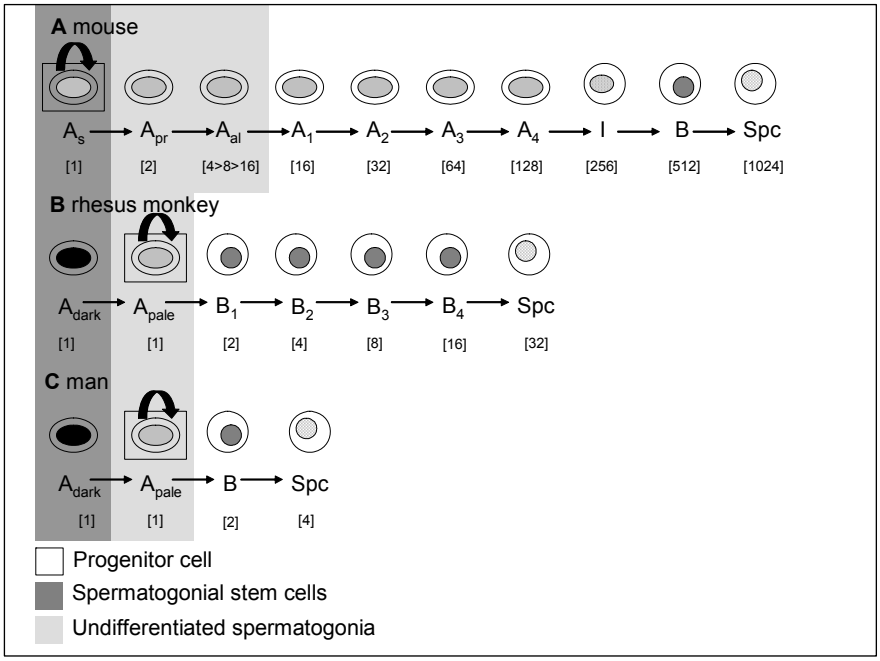


Figure 4.11 Schematic overview of the premeiotic steps of spermatogenesis in different species of mammals

Premeiotic steps of spermatogenesis are shown for **A.** mouse, **B.** rhesus monkey and **C.** man. The number given in brackets indicates the total number of daughter cells derived from any one progenitor cell that enters differentiation. Abbreviations: A_s, A_{single}; A_{pr}, A_{paired}; A_{al}, $A_{aligned}$; Spc, spermatocyte. [Adapted from Ehmcke *et al.* (2006).]

A role for SOX2 in spermatogenesis is supported by the phenotype of *Sox2*$^{\beta geo}$ heterozygous mice, which show reduced male fertility (Kelberman *et al.* 2006). These animals occasionally have smaller testes with sperm blockage in the seminiferous tubules, however the infertility is thought to be more likely due to a primary defect in developing sperm that compromises their motility and/or ability to fertilise the oocyte. However, in the mouse, undifferentiated spermatogonia also express Sox3, required for spermatogenesis (Raverot *et al.* 2005), which, as a member of the B1 group of SOX proteins, may function redundantly with Sox2 (Wegner and Stolt 2005).

SOX2 functions with partner proteins to activate transcription of target genes; for example with the class III POU factor Brain 2 (Brn2) to drive *Nestin* expression in the nervous system (Tanaka *et al.* 2004) and with Paired box gene 6 (Pax6) to activate the *δ*-

crystallin enhancer DC5 in the eye (Kamachi *et al.* 2001); thus it may have an, as yet unknown, partner protein in spermatogonia.

4.5.3 SOX2 Expression in CIS

SOX2 functions in concert with OCT4 and NANOG to maintain pluripotency in human and mouse ESCs (Boyer *et al.* 2005; Loh *et al.* 2006). However, only *OCT4* and *NANOG* have been shown to be up-regulated in CIS compared to normal testes (Almstrup *et al.* 2004). Work described in this chapter involved a more comprehensive analysis of both the transcription factor and its associated transcript in human CIS and TGCTs. A differential pattern of *SOX2*/SOX2 expression was found: in concert with other studies (Korkola *et al.* 2006; Santagata *et al.* 2007), clear expression was detected at both the mRNA and protein level in EC cells. However, variable SOX2 expression was detected at the mRNA and protein level in germ cells, CIS cells and seminomatous TGCTs. The latter was in concert with Santagata *et al.* (2007) who also noticed SOX2-positivity in a small subset of seminoma cells (<1%). In this study, weak staining was also observed in undifferentiated Sertoli cells in some CIS tubules, as with the fetal testes, consistent with tubules containing CIS demonstrating an arrest of development (Rajpert-De Meyts *et al.* 1998; Rajpert-De Meyts 2006). The presence of immature Sertoli cells could explain the weak *SOX2* expression observed in a Sertoli cell-only specimen by RT-PCR.

The heterogeneous expression of SOX2 in CIS cells, with occasional positive cells, is reminiscent of a pattern previously noted for another marker, TRA-1-60 (Rajpert-De Meyts *et al.* 1996). Human PGCs and fetal gonocytes are SOX2-negative (Perrett *et al.* 2008 and data herein); nonseminomatous TGCTs, derived from CIS, are SOX2-positive. It is possible that SOX2 (and TRA-1-60) expression in CIS may mark those cells in a process of re-programming towards malignancy associated with a stem cell compartment and a capacity for differentiation (i.e. an EC, CC or YST phenotype). The factors that affect this process can be considered extrinsically or intrinsically: it is perhaps noteworthy that CIS cells lie within a variable microenvironment consisting of several cell-types that secrete hormones and growth factors. The fact that Sertoli cells within CIS tubules are sometimes SOX2-positive along with the CIS cells themselves indicates that a diffusible growth factor may induce *SOX2* expression, and therefore may not be intrinsic to the tumour cell *per se*. Conversely, an up-regulation of *SOX2* gene expression may be caused by altered

epigenetic status. Methylation changes are a common feature in somatic cancers and may be indicative of increased malignant potential. SOX2 expression has been associated with poorly-differentiated tumours (Sanada *et al.* 2006; Rodriguez-Pinilla *et al.* 2007) and has been reported in a range of tumours derived from cells that are ordinarily SOX2-negative, such as sporadic breast cancer (Rodriguez-Pinilla *et al.* 2007) and pancreatic carcinoma (Sanada *et al.* 2006), as well as SOX2-positive parent cells, malignant glioma (Schmitz *et al.* 2007) and stomach adenocarcinomas (Tsukamoto *et al.* 2005). Alternatively, SOX2 expression by a subset of CIS cells could indicate derivation from postnatal SOX2-positive prespermatogonia.

While this study was in progress, SOX2 was reported to be absent in CIS cells (Korkola *et al.* 2006; Biermann *et al.* 2007), along with gonocytes and normal adult testis (de Jong *et al.* 2008). This inconsistency with results herein possibly relate to the use of a different antibody (Korkola, Biermann and de Jong used a monoclonal antibody from R&D Systems, while Santagata *et al.* used the same antibody used here) and problems of cross-reactivity with other epitopes or variable sensitivity of SOX2 detection. Therefore, findings were corroborated at the RNA level by ISH and RT-PCR. In accordance with the findings of de Jong *et al.* (2008), weak staining was observed in undifferentiated Sertoli cells in some CIS tubules, consistent with tubules containing CIS demonstrating an arrest of development (Rajpert-De Meyts *et al.* 1998; Rajpert-De Meyts 2006).

4.5.4 SOX2 Expression in TGCTs Derived From CIS

Findings regarding SOX2 expression in TGCTs were in accordance with other studies (Korkola *et al.* 2006; Santagata *et al.* 2007; de Jong *et al.* 2008): SOX2 protein was expressed in OCT4-, NANOG-positive ECs, in overlapping areas, but was absent from OCT4-, NANOG-positive seminomas. Contrastingly, by ISH, seminomas were all positive for *SOX2* expression with focal or heterogeneous staining. The lack of SOX2 protein expression, but presence of mRNA, suggests some form of post-transcriptional repression. Interestingly, mouse germline stem cells derived from newborn mouse testes (Kanatsu-Shinohara *et al.* 2003) express abundant transcripts but limited Sox2 protein (Imamura *et al.* 2006). Post-transcriptional suppression of Sox2 is implicated in their loss of pluripotency.

The only human seminoma cell line isolated, TCam-2, does not express SOX2 protein (de Jong *et al.* 2008; Eckert *et al.* 2008). In addition, microarray expression analysis demonstrated a lack of *SOX2* expression in TCam-2 along with three seminoma tumour samples (de Jong *et al.* 2008) This contrasts findings herein of *SOX2* expression in seminoma. Interestingly, high expression of miRNAs 145 and 324-5p were found in TCam-2 versus EC (de Jong *et al.* 2008); these target SOX2 and may be responsible for preventing its translation. miRNA could therefore be the mechanism of post-transcriptional repression of *SOX2* expression within these tumours.

Gene expression studies in seminoma are consistent with the OCT4-SOX2-NANOG transcription factor complex being absent compared to EC and thus being unable to activate expression of target genes. One such target in mouse is *Fgf4*, activated by the Sox2-Oct4 complex (Yuan *et al.* 1995). A study examining FGF4 expression in TGCTs found all nonseminomas (excluding teratomas) expressed FGF4, compared with a much lower incidence in seminomas (Suzuki *et al.* 2001). In addition TDGF1, a gene whose transcription is putatively activated by OCT4, SOX2 and NANOG in hESCs (Boyer *et al.* 2005), was expressed in all nonseminomas compared with a lower incidence in seminomas and with no expression in normal testes tissue (Baldassarre *et al.* 1997).

4.5.5 SOX2 Expression in TGCTs Not Derived From CIS

This study is the first described to examine SOX2 expression in tumours not derived from CIS cells - spermatocytic seminomas - where moderate expression of SOX2 protein was detected. These tumours are OCT4- and NANOG-negative and are believed to originate from adult spermatogonia (Rajpert-De Meyts *et al.* 2003) or spermatocytes (Looijenga *et al.* 2006). Interestingly, SOX2 expression in spermatocytic seminomas was predominantly cytoplasmic, in contrast to the nuclear localisation in normal adult germ cells, implying an inability to function as a transcription factor in this tumour.

4.5.6 Summary

This study describes SOX2 expression during testicular development, illustrating previously undescribed expression within infantile and adult spermatogonia. Here, SOX2 is expressed independently from its usual transcriptional activating partners OCT4 and NANOG, as is also the case in developing nervous and sensory systems and gut (Ishii *et al.* 1998; Wood and Episkopou 1999; Sakamoto *et al.* 2000; Graham *et al.* 2000; Kondoh *et al.* 2004; Kiernan *et al.* 2005; Okubo *et al.* 2006; Taranova *et al.* 2006; Donner *et al.* 2007 and Chapter 5). In these tissues it preserves progenitor cell identity; both by maintaining proliferative capacity and inhibiting differentiation. SOX2 functions in adult tissues: for example, in mouse brain (Ferri *et al.* 2004; Suh *et al.* 2007) and ear (Oesterle *et al.* 2008). The presence of SOX2 in selected adult spermatogonia may be indicative of the adult spermatogonial stem cell population that underpins male gametogenesis.

This study has demonstrated that a hitherto unappreciated subset of CIS cells express SOX2, at the RNA and protein level. This expression may be linked to invasive CIS progression towards a malignant pluripotent phenotype. Conversely, SOX2-negative CIS cells may be forerunners of SOX2-negative seminomas; a larger specimen study would help to determine this. The absence of SOX2 from seminomas, and thus absence of the pluripotency maintaining OCT4-SOX2-NANOG transcription factor complex, may explain the homogeneity of seminomas as compared to pluripotent nonseminomas, which in many respects resemble a developing embryo. In addition, the expression of *SOX2* in seminoma, but absence of protein, in contrast to the detection of SOX2 protein in EC, suggest differential regulation of *SOX2* expression at the post-transcriptional level likely related to different roles in germ cells during testis development and adult spermatogenesis.

CHAPTER 5: Investigating the Association of the OCT4-SOX2-NANOG Transcription Factor Complex with Pluripotency

5.1 ABSTRACT

As well as being expressed in pluripotent cells, SOX2 is also expressed in progenitor cells during development, in the epithelial lining of foregut-derived organs, including the lung and stomach, and within the nervous and sensory systems. In order to study SOX2 independently from OCT4 and NANOG, SOX2-positive cells (neuroprogenitors and stomach epithelial cells) were isolated from the human embryo/early fetus and cultured *in vitro*. These cells were found to express 13 arbitrarily selected OCT4-SOX2-NANOG target genes, despite the absence of OCT4 and NANOG. Expression of two of these genes, *TDGF1* and *DPPA4*, was analysed at the protein level. TDGF1 was detected in neuroprogenitors, but not in stomach epithelial cells *in vitro*. DPPA4 was present in neuroprogenitors and stomach epithelial cells, *in vitro* and *in vivo*. It was also expressed by germ cells, which are OCT4- and NANOG-positive, but SOX2-negative, and in mesonephros (negative for all three transcription factors). Therefore, in the case of TDGF and DPPA4, the combined presence of OCT4, SOX2 and NANOG is not required for gene expression. The expression of genes demonstrated to be direct targets of OCT4 and SOX2, namely *FGF4* and *UTF1*, correlated better with the combined presence of OCT4, SOX2 and NANOG, and were not expressed in the absence of OCT4 and NANOG.

The transduction of a few key transcription factors into somatic cells reverts them to ES-like cells, termed induced pluripotent stem (iPS) cells. Prior to publication of this recent discovery, OCT4 and NANOG were transduced into SOX2-positive cells; however, pluripotent cells were not generated. It has since been discovered that somatic cells require 10-12 days exposure to the exogenous factors to be reprogrammed, achieved using lentiviral vectors, whereas experiments herein used transient (48 hour) transfection.

In addition, it was discovered that the expression of cell surface antigens commonly used to confirm pluripotency in human embryonic stem cells; stage-specific antigen 3 (SSEA3), SSEA4, TRA-1-60 and TRA-1-81, were not specific to pluripotent cells, being expressed by neuroprogenitors and stomach epithelial cells. This highlights some limitations for their use as pluripotent markers; a combination of markers including those involved in maintaining pluripotency would provide a more robust criterion.

5.2 INTRODUCTION

5.2.1 The OCT4-SOX2-NANOG Transcription Factor Complex: Indispensable or Dispensable for Pluripotency?

Numerous studies have implicated the OCT4-SOX2-NANOG transcription factor complex as crucial in maintaining pluripotency. Knock-out of any one alone is embryonic-lethal (Nichols *et al.* 1998; Avilion *et al.* 2003; Mitsui *et al.* 2003). The OCT4-SOX2 complex, sometimes in combination with NANOG, activates transcription of a number of genes which either promote pluripotency, are essential for embryonic development, or both: for example, *FGF4* (Yuan *et al.* 1995) and *UTF1* (Nishimoto *et al.* 1999). The wide encompassing roles of the transcription factor complex were revealed in an elegant study by Boyer *et al.* (2005) which used genome-scale location analysis (chromatin immunoprecipitation [ChIP] coupled with DNA microarrays) to identify promoters (defined as within 8 kb upstream and 2 kb downstream of the transcript start site) bound by the transcription factor complex in hESCs. Numerous promoters were bound by all three, and the identity of these - genes which promoted pluripotency and proliferation and inhibited differentiation - suggested that these occupancies were functionally significant (Figure 1.6). Loh *et al.* (2006) reproduced these findings in mESCs, although with relatively little overlap with the target genes identified in hESCs.

Studies by the group of Shinya Yamanaka have to some extent disputed the essential role of the transcription factor complex, at least in the initial conversion to pluripotency. In August 2006 the group published a landmark paper in which they demonstrated that pluripotent stem cells (termed induced pluripotent stem [iPS] cells), could be generated from mouse embryonic or adult fibroblasts by introducing four factors - Oct4, Sox2, c-Myc and Klf4 - under ESC culture conditions (Takahashi and Yamanaka 2006). Unexpectedly, Nanog was dispensable, although selection for *Nanog* expression rather than *Fbx15* (both targets of Oct4 and Sox2) gave iPS cells which were more similar to ESCs, and notably were able to form adult chimaeras (Okita *et al.* 2007). Recently it has been found that the role of Nanog in pluripotent cells is more in resisting or reversing alternative gene expression states rather than in the housekeeping machinery of pluripotency (Chambers *et al.* 2007).

This technique has been reproduced by others (Wernig *et al.* 2007), also in human adult fibroblasts (Takahashi *et al.* 2007) and in fetal, neonatal and adult human primary

cells (Lowry *et al.* 2008; Park *et al.* 2008). The cells sequentially express pluripotency markers during the reprogramming process: AP first, followed by SSEA1, then Oct4, Sox2 and Nanog later (Brambrink *et al.* 2008; Stadtfeld *et al.* 2008). The reprogrammed fibroblasts show global epigenetic remodelling from the somatic epigenome to an ES-like state (Maherali *et al.* 2007). Three groups have since demonstrated that tumourigenic c-Myc is not required when transducing cells with Oct4, Sox2 and Klf4 (Blelloch *et al.* 2007; Nakagawa *et al.* 2008; Wernig *et al.* 2008). In addition, drug selection is not necessary (Blelloch *et al.* 2007; Meissner *et al.* 2007; Wernig *et al.* 2007). OCT4, NANOG, SOX2 and LIN28 reprogram human somatic cells to pluripotent stem cells (Yu *et al.* 2007). The only two transcription factors common to all studies were OCT4 and SOX2, although the transduction of these two alone was not sufficient to induce pluripotency in any study. Terminally differentiated mature B lymphocytes could also be reprogrammed, however along with Oct4, Sox2, Klf4, and c-Myc they also required either ectopic expression of the myeloid transcription factor CCAAT/enhancer-binding-protein-alpha (C/EBPalpha), or specific knockdown of the B-cell transcription factor Pax5 (Hanna *et al.* 2008). Most recently, it has been demonstrated that Sox2-positive neural progenitors isolated from neonatal mouse brains can be reprogrammed with Oct4, Klf4 and c-Myc, however the overall efficiency at generating iPS cells was 100-fold lower than when transducing the four factors into mouse fibroblasts (Eminli *et al.* 2008). In addition, iPS cells can be generated from adult mouse neural stem cells, which express higher endogenous levels of Sox2 and c-Myc than ESCs, with exogenous Oct4 and either Klf4 or c-Myc (Kim *et al.* 2008).

The OCT4-SOX2-NANOG transcription factor complex is not always co-expressed during human development; whilst all three transcription factors are co-expressed in the ICM and ESCs, hPGCs express OCT4 and NANOG without SOX2 (Perrett *et al.* 2008). This is the first cell type demonstrated thus far to do so and is fascinating given this transcription factor complex's proposed ability to act in a feed-forward loop activating its own transcription (Boyer *et al.* 2005). The major role for Sox2 in ESCs is proposed to be in maintaining *Oct4* expression (Masui *et al.* 2007). Therefore, *Oct4* must be activated/maintained by a different mechanism in hPGCs, possibly via Lrh1 (Gu *et al.* 2005), or Sall4 (Zhang *et al.* 2006), which maintains *Nanog* expression (Wu *et al.* 2006). SOX2 appears dispensable in the ability to activate target genes with OCT4 and NANOG, as without it, all 13 of the arbitrarily selected 'ESC-restricted' target genes from Boyer and

colleagues, as well as *UTF1* and *FBX15*, were expressed in the fetal ovary and testis (Perrett *et al.* 2008 and Chapter 3). In contrast, the data do support a pivotal role for SOX2 in promoting *FGF4* expression (Yuan *et al.* 1995; Ambrosetti *et al.* 1997): *FGF4* transcripts were undetected in the SOX2-negative germ cell lineage. hPGCs are nullipotent, and lack of SOX2 could be deemed a cause of this. However, upon hEGC derivation, with associated 'VP' ('vigorously proliferative') growth, SOX2 expression was not activated. Whilst hEGCs do differentiate *in vitro* to derivatives of all three germ layers, they have not been reported to generate teratomas in mice. Whether this is associated with lack of *SOX2* expression is unknown.

5.2.2 SOX2: Not Just a Regulator of Pluripotency

Whilst hPGCs are the only cells identified so far to express OCT4 and NANOG without SOX2, SOX2 is expressed by a variety of cells later on in development in the absence of OCT4 and NANOG, where, rather than maintaining pluripotency, it plays important roles in the regulation of organ development and cell-type specification, both during embryogenesis and in the adult.

5.2.2 (i) SOX2 in Foregut Derived Organs

The foregut of the embryo is initially an epithelial tube of endoderm surrounded by mesoderm. During development, the foregut gives rise to separate organs, including the trachea, esophagus, stomach and lungs. Sox2 is expressed at varying levels in epithelial cells of these organs in a variety of species (Ishii *et al.* 1998; Wood and Episkopou 1999; Park *et al.* 2006; Que *et al.* 2007). Here it may function to conserve progenitor cell characteristics. In the developing chick stomach, Sox2 expression is switched off in epithelia that are actively changing their morphology (Ishii *et al.* 1998), possibly linked to its ability to regulate the transcription and expression of gastric differentiation-related genes, for example, *Pepsinogen A* (Tani *et al.* 2007) and gastric foveolar mucin, *MUC5AC* (Li *et al.* 2004; Park *et al.* 2008). Heterozygosity for *SOX2* in humans is associated with anophthalmia-esophageal-genital (AEG) syndrome (Williamson *et al.* 2006), which in some instances is associated with failure of normal tracheo-esophageal separation, with the trachea being connected to the stomach by an abnormal distal esophagus. Using compound mutant mouse embryos in which the level of Sox2 varies below 50%, Que *et al.* (2007)

proposed that Sox2 regulates two independent processes in the developing foregut endoderm: 1. The early dorsal/ventral patterning of the anterior foregut that is coupled to the specification and morphogenesis of the trachea and eosophagus; 2. The patterning and differentiation of the foregut into keratinised, squamous eosophagus/forestomach versus posterior glandular hindstomach.

SOX2 is important for branching morphogenesis and epithelial cell differentiation in mouse and chick lung (Ishii *et al.* 1998; Gontan *et al.* 2008). In addition, SOX2 expression has been detected in a stem cell population in the adult human pancreas (Zhao *et al.* 2007).

5.2.2(ii) SOX2 in the Nervous System

The expression profile and function of SOX2 in the nervous system of many species, including *Drosophila*, *Xenopus*, zebrafish and chick, has been relatively well characterised (for reviews, see Pevny and Placzek 2005; Wegner and Stolt 2005). In the mouse nervous system, Sox2 is expressed in stem cells and early precursors, and in a few mature neurons (Zappone *et al.* 2000; Ferri *et al.* 2004). The role of Sox2 in maintaining neural progenitor identity extends from embryogenesis into adulthood: adult Sox2-deficient mice, in which *Sox2* expression is decreased by about 70%, exhibit neural stem/precursor cell proliferative defects in the hippocampus and periventricular zone (Ferri *et al.* 2004). Sox2 is thought to play an important role in maintaining the neural stem cell pool in the adult mouse brain (Suh *et al.* 2007) and adult human spinal cord (Dromard *et al.* 2008).

Heterozygous *SOX2* mutations in humans cause hippocampal defects and forebrain abnormalities, associated with anophthalmia (Fantes *et al.* 2003; Kelberman *et al.* 2006; Sisodiya *et al.* 2006). SOX2 functions to maintain neural progenitor identity in the chick (Graham *et al.* 2003), and conversion of rat oligodendrocyte precursor cells (OPCs) to neural stem cells depends on the reactivation of *Sox2* (Kondo and Raff 2004). It is also thought to have an additional function in early precursors committing themselves to neurogenesis: in 'programming' later neural differentiation events, and suppressing alternative (glial-specific) lineage markers (Cavallaro *et al.* 2008).

The mechanism of action of Sox2 in the nervous system has not yet been elucidated, it possibly counteracts neurogenic genes - the ability of basic-helix-loop-helix (bHLH) proteins to direct neuronal differentiation depends on their ability to suppress

Sox1-3 expression in chick CNS progenitors (Bylund *et al.* 2003). *SoxNeuro*, a putative *Drosophila* orthologue of the vertebrate Sox1, Sox2 and Sox3 proteins, acts upstream and in parallel to the proneural genes of the *achaete-scute* gene complex (Buescher *et al.* 2002; Overton *et al.* 2002). Alternatively, it may maintain neural progenitors in the cell cycle - inhibition of Sox2 in chick neural progenitor cells results in cell cycle exit (Bylund *et al.* 2003; Graham *et al.* 2003) and rat OPCs (Kondo and Raff 2004).

Nestin is the only known nervous system target of Sox2; the transcription of this intermediate filament protein is activated by the binding of Sox2 and the POU domain factor Brn2 to the *Nestin* enhancer (Tanaka *et al.* 2004). *Sox2* expression in turn is regulated by neural induction signals. An enhancer of chick *Sox2*, conserved in mammals, responds directly to neural inducing signals (Uchikawa *et al.* 2003). In both *Xenopus* and *Drosophila*, *Sox2* (or the ortholog *SoxNeuro* in *Drosophila*) is suppressed by BMP (*dpp* in *Drosophila*) or up-regulated by Chordin (*sog*) (Mizuseki *et al.* 1998; Cremazy *et al.* 2000; Buescher *et al.* 2002). Pax6 activates *Sox2* expression in neuroprogenitors of the cortical subventricular zone late on in mouse embryonic development (Wen *et al.* 2008). In addition, Stat3 directly regulates the *Sox2* promoter thereby initiating commitment to the neural precursor cell fate (Foshay and Gallicano 2008). Recently, a mechanism governing the onset of *Sox2* expression in the chick embryonic neural plate has been described (Papanayotou *et al.* 2008), involving the recruitment of transcriptional repressors to chromatin-remodelling complexes. The neural plate generates the entire nervous system, and one of its earliest molecular markers is Sox2.

5.2.2 (iii) SOX2 in the Sensory System

Mutations in *SOX2* cause anophthalmia (Fantes *et al.* 2003) and hearing loss (Hagstrom *et al.* 2005). Sox2 is expressed at all stages of eye development within zebrafish and chick in the optic vesicle, head surface ectoderm and derived lens placode (chick) or eye field (zebrafish) through to the neural retina (Kamachi *et al.* 1998). Here it activates the transcription of the *αB*- and *δ1-crystallin* genes, the latter in cooperation with Pax6 (Kamachi *et al.* 2001; Ijichi *et al.* 2004; Kondoh *et al.* 2004). It also acts with Oct1 to maintain *Pax6* expression during lens and nasal placode induction (Donner *et al.* 2007), and with Pax6 to regulate its own transcription (Inoue *et al.* 2007). Alterations in the levels of Sox2 regulate the choice between maintenance of retinal progenitor cell identity and

differentiation. Sox2 mediates its effects by directly regulating the expression levels of Notch1 (Taranova *et al.* 2006).

Levels of Sox2 regulate the differentiation of endodermal progenitor cells of the mouse tongue into taste bud sensory cells versus keratinocytes (Kiernan *et al.* 2005). In the mouse inner ear, alteration or loss of expression of Sox2 correlates with abnormal/absent hair and supporting cell differentiation, indicating a role for Sox2 in establishing sensory progenitors (Okubo *et al.* 2006). Sox2 is also expressed by some highly differentiated vestibular hair cells in the mature mouse ear, and given their limited proliferative capability, a role for maintaining a stem cell-like state appears unlikely (Oesterle *et al.* 2008). In the chick, Sox2 is proposed to specify a population of otic progenitors committed to a neural fate, giving rise to neurons and hair cells (Neves *et al.* 2007).

To summarise, therefore, when expressed in the absence of OCT4 and NANOG, SOX2 maintains progenitor cells during development, and occasionally in the adult, it has additional uncharacterised functions in some differentiated cells. To investigate these three regulators of pluripotency further, SOX2-positive, OCT4- and NANOG-negative cells were isolated and cultured from the human embryo/early fetus, providing the opportunity to study SOX2 in isolation from OCT4 and NANOG, with the following aims:

5.3 SPECIFIC AIMS

5.3.1 To determine the expression profile of SOX2 during human embryonic development.

SOX2 is reportedly expressed in a number of sites during human embryonic development, including neuroprogenitors and the stomach epithelial lining; this expression will be verified by RT-PCR and immunohistochemistry.

5.3.2 To successfully isolate, culture and characterise SOX2-positive, OCT4- and NANOG-negative cells from the human embryo/early fetus.

This will involve the dissection of the spinal cord and stomach from the human embryo/early fetus, isolation and *in vitro* culture of neuroprogenitors and stomach epithelial cells. Cells will be tested for SOX2 expression and also for neuroprogenitor markers (e.g. the intermediate filament protein Nestin) or stomach epithelial markers (e.g. cytokeratin 19 [CK19]).

5.3.3 To determine the expression profile of genes which reportedly rely on the presence of the OCT4-SOX2-NANOG transcription factor complex in these SOX2-positive cells.

The expression of genes previously recognised as targets of OCT4 and SOX2, and of those reported to be expressed in hESCs when OCT4, SOX2 and NANOG are bound to the respective promoter regions, will be examined in neuroprogenitors and stomach epithelial cells.

5.3.4 To determine whether inducing expression of the OCT4-SOX2-NANOG transcription factor complex causes cells to become pluripotent.

This will be determined by transfecting SOX2-positive cells with OCT4 and NANOG expression vectors and examining subsequent expression of neuroprogenitor/stomach epithelial and stem cell-specific markers.

5.4 RESULTS

5.4.1 SOX2 Expression in the Human Embryo

By RT-PCR, *SOX2* expression was detected in human fetal (56 dpc) brain, spinal cord, eye and stomach (Figure 5.1), consistent with reports in other species (Ishii *et al.* 1998; Kamachi *et al.* 1998; Wood and Episkopou 1999; Zappone *et al.* 2000; Graham *et al.* 2003; Que *et al.* 2007). It was absent from lung, contrasting previous findings in mouse and chick (Ishii *et al.* 1998; Gontan *et al.* 2008), and from pancreas. Embryonic mouse pancreas does not appear to contain Sox2-expressing stem cells, although mRNA has been detected at low levels (Wilson *et al.* 2005), and it is proposed to mark a stem cell population in the adult human pancreas (Zhao *et al.* 2007).

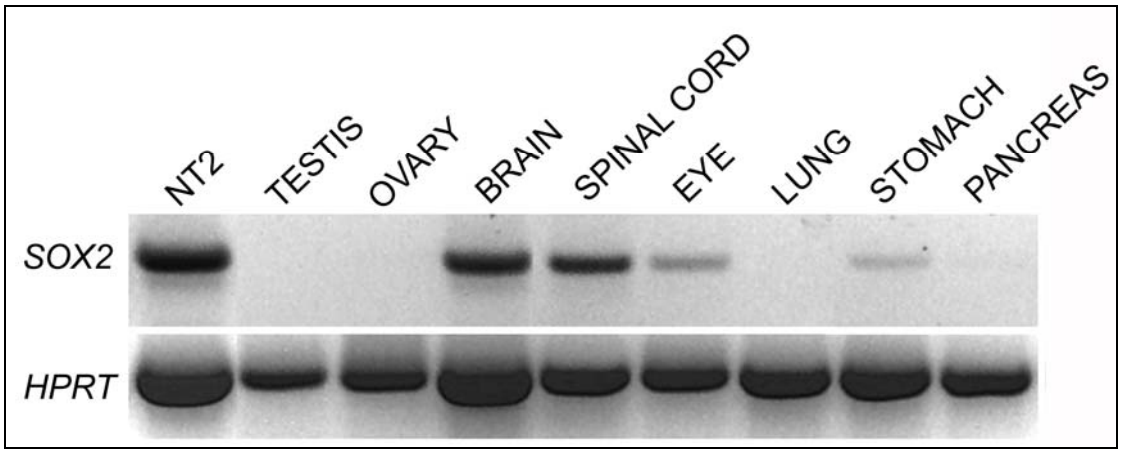


Figure 5.1 Expression of *SOX2* in human fetal tissues
RT-PCR panel for *SOX2* and the housekeeping gene *HPRT* in a range of human fetal (56 dpc) tissues following PCR for 35 cycles. NT2: NTERA2 cells (positive control for *SOX2* expression). Testis and ovary represent negative controls (Perrett *et al.* 2008 and Chapter 3). For primer sequences see Appendix 1.

By immunohistochemistry, strong nuclear SOX2 protein was detected in the developing spinal cord in a 48 dpc embryo (Figure 5.2) and in older embryonic and fetal sections (data not shown), throughout the progenitor domains (in the ventricular region close to the midline) from the roof plate to the floor plate. Strong nuclear staining was also detected throughout the epithelial lining of the stomach. At this stage of embryogenesis, the human gastric epithelium is stratified, composed of undifferentiated cells (Montgomery *et al.* 1999).

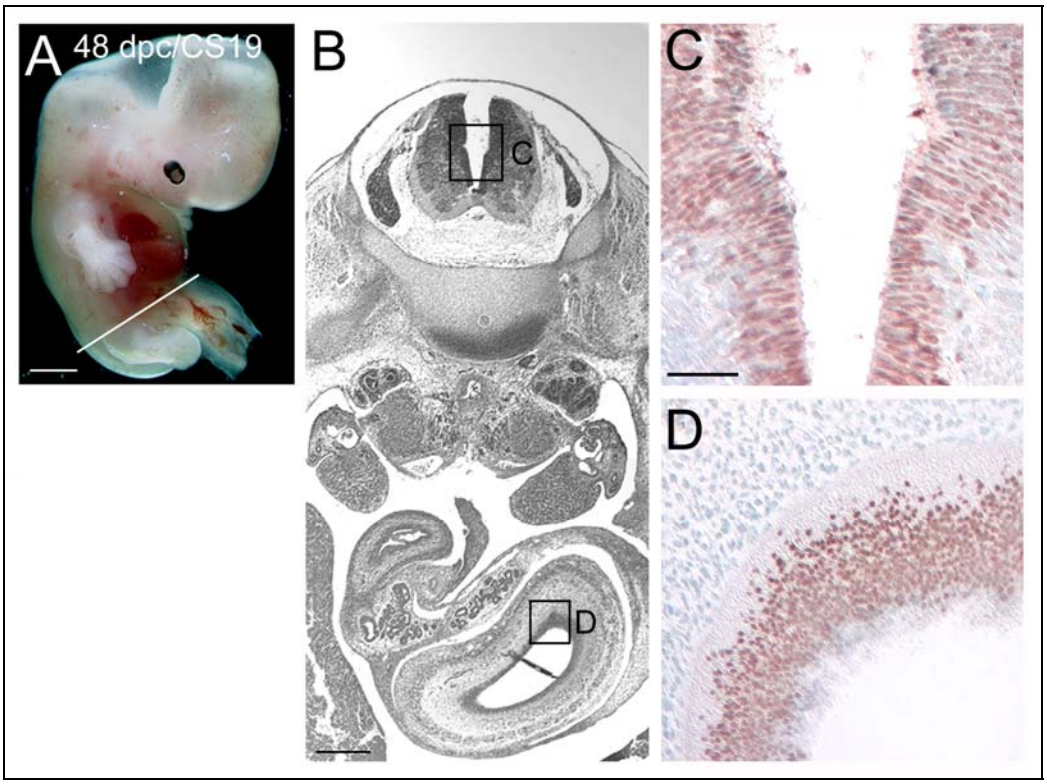


Figure 5.2 SOX2 expression during human embryogenesis at 48 dpc/CS19
A. Bright-field image of human embryo at 48 dpc/CS19. White line indicates plane of transverse section shown in B. **B.** Bright-field image of transverse section. SOX2 immunohistochemistry is shown for the boxed areas in C and D. **C-D.** Immunohistochemical staining of SOX2 in **C.** spinal cord and **D.** stomach. Size bars represent 2 mm (A), 250 μm (B) and 50 μm (C).

5.4.2 Isolation and Culture of SOX2-Expressing Cells from the Human Embryo/Early Fetus

With confirmation that embryonic and fetal neuroprogenitors and stomach epithelial cells expressed SOX2, they were isolated from human embryonic/fetal material and culture methods employed aiming to retain SOX2 and cell-specific marker expression *in vitro*.

5.4.2(i) Neuroprogenitors

Freshly collected spinal cord (6-10 wpc) was isolated and immediately processed. A modified protocol from Austin Smith's group was used to culture the cells (Conti *et al.* 2005; Pollard *et al.* 2006). Under a dissecting microscope, the spinal cord was isolated,

separated from associated material (Figure 5.3 B), and the meninges removed (Figure 5.3 C). Tissue was repeatedly pipetted with a P1000 pipette until completely homogenised, and then plated in 'expansion' media, comprising DMEM/Ham's F-12 containing N2 and B27 supplements and 10ng/ml of FGF2 and epidermal growth factor ([EGF], all Invitrogen). The combination of FGF2 and EGF is sufficient for the derivation and continuous expansion of pure cultures of neural stem cells (Suslov *et al.* 2002; Bez *et al.* 2003). Cells were left for approximately 3-4 days, until neurospheres became visible (Figure 5.3 D). Neurospheres contain a mixture of stem cells, committed progenitors, and differentiated cells (Aleksandrova *et al.* 2005); however, when grown under adherent conditions, only neural stem cells remain (Pollard *et al.* 2006). These spheres were allowed to settle for 10 minutes, then plated onto a dish pre-coated in 0.1% gelatin. Attachment and outgrowth occurred within 4-5 days. At 70% confluence, cells were passaged 1:3 to 1:5 using trypsin and grown under adherent conditions.

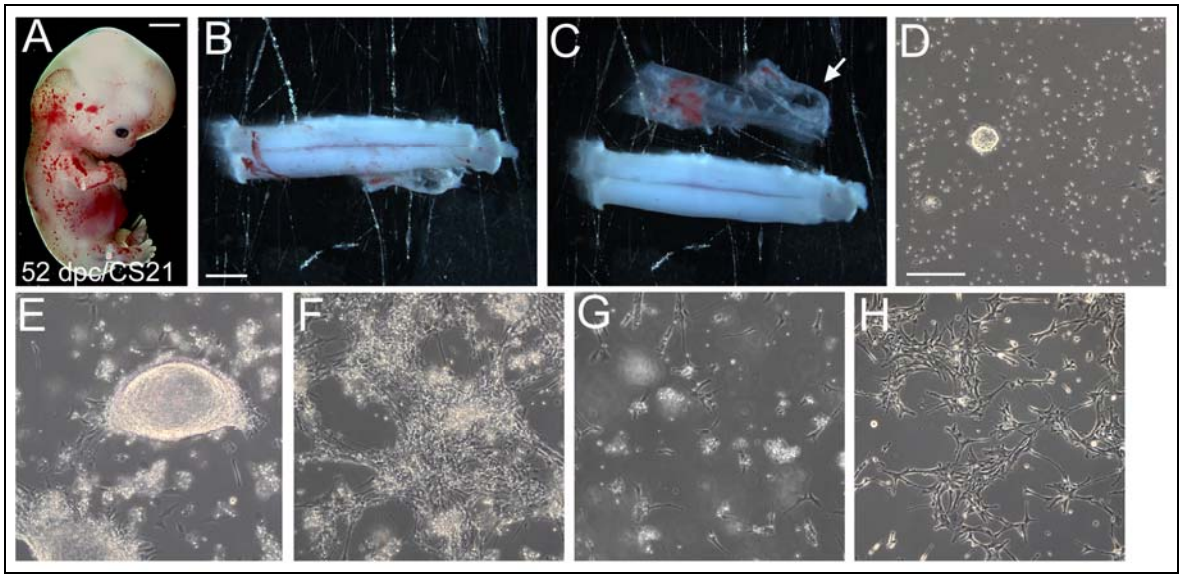


Figure 5.3 Dissection and culture of human embryonic/early fetal spinal cord

A. Collected embryo at 52 dpc/CS21. **B.** Isolated spinal cord. **C.** Spinal cord with meninges removed (arrow). **D.** Phase-contrast of spinal cord following mechanical disaggregation and culture for 2 days in suspension. Note formation of small neurosphere. **E.** Neurosphere after culture for 2 days on gelatin-coated plastic. Neurosphere has enlarged and attached to growth surface with outgrowths. **F.** Neurosphere following culture for 7 days on gelatin-coated plastic. Outgrowths have extended across growth surface. **G.** Neuroprogenitors 1 day following trypsinisation and culture on gelatin-coated plastic. **H.** The same culture following 5 days of growth. Size bars represent 3 mm (A) 1 mm (B-C) and 200 µm (D-H). The dissection was performed by Prof. Neil Hanley (Human Genetics Division, University of Southampton).

5.4.2(ii) Stomach Epithelial Cells

Freshly collected material (6-10 wpc) was immediately processed. Under a dissecting microscope, an incision was made that completely exposed the abdominal cavity, confirming the anatomical orientations of the liver, stomach, gall bladder and spleen (Figure 5.4 B). The entire stomach was removed from the associated organs (Figure 5.4 C and D). The white dashed lines indicate the location of the incision made to gain access to the inner lining of the stomach (Figure 5.4 E) and the stomach flattened out (Figure 5.4 F) to expose the inner surface, which was then gently scraped, removing the epithelial layer of cells (Figure 5.4 G).

Increasing cell density arrests epithelial cell proliferation by a process termed contact inhibition. As the initial dissection of cells provided them as a layer (Figure 5.4 G), cells were incubated in a cell dissociation mix, consisting of 0.25% collagenase, 20 U/ml DNase I, 60 μg/ml $CaCl_2$ (all Sigma), 2% heat-inactivated newborn calf serum (Invitrogen), in HBSS (PAA Laboratories) at 37°C for 1 hour, with repeated trituration, to encourage dispersion of cells. They were then plated in DMEM containing 10% FCS [media used by Basque *et al.* (1999) in their culture of human fetal stomach epithelium on plastic, with resultant retention of all gastric epithelial cell-types for one week]. Although cells attached and proliferated as 'plaques' (Figure 5.4 H), growth ceased after approximately 7 days, with detachment from the growth surface. Trypsinisation and subsequent replating did not encourage any further growth.

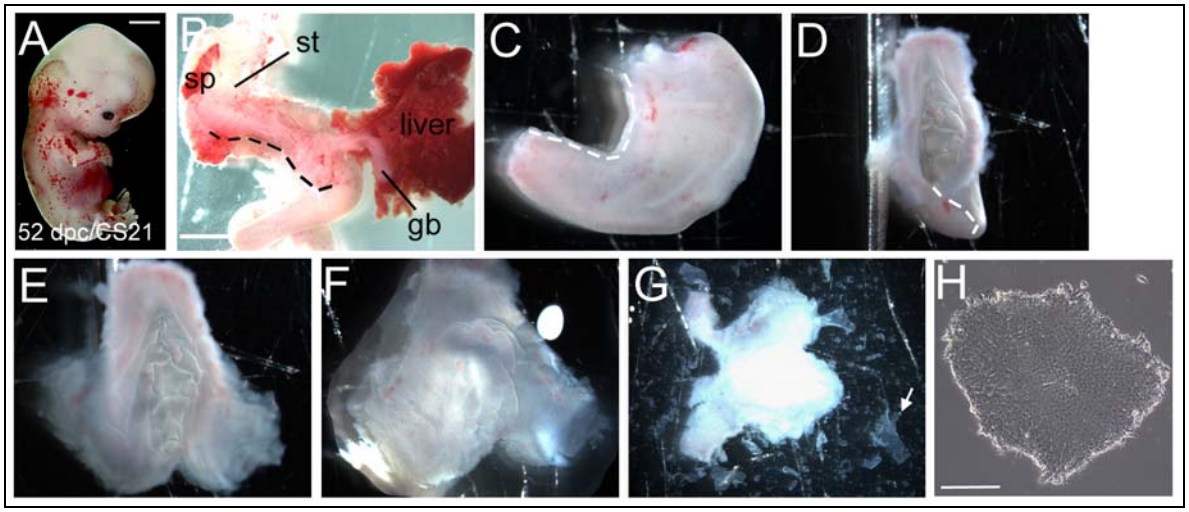


Figure 5.4 Dissection and culture of human embryonic/early fetal stomach

A. Collected embryo at 52 dpc/CS21. **B.** Posterior view of preliminary dissection. **C.** Further dissection has removed associated organs. Dotted line indicates site of incision. **D.** View from the top of the stomach. **E.** Dotted line has been opened up. **F.** Further opening and flattening. **G.** Inner lining of stomach has been scraped leaving epithelial cells (arrow) which were enzymatically digested before plating. **H.** Phase-contrast morphology of stomach epithelial cells following 4 days in culture. Gb, gall bladder; st, stomach; sp, spleen. Size bars represent 3 mm (A), 1 mm (B-G) and 200 μm (H). The dissection was performed by Prof. Neil Hanley (Human Genetics Division, University of Southampton).

5.4.3 Expression of SOX2 and Cell-Specific Markers in Human Fetal Neuroprogenitors and Stomach Epithelial Cells *in vitro*

The cultured cells were tested to ensure that they retained SOX2 and cell-specific marker expression *in vitro*.

5.4.3 (i) Neuroprogenitors

Cells were isolated, cultured under non-adherent then adherent conditions, and then plated onto chamber slides coated with fibronectin and fixed with 4% PFA three days later. Therefore, all cells had been *ex vivo* for approximately thirteen days when characterised. Approximately 80% of cells expressed SOX2, β-tubulin, nestin and vimentin (Figure 5.5 A-E). Between 10-20% of cells expressed glial fibrillary acidic protein (GFAP), in some cases co-expressing SOX2 (Figure 5.5 F). The expression of vimentin and nestin is

consistent with the cells being neural precursors/stem cells; however, expression of β-tubulin III, a marker for newly-formed neurons, is indicative of differentiation towards a neuronal fate. In addition, SOX2 marks neural precursors, whereas GFAP marks glial cells; therefore, dual expression seems paradoxical (Figure 5.5 F). However, a population of GFAP-positive cells have been derived from human fetal brain, which, during their initial proliferative phase, co-express GFAP, nestin, vimentin and β-tubulin III. These cells differentiate into two distinct populations of cells, either restricted glial precursors ($GFAP^+$/$nestin^+$) or neuronal precursors (β-tubulin III^+/$nestin^+$) (Rieske *et al.* 2007). In addition, neurospheres obtained from human embryonic brain were found to co-express nestin and β-tubulin III (Aleksandrova *et al.* 2005). As well as being expressed by precursors, SOX2 is expressed in differentiating neural cells *in vitro* (Cavallaro *et al.* 2008) and *in vivo* (Bani-Yaghoub *et al.* 2006). Therefore, the cell type boundaries drawn by marker expression may not be distinct and it appears that some neural cells co-express a combination of precursor, neuronal and glial markers before final commitment. Despite the protocol for generation of neural stem cells being used (Conti *et al.* 2005; Pollard *et al.* 2006), expression of β-tubulin III indicates further commitment down the neuronal lineage. Therefore, the cells isolated here are referred to as 'neuroprogenitors' rather than neural stem cells.

5.4.3 (ii) Stomach Epithelial Cells

Stomach epithelial cells were isolated, enzymatically digested, directly plated onto fibronectin coated chamber slides, and fixed four days later with 4% PFA. Approximately 70% of cells expressed nuclear SOX2 (Figure 5.6 A-B), although diffuse cytoplasmic staining was also apparent, and all cells were positive for the epithelial cell marker CK19 (Figure 5.6 C) and the gap junction protein Connexin 43 (CX43) (Figure 5.6 D).

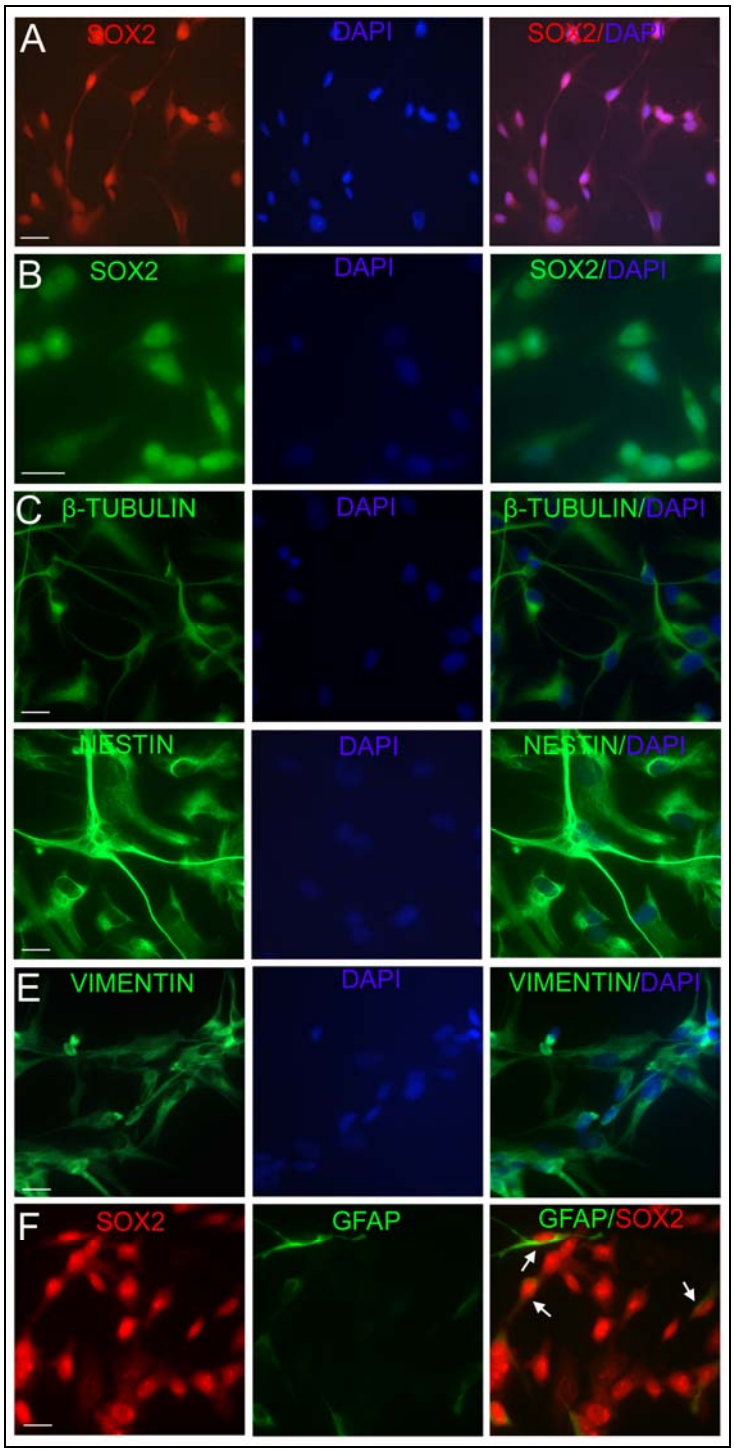


Figure 5.5 Expression of neural markers in cultured human fetal neuroprogenitors

Immunocytochemistry for **A-B.** SOX2, **C.** β-tubulin III, **D.** nestin, **E.** vimentin in fetal neuroprogenitors. **A-E.** Individual band pass images are shown for Texas Red or FITC (left panel), DAPI (middle panel); with dual band pass imaging (right panel). **F.** SOX2 (red)/GFAP (green); individual band pass images are shown for Texas Red (left panel), FITC (middle panel), with dual band pass imaging (right panel). Arrows point to cells exhibiting dual expression. Cells were cultured for 13 days before staining. Size bars represent 20 μm.

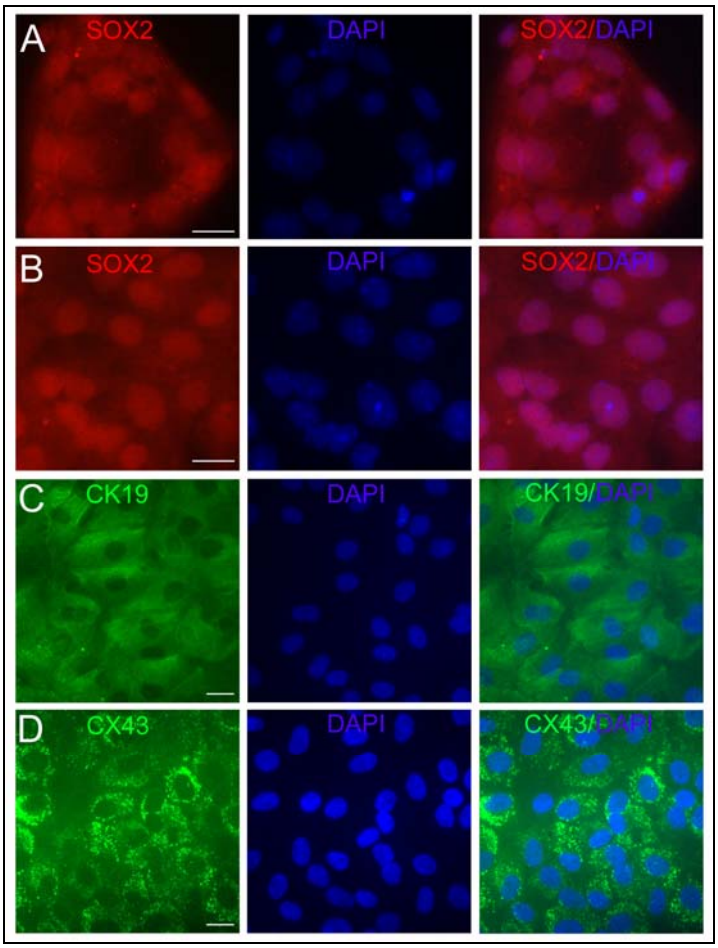


Figure 5.6 SOX2, CK19 and CX43 immunoreactivity in cultured human fetal stomach epithelial cells

Immunocytochemistry for **A-B.** SOX2, **C.** CK19 and **D.** CX43 in cultured human fetal stomach epithelial cells. Individual band pass images are shown for Texas Red or FITC (left panel), DAPI (middle panel), with dual band pass imaging (right panel). Cells were cultured for 4 days before staining. Size bars represent 20 μm.

5.4.4 Expression of OCT4-SOX2-NANOG Target Genes in Human Fetal Neuroprogenitors and Stomach Epithelial Cells *in vitro* and *in vivo*

Boyer *et al.* (2005) identified a number of genes expressed in hESCs when OCT4, NANOG and SOX2 were collectively bound to their respective promoter regions. Thirteen were arbitrarily selected, and, along with genes known to be up-regulated by OCT4 and SOX2 (Yuan *et al.* 1995; Nishimoto *et al.* 1999; Tokuzawa *et al.* 2003), their expression tested for by RT-PCR in hPGCs (Perrett *et al.* 2008 and Chapter 3). Surprisingly, despite the absence of SOX2, all genes were expressed in fetal ovary and testis, indicating OCT4 and NANOG expression may be sufficient to maintain expression of these genes. Here, the

expression of these target genes was also tested in neuroprogenitors and stomach epithelial cells. RT-PCR confirmed the presence of SOX2 and absence of OCT4 and NANOG in the cells (Figure 5.7 A). FGF4 and UTF1, transcriptional targets of the OCT4-SOX2 complex (Yuan *et al.* 1995; Nishimoto *et al.* 1999), were not expressed; however, FBX15, also a target (Tokuzawa *et al.* 2003), was (Figure 5.7 A), along with the 13 arbitrarily selected genes proposed to be targets of OCT4, SOX2 and NANOG in hESCs (Figure 5.7 B).

Extending this analysis to protein expression, TDGF1 was detected in neuroprogenitors (Figure 5.8 A), but not stomach epithelial cells (Figure 5.8 B), despite *TDGF1* expression being detected by RT-PCR in stomach epithelial cells (Figure 5.7 B). It is possible that protein levels were too low for detection by immunohistochemistry, or translation of the transcript is repressed.

Nuclear DPPA4 protein was detected, consistent with *DPPA4* expression, in neuroprogenitors (Figure 5.9 A), with expression stronger in some cells (arrows) than others (arrowheads). Weak expression was detected in stomach epithelial cells (Figure 5.9 B), with a distinct subnuclear localisation, presumably nucleolar (arrows), similar to the staining pattern observed in NTERA-2 D1 cells (arrows Figure 5.9 D).

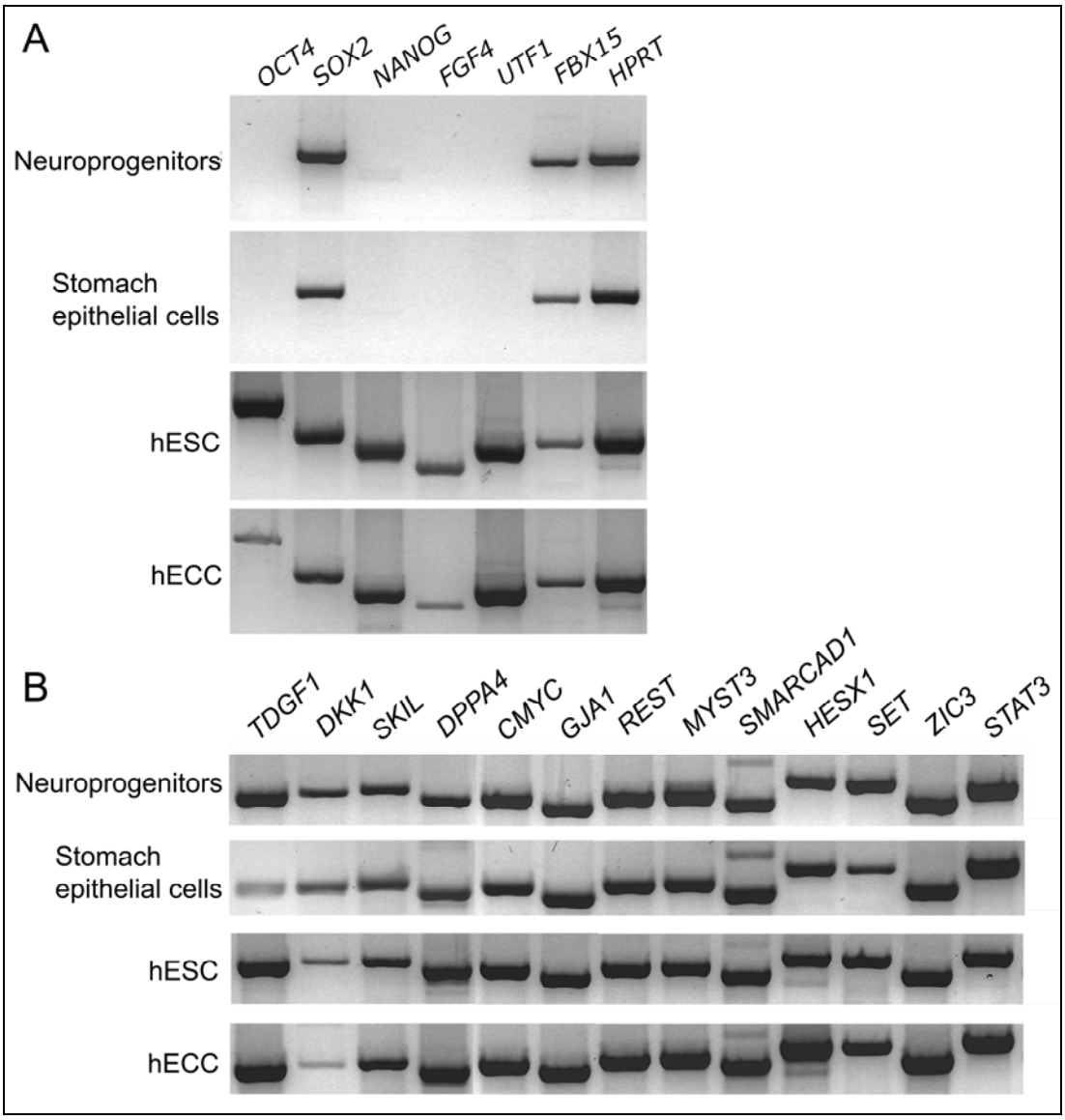


Figure 5.7 RT-PCR analysis for expression of genes regulated by OCT-SOX enhancers and those identified by bioinformatic analysis as targets of OCT4, SOX2 and NANOG in human fetal neuroprogenitors and stomach epithelial cells

A. *OCT4*, *SOX2*, *NANOG* and genes previously recognised as targets of OCT4 and SOX2 (Yuan *et al.* 1995; Nishimoto *et al.* 1999; Tokuzawa *et al.* 2003), and **B.** genes previously described to be expressed in hESCs when OCT4, NANOG and SOX2 are bound to the respective promoter regions (Boyer *et al.* 2005) following PCR for 35 cycles. Abbreviations[28]. For primer sequences see Appendix 1.

[28] FGF4, fibroblast growth factor 4; UTF1, undifferentiated embryonic cell transcription factor 1; FBX15, F-box only protein 15; HPRT, hypoxanthine-guanine phosphoribosyl-transferase; TDGF1, teratocarcinoma-derived growth factor 1; DKK1, Dickkopf Xenopus, homolog of 1; SKIL, SKI-like; DPPA4, developmental pluripotency associated gene 4; CMYC, myelocytomatosis oncogene; GJA1, gap junction protein, alpha 1; REST, RE-1 silencing transcription factor; MYST3, MYST histone acetyltransferase; SMARCAD1, SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1; HESX1, homeobox gene expressed in ESCs; SET, suppressor of variegation, enhancer of zeste and Trithorax; ZIC3, zinc finger protein of cerebellum; STAT3, signal transducer and activator of transcription 3; ECC, embryonal carcinoma cell; ESC, embryonic stem cell.

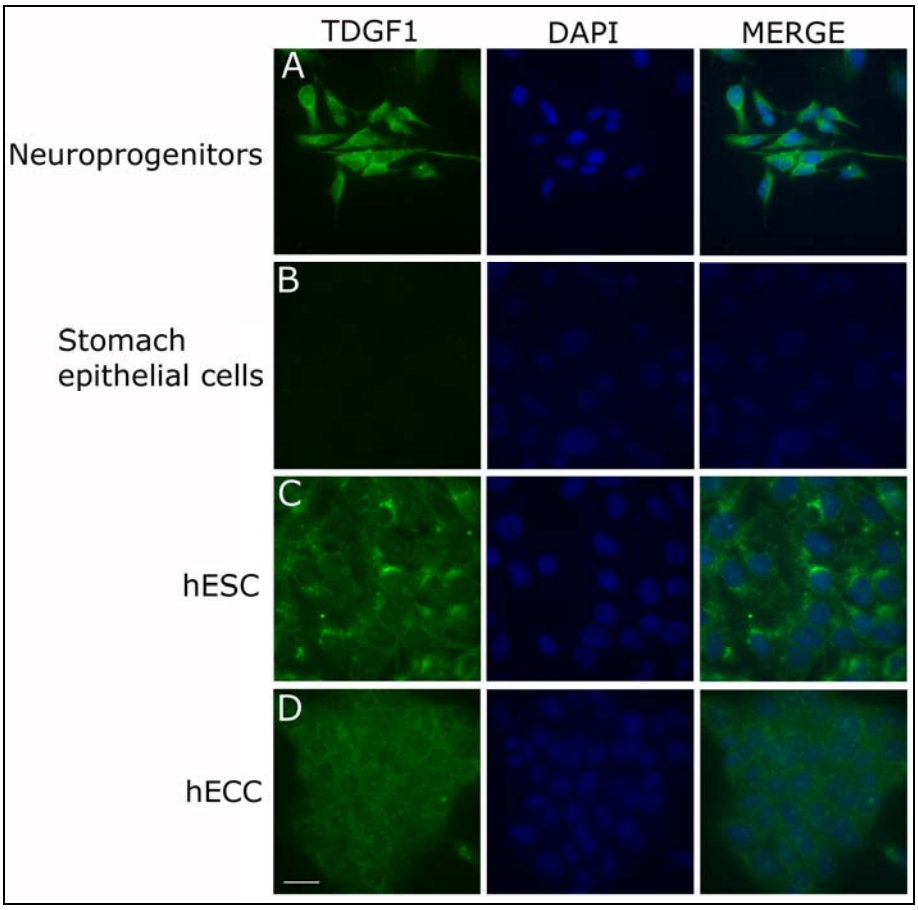


Figure 5.8 TDGF1 immunoreactivity in cultured human fetal neuroprogenitors and stomach epithelial cells

Immunocytochemistry for TDGF1 in **A.** cultured fetal neuroprogenitors, **B.** cultured fetal stomach epithelial cells, **C.** hESCs (HUES1) and **D.** hECCs (NTERA-2 D1 cells, both positive controls). Individual band pass images are shown for FITC (left panel), DAPI (middle panel), with dual band pass imaging (right panel). Size bar represents 20 μm.

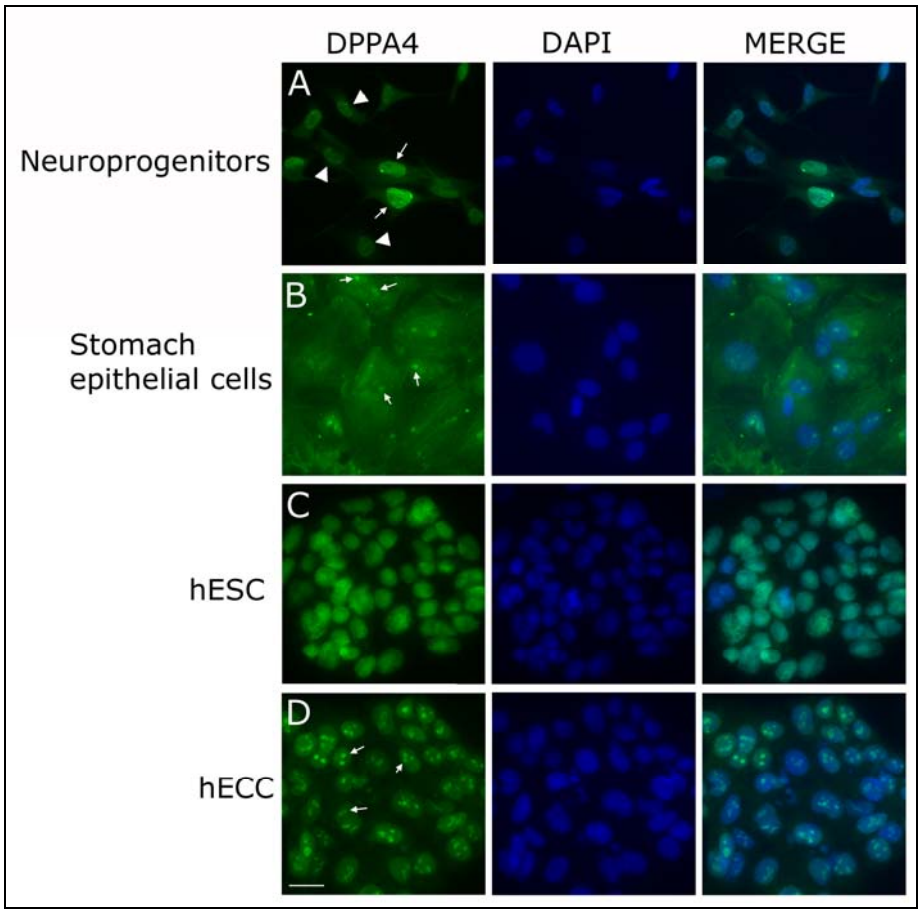


Figure 5.9 DPPA4 immunoreactivity in cultured human fetal neuroprogenitors and stomach epithelial cells

Immunocytochemistry for DPPA4 in **A.** cultured fetal neuroprogenitors (arrows indicate cells demonstrating strong expression, arrowheads weaker), **B.** cultured fetal stomach epithelial cells (arrows indicate presumed nucleolar expression), **C.** hESCs (HUES1) and **D.** hECCs (NTERA-2 D1 cells, arrows indicate presumed nucleolar expression). hESCs and hECCs are positive controls. Individual band pass images are shown for FITC (left panel), DAPI (middle panel), with dual band pass imaging (right panel). Size bar represents 20 μm.

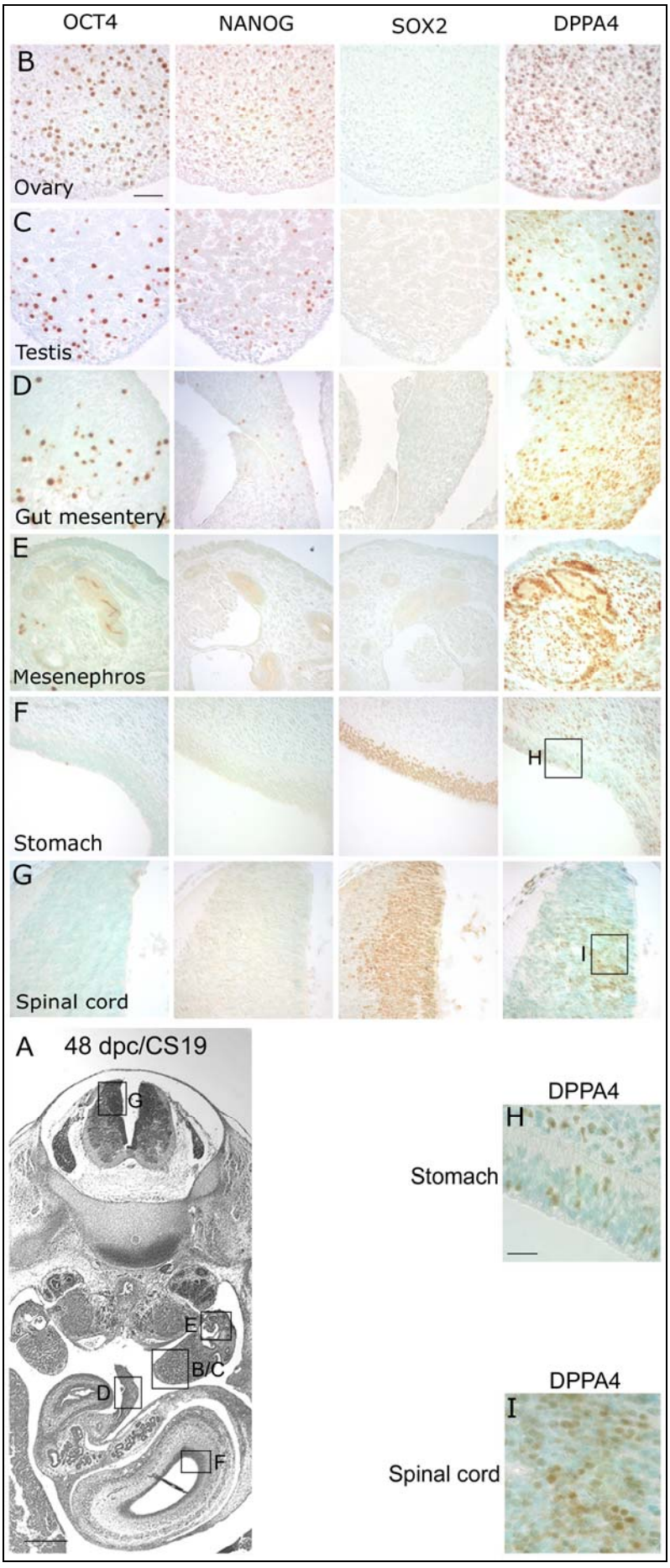
For comparison with these *in vitro* expression profiles, DPPA4 expression was investigated *in situ* in the human embryo at 48 dpc/CS19. Nuclear DPPA4 protein was detected in OCT4- and NANOG-positive germ cells in the ovary and testis (Figure 5.10 B-C) and gut mesentery (Figure 5.10 D). Significant expression was noted in the mesonephros (Figure 5.10 E), which was negative for OCT4, NANOG and SOX2. Nuclear DPPA4 expression was apparent in the stomach (Figure 5.10 F, enlarged in H). Although this is a site of SOX2 expression, the pattern of DPPA4 expression was not identical, being expressed in a proportion of epithelial cells only and additionally in some cells of the

underlying mesenchyme. DPPA4 was expressed in the spinal cord (Figure 5.10 G, enlarged in I). Again, expression did not match that of SOX2 (Figure 5.11): SOX2 was expressed by all neuroprogenitors, whereas DPPA4 was specifically expressed in some dorsal progenitors in regions 3-6 (marked with the upper bracket in Figure 5.11), and also ventral progenitors in region 3 and in the motor neuron precursors (marked with the lower bracket in Figure 5.11). Overall, therefore, DPPA4 expression overlapped but did not exclusively correlate with expression of either OCT4, NANOG or SOX2.

DPPA4 expression *in situ* was validated by immunoblotting of protein isolated from the human embryo (54 dpc; Figure 5.12). DPPA4 was present as a band of 34 kDa in gonads, with more detected in ovary presumably due to increased germ cell number, and also detected in mesonephros and hESCs (positive control). Weak expression was detected in spinal cord, cultured stomach and neuroprogenitors.

Figure 5.10 DPPA4 expression during human embryogenesis at 48 dpc/CS19

A. Bright-field image of transverse section of human embryo at 48 dpc/CS19. DPPA4 immunohistochemistry is shown for the boxed areas in B-G. DPPA4-positive (OCT4-, NANOG-positive, SOX2-negative) germ cells in **B.** fetal ovary, **C.** testis and **D.** gut mesentery. **E.** DPPA4-positive cells in mesonephros. The same cells are OCT4-, NANOG- and SOX2-negative. DPPA4 immunoreactivity is observed in fetal stomach (**F**, boxed area magnified in **H**) and spinal cord (**G**, boxed area magnified in **I**), but does not directly overlap with SOX2 expression. Size bars represent 250 μm (A), 50 μm (B-G) and 20 μm (H-I).


OCT4
NANOG
SOX2
DPPA4
B
Ovary
C
Testis
D
Gut mesentery
E
Mesenephros
F
H
Stomach
G
I
Spinal cord
A 48 dpc/CS19
G
E
B/C
D
F
DPPA4
H
Stomach
DPPA4
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Spinal cord

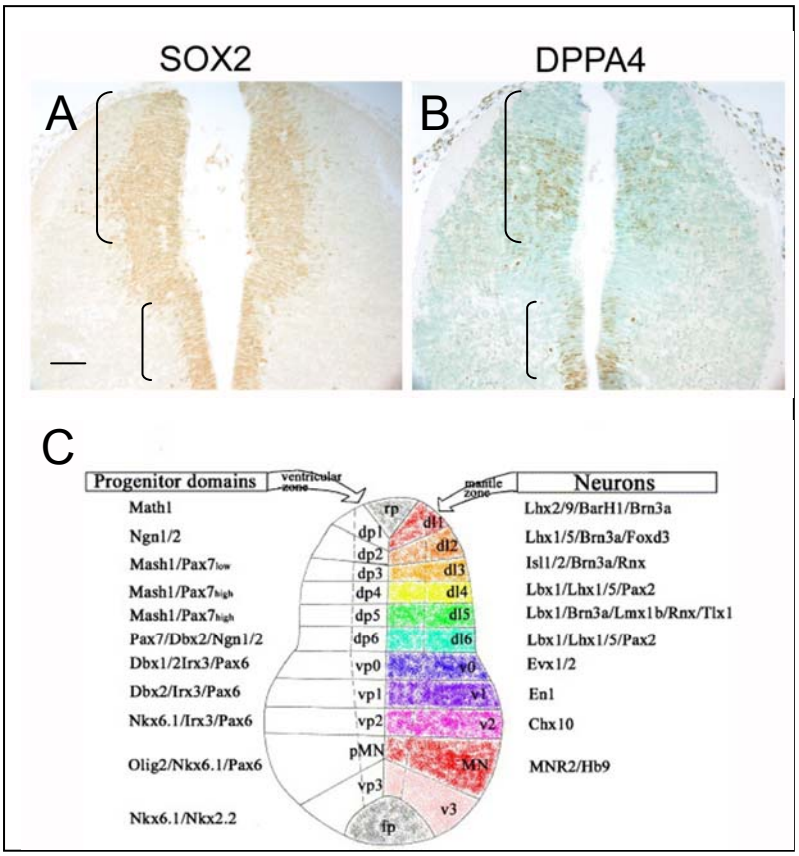


Figure 5.11 DPPA4 and SOX2 expression in the developing human spinal cord at 48 dpc/CS19

A. SOX2 and **B.** DPPA4 immunoreactivity in spinal cord. Upper and lower brackets indicate areas of co-expression; however, whilst all neuroprogenitors in these regions are SOX2-positive, only approximately 30% are DPPA4-positive. Size bar represents 50 µm. **C.** Diagram showing the regionalisation of the 6 types of dorsal neurons (dI1–dI6) and the 5 types of ventral neurons (v0–v3 + mn) in the developing spinal cord (from Wilson and Maden 2005). On the left are the gene and protein markers used to identify the progenitor domains (in the ventricular region close to the midline) of these different dorsoventral regions. On the right are the gene and protein markers which are used to identify the neuronal types (in the mantle region where neurons differentiate).

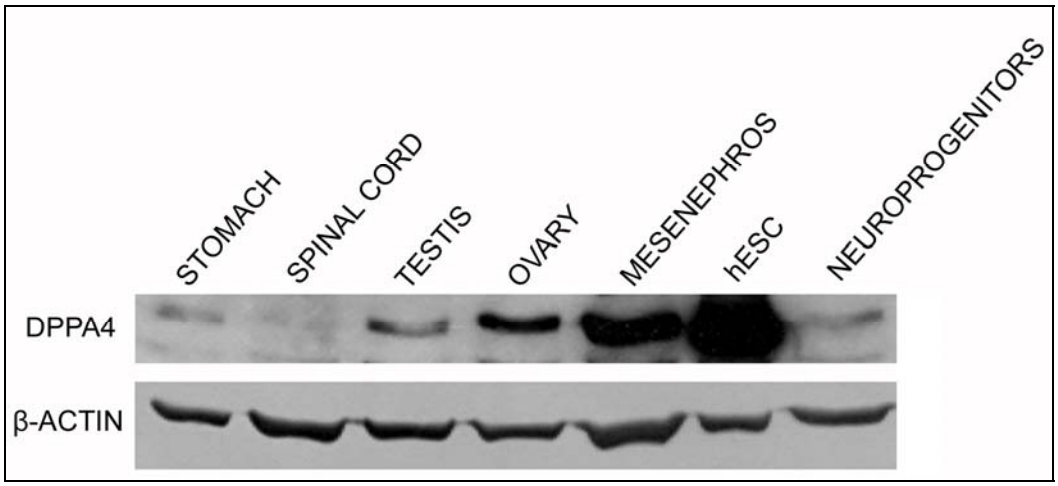


Figure 5.12 Immunoblot for DPPA4 in human embryonic tissues
Immunoblot for DPPA4 and β-actin in protein extracted from human embryonic (54 dpc) tissues (spinal cord, testis, ovary and mesonephros), hESCs (HUES1, positive control), and cultured fetal stomach and neuroprogenitors.

5.4.5 Introduction of OCT4 and NANOG Does Not Induce Pluripotency in SOX2-Positive Neuroprogenitors/Stomach Epithelial Cells

Recent studies have reported success in the reprogramming of somatic cells to pluripotency through the transduction of a combination of key transcription factors, including OCT4, SOX2 and NANOG, although most studies have found that NANOG is dispensable (Takahashi and Yamanaka 2006; Meissner *et al.* 2007; Okita *et al.* 2007; Takahashi *et al.* 2007; Wernig *et al.* 2007; Yu *et al.* 2007; Eminli *et al.* 2008; Kim *et al.* 2008; Nakagawa *et al.* 2008; Park *et al.* 2008). The coding regions of OCT4 and NANOG were amplified from I.M.A.G.E. clones and cloned into the expression vector pcDNA3.1Zeo+ [Invitrogen; Appendix 3 (i)].

To select a 'read-out' pluripotent stem cell marker which would indicate reversion of neuroprogenitors/stomach epithelial cells to pluripotency, the expression profile of so-called 'pluripotent' cell surface markers was firstly determined. Surprisingly, many of these surface antigens were already expressed by the differentiated cells (Figure 5.13). Neuroprogenitors were $SSEA^{+}$, $EMA1^{weak}$, $SSEA3^{weak}$, $SSEA4^{+}$, $TRA\text{-}1\text{-}60^{+}$ and $TRA\text{-}1\text{-}81^{+}$ (Figure 5.13 A). Stomach epithelial cells were $SSEA1^{+}$, $EMA1^{+}$, $SSEA3^{-}$, $SSEA4^{-}$, $TRA\text{-}1\text{-}60^{weak}$ and $TRA\text{-}1\text{-}81^{+}$ (Figure 5.13 B). hESCs were $SSEA1^{-}$, $EMA1^{-}$, $SSEA3^{+}$, $SSEA4^{+}$, $TRA\text{-}1\text{-}60^{+}$ and $TRA\text{-}1\text{-}81^{+}$ (Figure 5.13 C); hECCs $SSEA1^{weak}$ (possibly due to

low level differentiation), EMA1^{-}, SSEA3^{weak} (again possibly due to early differentiation), SSEA4^{weak}, TRA-1-60^{+} and TRA-1-81^{+} (Figure 5.13 D).

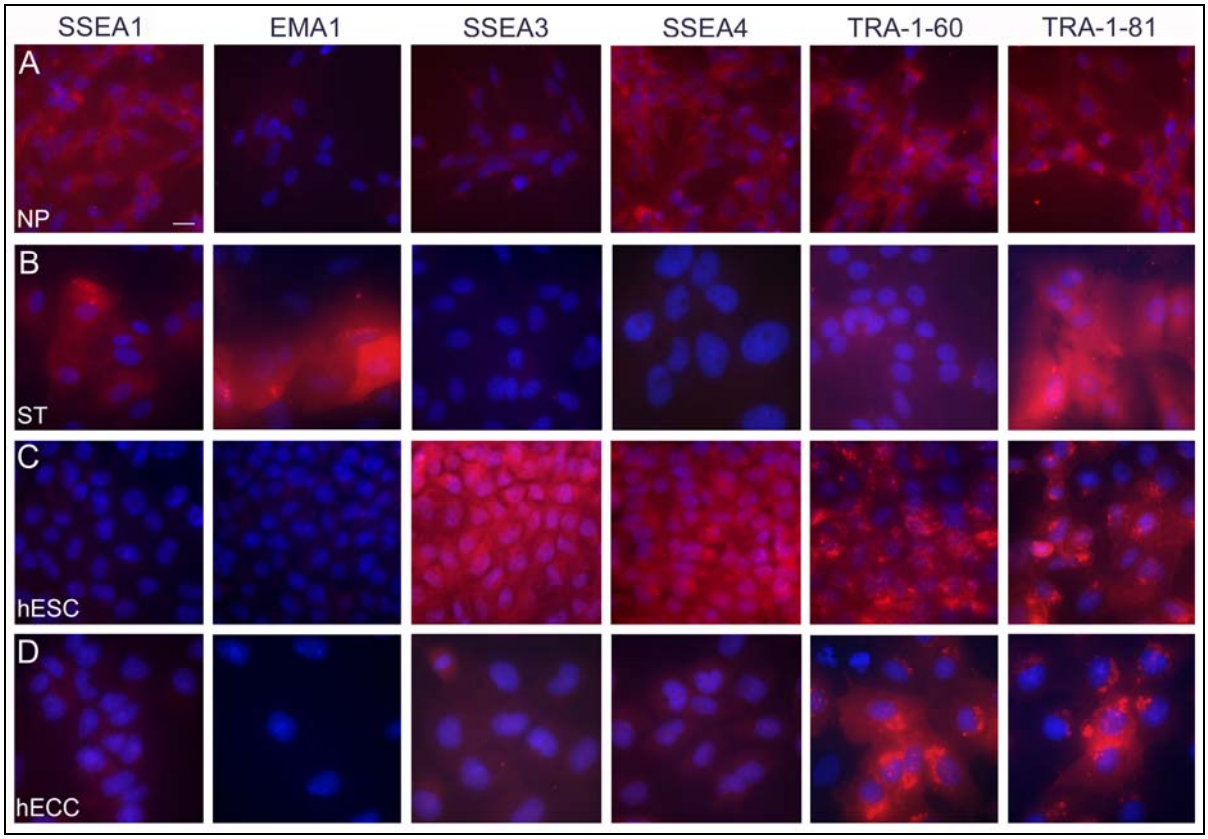


Figure 5.13 Immunocytochemistry for pluripotent stem cell markers in cultured human fetal neuroprogenitors and stomach epithelial cells

Immunocytochemistry for SSEA1, EMA1, SSEA3, SSEA4, TRA-1-60 and TRA-1-81 in cultured **A.** fetal neuroprogenitors, **B.** fetal stomach epithelial cells, **C.** hESCs (HUES1) and **D.** hECCs (NTERA2 D1 cells). Dual band pass images are shown for Texas Red and DAPI (to mark nuclei). Abbreviations; hECC, human embryonal carcinoma cell; hESC, human embryonic stem cell, NP; neuroprogenitors, ST; stomach epithelial cells. Size bar represents 20 μm.

As the neuroprogenitors and stomach epithelial cells already expressed pluripotent cell surface markers, another marker was selected. FGF4 was chosen, a SOX2-OCT4 target gene (Yuan *et al.* 1995) which is expressed by hESCs and hECCs (Figure 5.14 A-B) but not neuroprogenitors and stomach epithelial cells (Figure 5.14 C-D). Cells were co-transfected with OCT4 and NANOG expression vectors by nucleofection and plated onto fibronectin-coated chamber slides for 48 hours. Cells were then fixed with 4% PFA, and immunocytochemistry carried out. One chamber was always tested to confirm SOX2

expression (not shown). Dual immunocytochemistry for OCT4 and NANOG revealed nuclear co-staining of both transcription factors in the majority of transfected cells (Figure 5.14 E-F); although some cells only expressed one protein. However, dual immunocytochemistry demonstrated that no OCT4-positive cells expressed FGF4 (Figure 5.14 G-H). In addition, OCT4-positive cells still expressed differentiation markers: β-tubulin III for neuroprogenitors (Figure 5.14 I), and CK19 for stomach epithelial cells (Figure 5.14 J). Therefore, in these experiments, expression of the OCT4-SOX2-NANOG transcription factor complex in cultured neuroprogenitors and stomach epithelial cells did not induce pluripotency. Interestingly, though, cells expressing OCT4 and NANOG were often present as groups of cells rather than single cells, particularly notable for stomach epithelial cells, indicating a possible effect on cell proliferation worthy of further investigation.

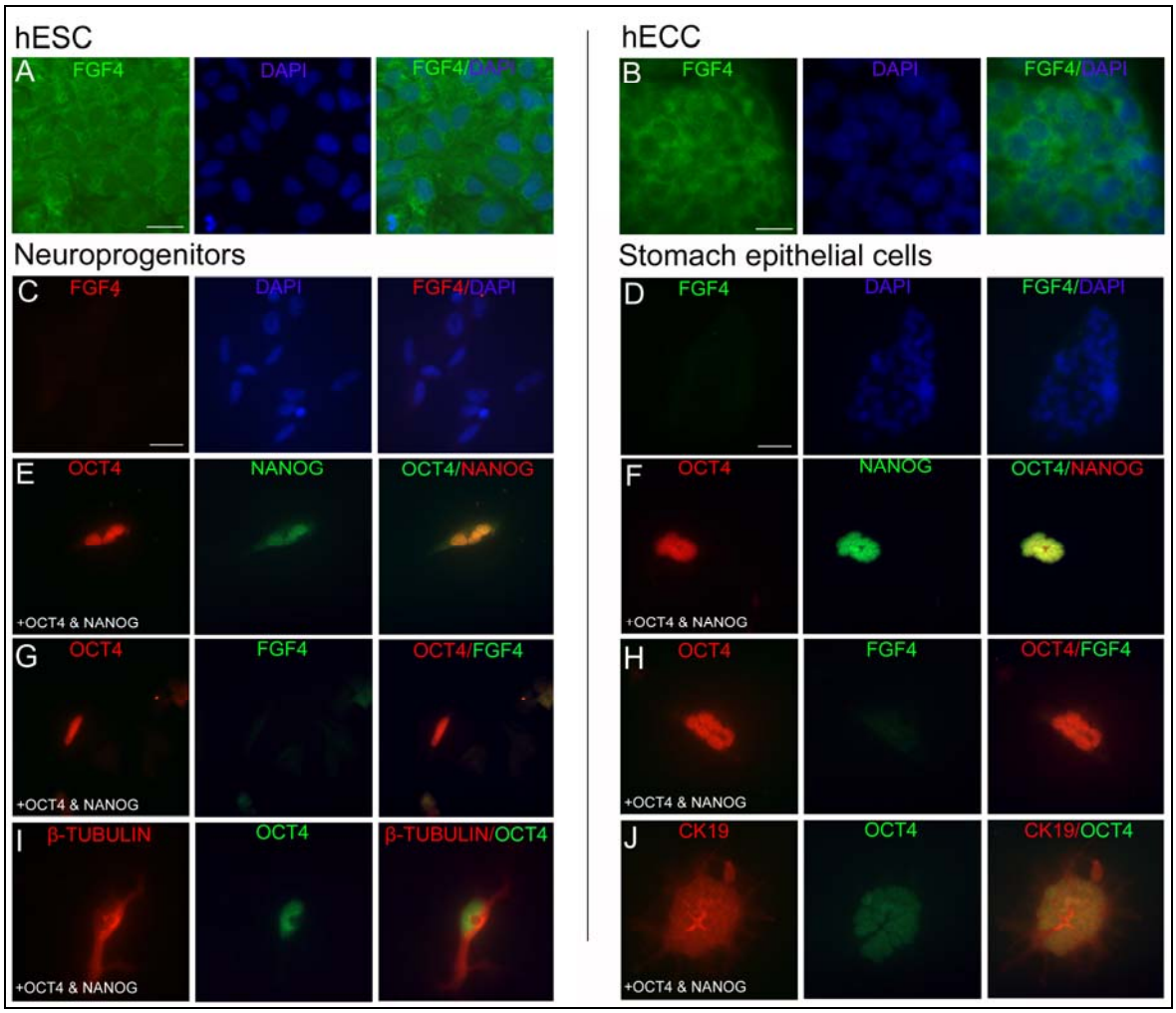


Figure 5.14 Immunocytochemistry for stem cell/tissue-specific markers in cultured human fetal neuroprogenitors and stomach epithelial cells 48 hours after co-transfection with OCT4 and NANOG expression vectors

A-D. Immunofluorescence staining of FGF4 in cultured hESCs (**A**), hECCs (**B**), neuroprogenitors (**C**) and stomach epithelial cells (**D**). **E-F.** Staining for OCT4 and NANOG in cultured neuroprogenitors (**E**) and stomach epithelial cells (**F**) 48 hours after co-transfection with OCT4- and NANOG-pcDNA3.1Zeo+. Note nuclear and overlapping staining for both. **G-H.** Staining for OCT4 and FGF4 in cultured neuroprogenitors (**G**) and stomach epithelial cells (**H**) 48 hours after co-transfection with OCT4- and NANOG-pcDNA3.1Zeo+. All cells were negative for FGF4 despite OCT4-positive cells being present. **I-J.** Staining for OCT4 and tissue-specific markers in cultured neuroprogenitors (**I.** β-tubulin III) and stomach epithelial cells (**J.** CK19) 48 hours after co-transfection with OCT4- and NANOG-pcDNA3.1Zeo+. Cells remained positive for tissue-specific markers despite acquired immunoreactivity for OCT4. Size bars represent 20 μm.

5.5 DISCUSSION

5.5.1 Successful Isolation and Culture of Human Fetal Stomach Epithelial Cells

Stomach epithelial cells were successfully isolated from human embryonic and early fetal (6-10 wpc) stomach and cultured with retention of SOX2, CK19 and CX43 expression for one week, with the avoidance of any obvious mesenchymal contamination. This is the first report of undifferentiated, stratified gastric epithelial cell culture from human embryonic and early fetal stomach, with retention of undifferentiated status, as marked by SOX2 expression. Stomach epithelium has been successfully cultured from human fetal material aged 15-18 wpc, again only for one week (Basque *et al.* 1999). However, Basque *et al.* used older fetal material: the epithelia at this age are differentiated simple columnar, which begins to replace stratified epithelium at around 9-10 wpc, with total replacement occurring at 13-15 wpc (Basque and Menard 2000). By 13 wpc, the human fetal stomach is representative of the adult stomach with all differentiated epithelial cell types being present (Menard *et al.* 1995; Tremblay and Menard 1996; Tremblay *et al.* 1997). Previous reports have not had success in culturing gastric epithelium using a scraping technique, as used here, with overgrowth by mucous cells occurring within a few days (Terano *et al.* 1983; Fukamachi *et al.* 1994). The success in this study is likely due to a homogenous starting cell population; the late fetal and adult specimens used in other studies contain differentiated cells which can contaminate the culture. The cessation of proliferation observed after approximately one week of culture found in this study and by Basque *et al.* is likely due to contact inhibition.

5.5.2 The OCT4-SOX2-NANOG Transcription Factor Complex is Not Required for Expression of Genes Identified as Targets by Bioinformatic Analysis

OCT4, NANOG and SOX2 are thought to establish the initial genomic state from which all other gene expression patterns are derived during development (Boyer *et al.* 2006). However, somatic cells do not require induction of NANOG to reprogram, whereas OCT4 and SOX2, along with KLF4 or LIN28 are required (Yu *et al.* 2007; Nakagawa *et al.* 2008). This suggests that the action of OCT4 and SOX2 is sufficient to generate a cascade of events which induce NANOG expression and pluripotency. Consistent with this

notion, Nanog is dispensable for pluripotency in mESCs; it acts primarily in the construction of the ICM, and stabilises mESCs in culture rather than playing a primary role in maintaining pluripotency (Chambers *et al.* 2007).

All 13 of the arbitrarily selected OCT4-SOX2-NANOG target genes proposed by Boyer *et al.* were expressed in neuroprogenitors and stomach epithelial cells, and in SOX2-negative hPGCs (Perrett *et al.* 2008 and Chapter 3). It is likely, therefore, that these genes are regulated by additional transcription factors. The analysis of target gene expression was extended to the protein level for DPPA4 and TDGF1. DPPA4 has recently been confirmed to be a direct target of Oct4 and Sox2 in mESCs (Chakravarthy *et al.* 2008), and is proposed to be a direct target of OCT4 (Babaie *et al.* 2007), and SOX2 and NANOG according to bioinformatic studies in hESCs (Boyer *et al.* 2005). DPPA4 is expressed in human and mouse ESCs, PGCs and ECCs (Maldonado-Saldivia *et al.* 2007). Consistent with these findings, it was detected in OCT4-positive hESCs, hECCs and hPGCs. However, DPPA4 was also detected in human tissues during embryonic development in the absence of OCT4, SOX2 and NANOG, indicating differences in transcriptional regulation between ESCs and cell types that arise later in embryogenesis. This is similar to SOX2 itself: within ESCs, expression is in part maintained by OCT4, but it is also expressed in differentiated tissues, where its regulators are currently unknown. DPPA4 was expressed within neuroprogenitors, however expression within the spinal cord has not been previously described, and the functional significance is unclear. Indeed, Dppa4 function in ESCs has not been characterised. One study found it inhibited differentiation into an ectoderm lineage in mESCs (Masaki *et al.* 2007); however, another found that over- or under-expression had no effect on differentiation (Ivanova *et al.* 2006).

The promoter of TDGF1 was also bound by OCT4, SOX2 and NANOG in hESCs (Boyer *et al.* 2005). Expression was detected in cultured neuroprogenitors in this study. Although Tdgf1 (also known as Cripto1) is required for commitment of ESCs to a cardiac fate (Parisi *et al.* 2003), Tdgf1 protein expression has been widely detected in the primate nervous system (Stephens *et al.* 2006), and knock-out mice have a disordered neuroepithelium and fail to produce a recognisable spinal cord or head-fold (Xu *et al.* 1999). This indicates a role in neurogenesis.

Of the genes demonstrated to be regulated by OCT-SOX enhancers, two were absent from cultured neuroprogenitors and stomach epithelial cells: *UTF1* and *FGF4* (Yuan *et al.* 1995; Nishimoto *et al.* 1999), indicating a requirement for OCT4 in their transcription. *UTF1* was detected in SOX2-negative, OCT4-positive hPGCs (Perrett *et al.*

2008 and Chapter 3), indicating SOX2 is dispensable for its activation. However, *FGF4* was also absent from hPGCs, indicating that both OCT4 and SOX2 need to be present for transcription (Yuan *et al.* 1995). *FBX15*, a SOX2/OCT4 target gene (Tokuzawa *et al.* 2003), was detected in neuroprogenitors and stomach epithelial cells. This is surprising as, in mice, the expression profile of Fbx15 is nearly identical to that of Oct4, restricted to ESCs, early embryos and testis tissue (Tokuzawa *et al.* 2003). Fbx15 is not required for mESC pluripotency or differentiation. Its role is unknown, but may function as an E3 ubiquitin ligase and play a role in determining the expression levels of regulatory proteins necessary for pluripotency. Its expression during human development has not been examined.

5.5.3 Neuroprogenitors and Stomach Epithelial Cells Express 'Stem Cell-Specific' Markers: Implications for Stem Cell Research

The glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens TRA-1-60 and TRA-1-81 are the cell surface antigens widely used in the ESC research community as indicators of pluripotent cells (for example Henderson *et al.* 2002), and to isolate pluripotent cells using cell sorting (for example SSEA4: Schulz *et al.* 2004; SSEA3: Enver *et al.* 2005). They were recently used in the characterisation of 59 hESC lines from 17 laboratories worldwide by the International Stem Cell Initiative (Adewumi *et al.* 2007). Immunoreactivity was detected in all lines, with the conclusion that they are thus valuable markers of undifferentiated cells. Whilst this is true, the finding that differentiated cells during development express these markers advises some caution for their use in marking a cell as undifferentiated. Specifically, neuroprogenitors were SSEA3^{weak}, SSEA4^{+}, TRA-1-60^{+}, TRA-1-80^{+}, and stomach epithelial cells SSEA3^{-}, SSEA4^{-}, TRA-1-60^{weak} and TRA-1-81^{+}. SSEA-3 and SSEA-4 are epitopes on related glycosphingolipids, termed GL-5 and GL-7 (Kannagi *et al.* 1983). They were recently shown not to be required for hESC pluripotency as their depletion using glycolipid biosynthesis inhibitors had no effect on retention of an undifferentiated state (Brimble *et al.* 2007). The exact molecular identity of the epitopes of TRA-1-60 and TRA-1-81 are unknown, but they have been shown to recognise a keratan-sulphated proteoglycan in neuraminidase-sensitive and neuraminidase-insensitive fashion, respectively (Badcock *et al.* 1999). The search for the carrier of the TRA family antigens in ECC has led to the identification of podocalyxin, a heavily sialylated membrane protein structurally similar to CD34. EC expresses two distinct forms

of podocalyxin, and the larger version is a molecular carrier of the TRA-1-60 and TRA-1-81 antigens (Schopperle and DeWolf 2007).

Given these data, it is candid to use other, more functionally relevant, markers to denote pluripotency. A combination of genes expressed in hESCs is required. Recently, quantitative RT-PCR using 12 selected genes was demonstrated to reliably distinguish differentiated cells from undifferentiated hESC populations (Cai *et al.* 2006).

5.5.4 Induction of the OCT4-SOX2-NANOG Transcription Factor Complex in Neuroprogenitors/Stomach Epithelial Cells Did Not Induce Pluripotency

The technique of converting somatic cells to iPS cells holds great promise therapeutically and ethically. Using a mouse model of sickle cell anaemia, mice could be rescued after transplantation with haematopoietic progenitors generated *in vitro* from autologous iPS cells (Hanna *et al.* 2007). Although the tumourigenic c-Myc was used, the mice did not show any evidence of tumour formation. iPS cells can now be generated without the use of c-Myc (Nakagawa *et al.* 2008; Wernig *et al.* 2008). The requirement for Klf4 is surprising: knock-out mice have no abnormalities in their pluripotent cell population during embryogenesis, and die within 15 hours of birth due to a skin barrier defect (Segre *et al.* 1999). In fact, iPS cells generated from human fibroblasts did not require KLF4, but rather SOX2, OCT4, NANOG, and LIN28, although the latter was not absolutely required for initial reprogramming or stable expansion. In addition, NANOG was not required for initial reprogramming, providing a beneficial effect in clone recovery (Yu *et al.* 2007).

Despite this evidence suggesting that introduction of OCT4, NANOG and SOX2 would be sufficient to reprogram somatic cells, the transduction of OCT4 and NANOG into SOX2-positive cells in this study proved insufficient. Differences in methodology may explain the lack of success. It has since been proven that the exogenous factors are required for at least 10-12 days in order to generate iPS cells (Brambrink *et al.* 2008; Stadtfeld *et al.* 2008). Experiments herein involved only transient expression with testing 48 hours later. Studies by Yamankana's group obtained iPS cells from only a small portion of cells that expressed the four factors. Too few cells may have been transfected, and only approximately 30% of cells were transfected with both vectors. In addition, the levels of OCT4 and NANOG generated from the vectors, or even the endogenous SOX2, may not

have been ‘correct’. A two-fold increase in Oct4 levels causes mESCs to differentiate into endoderm and mesoderm, whereas knockdown of Oct4 causes differentiation into trophectoderm-like cells (Niwa *et al.* 2000). Elevating the levels of Sox2 inhibits expression of Sox2-Oct4 target genes (Boer *et al.* 2007), and increasing or decreasing levels causes mESC differentiation (Chew *et al.* 2005; Kopp *et al.* 2008). Over-expression of Nanog in mESCs negates their need for LIF (Mitsui *et al.* 2003), and suppression results in differentiation to endoderm (Mitsui *et al.* 2003; Hough *et al.* 2006). Therefore, levels are precise and those generated by the pcDNAZeo3.1+ vector, with gene transcription under the control of the cytomegalovirus enhancer-promoter which gives high-level expression, may have been too high.

Another possibility is that SOX2 in the differentiated cells may be in an alternate ‘form’ or ‘state’, for example due to different post-translation modifications, which may render it incapable of complexing with the introduced OCT4 or NANOG. The mere presence of OCT4, SOX2 and NANOG in the transfected cells did not correlate with FGF4 expression, indicating that this was not enough to initiate target gene expression.

5.5.5 Fetal Tissue Culture Provides Insight into Developmental Pathways for ESC Differentiation Purposes

hESCs and hEGCs hold great promise therapeutically; however, only if they can be successfully differentiated in culture to specific cell types. The work and culture methodologies elucidated here provide insights into the normal developmental pathways for these cells, and thus provide clues as to the methodologies required to differentiate ESCs to these lineages.

5.5.6 Summary

This study describes the successful isolation and culture for one week of human fetal gastric epithelial cells. The starting cells were undifferentiated stratified epithelia, rather than differentiated epithelial cells, which have been isolated later in fetal development (Basque *et al.* 1999). Data within this chapter and Chapter 3 have demonstrated that the expression of the OCT4-SOX2-NANOG transcription factor complex is not required for expression of target genes identified by biometric analysis (Boyer *et al.* 2005). $SOX2^-$, $OCT4^+$, $NANOG^+$ germ cells and $SOX2^+$, $OCT4^-$, $NANOG^-$ neuroprogenitors and stomach epithelial cells expressed all 13 genes arbitrarily selected from this study. Expression of genes identified from functional studies as being activated by the SOX2-OCT4 complex showed better correlation with OCT4, SOX2 and NANOG expression: *FGF4* was not expressed within $SOX2^-$, $OCT4^+$, $NANOG^+$ germ cells and $SOX2^+$, $OCT4^-$, $NANOG^-$ neuroprogenitors and stomach epithelial cells, consistent with the absence of OCT4 and SOX2 (Ambrosetti *et al.* 1997). *UTF1*, also activated by OCT4 and SOX2 (Nishimoto *et al.* 1999), was expressed in $SOX2^-$, $OCT4^+$, $NANOG^+$ germ cells but not in $SOX2^+$, $OCT4^-$, $NANOG^-$ neuroprogenitors and stomach epithelial cells. Therefore, conclusions drawn regarding transcriptional activation of genes based on promoter binding require extensive validation by *in vitro* studies.

The potential shortcomings of the use of SSEA3, SSEA4, TRA-1-60 and TRA-1-81 to mark pluripotent cells have been identified by data presented within this chapter. Differentiated cells also express these markers. Therefore their use to characterise stem cell lines is limited and a combination of functionally relevant markers would perhaps prove more robust. In addition, initial experiments attempting to transduce OCT4 and NANOG into SOX2-positive neuroprogenitors and stomach epithelial cells did not reprogram the cells to a pluripotent state, contrasting subsequent reports using the same factors (Takahashi and Yamanaka 2006; Takahashi *et al.* 2007; Wernig *et al.* 2007; Yu *et al.* 2007; Lowry *et al.* 2008; Park *et al.* 2008).

CHAPTER 6: Identification of Candidate Genes for Germ Cell Proliferation

6.1 ABSTRACT

Alongside ESCs and ECCs, EGCs are a third pluripotent stem cell type which can be derived from the embryo. Human EGCs are poorly characterised, having only been derived by a handful of groups to date. Relative to hESCs, *in vitro* culture of hEGCs is problematic, with cells only remaining undifferentiated for a limited period. Mouse EGCs, on the other hand, remain undifferentiated long-term and in many respects resemble mESCs. However, the same culture conditions do not also maintain hEGCs indefinitely. This chapter aimed to identify factors which might promote human germ cell proliferation and thus which could aid in prolonging hEGC culture. Expression microarray was conducted on RNA isolated from developing ovaries and testes at the stage of mitotic arrest in the male; at this point female germ cells are still proliferating. Genes more highly expressed in female samples might promote germ cell proliferation, and those with higher expression in the male may be involved in inducing or maintaining mitotic arrest. Of the 20,000 genes analysed in the microarray, thirty genes were chosen from the microarray data and an initial screen performed by RT-PCR to confirm differential expression between ovary and testis. A number of interesting genes were identified which may potentially play a role in promoting germ cell proliferation, and could provide clues as to new factors to add to the media to improve hEGC culture.

6.2 INTRODUCTION

6.2.1 PGC Proliferation and Survival

Genetic studies in *Drosophila* and mice, along with cellular investigations using germ cell culture models, have revealed a number of genes and signalling molecules involved in promoting PGC proliferation and survival.

6.2.1(i) Genetic Analysis – Mouse Mutants

A number of mouse mutants are sterile or have reduced fertility, and the identification of the affected genes has implicated their corresponding products as playing important roles in PGC proliferation and/or survival. As described [previously in Section 1.10.2 and in Section 6.2.1(iv) of this chapter], mutants of *W* and *Sl*, which affect the genes encoding c-Kit and SCF, respectively, are sterile with reduced PGC number at 9-10 dpc (Godin *et al.* 1991). The *an* mutation (Hertwig's anaemia) causes sterility, with reduced germ cell number at 12 dpc (Russell *et al.* 1985). It is not clear whether this is due to a defect in migration, proliferation or survival, although no PGC proliferation is observed from 12 to 15 dpc. The identity of the affected gene(s) is not known.

The *ter* (teratoma) mutation inactivates the *Dead-end 1 (Dnd1)* gene on chromosome 18 and causes sterility, due to a defect in PGC proliferation which begins at 8 dpc (Sakurai *et al.* 1994); (Youngren *et al.* 2005). Dnd1 binds to the 3' UTR of mRNAs to displace miRNAs that bind to adjacent sites on the same mRNA (Kedde *et al.* 2007). Mouse Dnd1 protein shows highest homology to APOBEC1 complementation factor (ACF) (Youngren *et al.* 2005; Matin 2007). ACF is the RNA-binding co-factor of APOBEC1 and together they comprise the RNA editing enzyme complex (editosome) (Lellek *et al.* 2000; Mehta *et al.* 2000). Mouse Dnd1 interacts with APOBEC3; both are expressed in germ cells, and, in common with Dnd1, APOBEC3 can inhibit miRNA-mediated repression of mRNA (Huang *et al.* 2007).

The *gcd* (germ cell deficient) mutant is sterile with reduced PGC number at 11.5 dpc (Pellas *et al.* 1991). It is caused by an insertational mutation which disrupts two genes on chromosome 11: *Proliferation of germ cells (Pog)* and *Vaccinia virus-related protein kinase 2 (Vrk2)* (Agoulnik *et al.* 2002). *Pog* expression is required for mPGC proliferation starting between E9.5-10.25, just as germ cells begin to enter the gonadal ridge (Agoulnik

et al. 2002). *Vrk2* is highly expressed in actively proliferating cells (Nezu *et al.* 1997). Finally, the *at* (atrichosis) mutation causes depletion of germ cells (Handel and Eppig 1979), however, it is not known which gene(s) is affected.

6.2.1(ii) Genetic Analysis – Knock-out Mice

Along with the sterile mouse mutants, the phenotypes of various knock-out mice have also unearthed further genes involved in germ cell proliferation. Pin1, a peptidyl-prolyl isomerase, regulates the timing of PGC proliferation during mouse embryonic development (Atchison *et al.* 2003; Atchison and Means 2004). It promotes the degradation of proteins phosphorylated at Ser/Thr-Pro motifs, and is implicated in control of the cell cycle (Lu *et al.* 1996; Yaffe *et al.* 1997). PGCs have a prolonged cell cycle in the absence of *Pin1*, therefore there are fewer cell divisions and thus fewer *Pin1*$^{-/-}$ PGCs by the end of the proliferative phase. An inhibitor of Pin1 - PinB - reduces mPGC growth in culture (De Felici *et al.* 2004). Knock-out of the *Zfx* gene, which encodes a zinc finger protein, results in reduced PGC number at 11.5 dpc, with no defect in migration (Luoh *et al.* 1997). Therefore the gene may act to promote PGC proliferation and/or prevent PGC death.

Knock-out/reduction of *Pten* increases mPGC proliferation (Kimura *et al.* 2003; Moe-Behrens *et al.* 2003). Pten induces G1 arrest through suppression of the PI3K-Akt pathway (Li and Sun 1998; Ramaswamy *et al.* 1999; Sun *et al.* 1999). Disruption of the RNA-binding protein Tiar (T-cell restricted intracellular antigen-related protein) causes adult sterility; PGCs are reduced at 11.5 dpc and completely absent by 13.5 dpc (Beck *et al.* 1998). This is not due to a defect in migration. *Tiar*-deficient mESCs do not proliferate in the absence of LIF, supporting a role for Tiar in regulating cell proliferation (Beck *et al.* 1998).

Dazl knock-out mice are infertile, with a reduction in germ cells by 19 dpc, and a failure to progress from $A_{aligned}$ to A_1 spermatogonia (Ruggiu *et al.* 1997; Schrans-Stassen *et al.* 2001). Dazl binds to mRNA in the cytoplasm, and this mRNA might be translationally active (Tsui *et al.* 2000; Maegawa *et al.* 2002; Collier *et al.* 2005). It interacts with Pum2, a translational repressor (Moore *et al.* 2003), and may play a role in RNA transport (Lee *et al.* 2006).

Knock-out of *Desert hedgehog (Dhh)*, a cell-signalling molecule first discovered in *Drosophila*, causes sterility, with a reduction in testis size from 18.5 dpc (Bitgood *et al.*

1996). The source of Dhh is the Sertoli cells, with receptors on peritubular cells and possibly Leydig cells (Pierucci-Alves *et al.* 2001). Inactivation of *Cx43*, the predominant testicular gap junction protein (Steger *et al.* 1999), causes a severe depletion in germ cell number in fetuses and neonates, beginning at 11.5 dpc (Juneja *et al.* 1999).

6.2.1(iii) TGFβ Family

TGFβ family members have been implicated in PGC and gonocyte development in a number of studies, reviewed in (Itman *et al.* 2006; Knight and Glister 2006). In *Drosophila*, two homologues of mammalian BMPs, *decapentaplegic (dpp)* and *glass bottom boat (gbb*, also called *60A)*, function in the maintenance and proliferation of germline stem cells (Xie and Spradling 1998; Shivdasani and Ingham 2003; Kawase *et al.* 2004). As previously described (Section 1.10.1), Bmp2, 4 and 8b are all involved in mouse germ cell specification *in vivo* (Lawson *et al.* 1999; Ying *et al.* 2000; Ying and Zhao 2001). Bmp4 promotes mPGC proliferation in culture, through Smad1/4 signalling (Pesce *et al.* 2002). Disruption of *Bmp8b* causes male infertility due to a reduction in germ cell proliferation followed by an increase in death (Zhao *et al.* 1996). In addition, Bmp7 is required for mPGC proliferation between E10.5-11.5 (Ross *et al.* 2007). Knock-out mice for *Smad1* (Tremblay *et al.* 2001) or *Smad5* (Chang and Matzuk 2001) show reduced PGC number. Exogenous Activin and Tgfβ limit mPGC proliferation (Richards *et al.* 1999), and Tgfβ2 suppresses chick PGC proliferation (Fujioka *et al.* 2004).

6.2.1(iv) Role of c-Kit and Stem Cell Factor

The sterile mouse mutants *Sl (Steel)* and *W (Dominant White Spotting)* arose from mutations affecting SCF and its receptor, c-Kit, respectively, and revealed a role for SCF in the proliferation and maintenance of mPGCs (Godin *et al.* 1991). c-Kit is present on the surface of mPGCs and its ligand SCF can either exist as a membrane-bound (mbSCF) or a soluble (sSCF) isoform. mbSCF is required for mPGC proliferation and migration *in vivo* (Lev *et al.* 1994; Runyan *et al.* 2006), and *in vitro* it promotes survival for longer than sSCF (Dolci *et al.* 1991; Pesce *et al.* 1993; Yan *et al.* 2000; De Miguel *et al.* 2002). However, the latter increases telomerase activity *in vitro* (Dolci *et al.* 2002).

6.2.1(v) Role of LIF and Related Factors

LIF increases the number and mitotic activity of mPGCs (De Felici and Dolci 1991; Matsui *et al.* 1991; Resnick *et al.* 1992; Dolci *et al.* 1993; Koshimizu *et al.* 1996), and enhances the survival of mouse fetal gonocytes (Cheng *et al.* 1994) by reducing apoptosis (Pesce *et al.* 1993). LIF acts on the LIF receptor (LIFR) and the gp130 subunit (Cheng *et al.* 1994), which are both expressed in mouse gonads (Koshimizu *et al.* 1996). Addition of anti-LIFR or anti-gp130 antibodies to mPGC culture medium reduces PGC number (Cheng *et al.* 1994; Koshimizu *et al.* 1996). However, PGC development in LIF- or LIFR-deficient mice is normal (Stewart *et al.* 1992; Li *et al.* 1995; Ware *et al.* 1995). It is likely there is redundancy between related cytokines, which may be able to bind to gp130 and not require the presence of LIFR: inactivation of gp130 does reduce PGC number (Hara *et al.* 1998). Indeed, oncostatin M (OSM) can substitute for LIF in affecting survival and/or proliferation of mPGCs in culture (Cheng *et al.* 1994; Koshimizu *et al.* 1996; Hara *et al.* 1998). Therefore, LIF itself may be dispensable. LIF maintains mESCs in the absence of feeders (Smith *et al.* 1988; Williams *et al.* 1988), but not hESCs (Daheron *et al.* 2004; Humphrey *et al.* 2004). Although all groups culturing hEGCs have included LIF, none has proven its necessity in derivation/growth.

Knock-out mice for ciliary neurotrophic receptor (CNTF), another gp130 ligand, show no defects in germline development (Masu *et al.* 1993). *In vitro* CNTF promotes the survival of fetal gonocytes, and enhances the formation of germ cell colonies in neonatal mouse testis culture, but has no effect on mPGCs (Cheng *et al.* 1994; De Miguel *et al.* 1996; Koshimizu *et al.* 1996; Kanatsu-Shinohara *et al.* 2007). Interleukin 6 (IL6), also a gp130 ligand, has no effect on mPGC or gonocyte proliferation *in vitro* (De Felici and Dolci 1991; De Miguel *et al.* 1996; Koshimizu *et al.* 1996), although it, IL2, IL4 and IL11 enhance mPGC survival (Cooke *et al.* 1996; Koshimizu *et al.* 1996; Eguizabal *et al.* 2007).

6.2.1(vi) Other Local Factors

FGF2 supplementation to mPGC cultures already containing LIF and SCF led to the first reports of mEGC derivation (Matsui *et al.* 1992; Resnick *et al.* 1992). Only 12 hour exposure is required for conversion to mEGCs; such a short time-frame indicating a direct rather than indirect effect (i.e. via feeders) (Donovan and de Miguel 2003). Once mEGCs are established, FGF2 and SCF are no longer required (Matsui *et al.* 1992). In addition, some studies suggest that FGF2 can be replaced by retinoic acid or agents that

activate cAMP such as forskolin (Koshimizu *et al.* 1996). cAMP has a proliferative effect on mPGCs and gonocytes (De Felici *et al.* 1993; Dolci *et al.* 1993; Pesce *et al.* 1996). Pituitary adenylate cyclase-activating peptide (PACAP) promotes mPGC proliferation via the activation of adenylate cyclase (Pesce *et al.* 1996). Forskolin raises intracellular cAMP levels and stimulates mitosis in cultured mPGCs (Dolci *et al.* 1993), and has been included by all groups reporting hEGC derivation. Its effect in mEGCs does not substitute for the survival mediated by LIF and SCF (De Felici *et al.* 1993).

Growth arrest-specific 6 (Gas6) is expressed in both female and male mouse genital ridges after 11.5 dpc and it promotes mPGC proliferation and/or survival in culture (Matsubara *et al.* 1996). It has no effect on mPGCs cultured on feeders lacking the SCF gene, indicating it enhances SCF-induced growth or survival of mPGCs. The Sdf1/Cxcr4 interaction, as well as regulating mPGC migration (Section 1.10.2), also mediates germ cell growth and survival (Molyneaux *et al.* 2003).

The receptor tyrosine kinase ErbB3, its coreceptor ErbB2 (named for their similarity to avian erythroblastosis oncogene B), and their ligand Neuregulin-β, are expressed in mouse genital ridges at 12.5 dpc, and downregulated at 14.5 dpc when germ cells undergo growth cessation. Neuregulin-β enhances mPGC proliferation at 12.5 dpc (Toyoda-Ohno *et al.* 1999). Tumour necrosis factor α (TNFα) stimulates mPGC proliferation (extracted before and during migration) *in vitro* (Kawase *et al.* 1994). TNFα and its receptors TNFRI and TNFRII are expressed in most embryonic tissues (Kohchi *et al.* 1994), therefore a role *in vivo* could be anticipated.

Suppression of Wnt/β-catenin signalling is required for normal mPGC development. Aberrant activation of Wnt/β-catenin signalling causes germ cell deficiency due to delayed cell cycle progression, followed by apoptosis (Kim *et al.* 2000; Kimura *et al.* 2006). The opposite is true in *Drosophila*, where Wingless (Wg) signalling initiates mitosis in PGCs (Sato *et al.* 2008). Finally, knock-out of the mouse *Vasa* homologue gene (*Mvh,* also known as *Ddx4*), which encodes a cytoplasmic RNA helicase, causes reduced male PGC proliferation and differentiation (Tanaka *et al.* 2000).

6.2.1(vii) Human PGCs

hEGCs can be derived using the same cocktail of factors as mEGCs, i.e. forskolin, FGF2, LIF and SCF (Shamblott *et al.* 1998). However, unlike mEGCs, hEGCs do not remain undifferentiated long-term in their presence, and their derivation efficiency is

lower. This indicates that while the mechanisms controlling initial PGC growth may be largely conserved between the two species, differences do exist, particularly relating to longer term growth.

c-KIT, mbSCF and sSCF are expressed in human fetal gonads (Hoyer *et al.* 2005). However, in contrast to the mouse, mutations in c-KIT do not affect fertility, instead they are associated with mast cell neoplasms (Nagata *et al.* 1995) and GI stromal tumours (Hirota *et al.* 1998). c-KIT mutations are found in some types of TGCT (Tian *et al.* 1999), which are believed to originate from PGCs, suggesting that, in common with the mouse, c-KIT plays a role in hPGC survival and/or proliferation. Individuals suffering from Fancoli's anaemia exhibit reduced fertility (D'Andrea and Grompe 2003). Mice carrying one of the disease-causing mutations, within the Fanconi anaemia complementation group C locus *(Fancc)*, show reduced mPGC proliferation, beginning at E12.5 (Nadler and Braun 2000), again indicating similarities exist in the control of proliferation. In addition, a mouse model of Down's syndrome (trisomy 16), human sufferers of which have reduced fertility, has fewer PGCs than normal (Leffler *et al.* 1999).

Therefore, while there are some similarities in the genes and signalling molecules controlling mouse and human PGC proliferation, differences are apparent. Work undertaken in this chapter attempted to identify factors which might promote hPGC/hEGC proliferation, and thus extend culture, as detailed below.

6.3 SPECIFIC AIMS

6.3.1 To identify endogenous factors which promote human primordial germ cell proliferation.

hPGC-EGC cultures generally acquire one of two differing characteristics, designated either 'PP' ('poorly proliferating') or 'VP' ('vigorously proliferating') (Turnpenny *et al.* 2003). VP cultures arise from approximately 15% of cultures, in our group's experience, and, along with fulfilment of other criteria, including pluripotent marker expression and *in vitro* pluripotency, are taken as conversion to hEGCs. However, they are difficult to maintain undifferentiated long-term. The proportion of cells maintaining expression of pluripotent markers, such as OCT4 and SSEA family members, declines over time and is exacerbated by freeze-thaw routines (Turnpenny *et al.* 2003). This study sought to identify endogenous factors promoting hPGC proliferation, which might potentially unearth factors which have the same effect in hEGCs.

In the developing human testis, PGCs enter mitotic arrest around 7 wpc, whereas PGCs in the developing ovary increase in number until approximately 12 wpc (Gondos and Hobel 1971). Expression microarray was used to identify genes whose detection level differed between testis and ovary at the time of this mitotic arrest. Genes more strongly detected in the ovary could potentially be involved in promoting germ cell proliferation. RT-PCR verification of differential expression validated a number of interesting candidate genes selected from the microarray data.

6.4 RESULTS

6.4.1 Selection of Appropriately Aged Material for Expression Microarray

Before RNA was isolated from ovary and testis for the expression microarray, dual immunohistochemistry was undertaken on gonads from embryos of different stages using an OCT4 antibody, which marks germ cells, and a Ki67 antibody, which recognises the proliferation marker MK167, to determine at which age germ cells in the testis would be in mitotic arrest, but those in the ovary would still be proliferating. In 54 dpc ovary (Figure 6.1 A-B), many dual stained cells were visible (cells with yellow nuclei, indicated by arrows), approximately 75% of germ cells were proliferating[29]. However, in 56 dpc testis (Figure 6.1 C-D) and 64 dpc testis (Figure 6.1 E-F), fewer dual stained cells could be detected; 37% and 36% of germ cells were proliferating, respectively, indicating that germ cells were entering mitotic arrest at this stage.

Therefore, for the purposes of RNA preparation, gonads were collected from embryos/early fetuses aged 50-60 dpc and stored in RNA later (QIAGEN) at -80^{o}C. Sex was determined by fluorescent *in situ* hybridisation (FISH) on placental tissue, using probes specific for the X and Y chromosomes (performed by Louise Williams, Human Genetics Division, University of Southampton; data not shown). Four ovaries (average age 55 dpc) and four testes (average age 56 dpc) were separately pooled, RNA extracted, purified using the RNeasy MinElute Cleanup Kit (QIAGEN) and concentration and integrity determined using an Agilent 2100 Bioanalyzer (Figure 6.2). RNA yield and quality were deemed sufficient to proceed with the expression microarray.

[29] Determined by counting the number of dual stained cells, and dividing it by the total number of germ cells (OCT4-positive cells) in ten fields of vision (× 20 magnification).

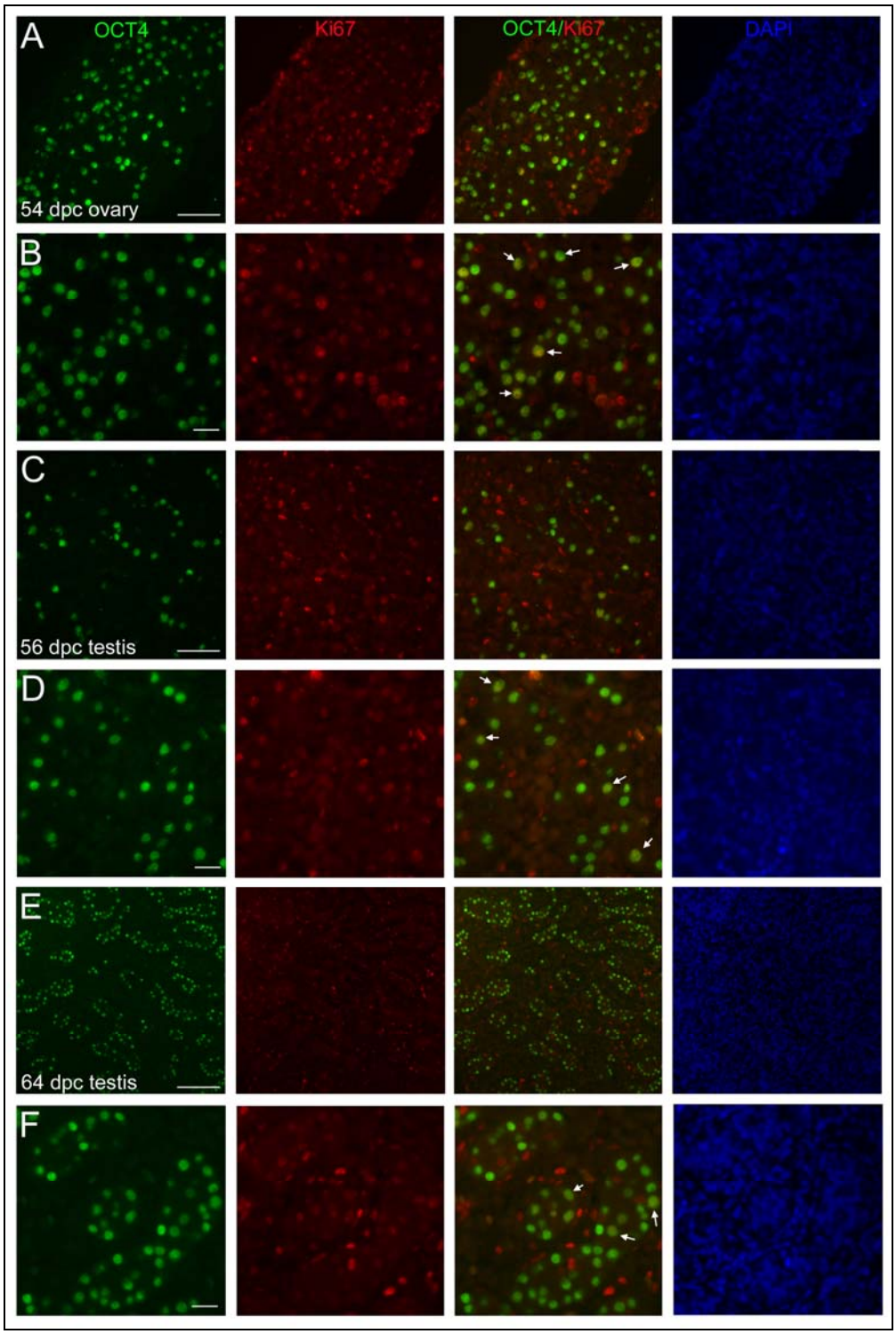


Figure 6.1 Dual immunohistochemistry for OCT4 (green) and the proliferation marker MK167, identified using the Ki67 antibody (red), in human embryonic ovary and embryonic/early fetal testes

IHC was performed in 54 dpc ovary (**A-B**), 56 dpc testis (**C-D**) and 64 dpc testis (**E-F**). Arrows point to examples of PGCs that are dual stained and therefore proliferating. Size bar represents 50 μm (A, C, E) and 20 μm (B, D, F). From left to right, individual band pass images are shown for FITC (OCT4), Texas Red (Ki67), dual band pass imaging and DAPI, at low and high magnification for each stage.

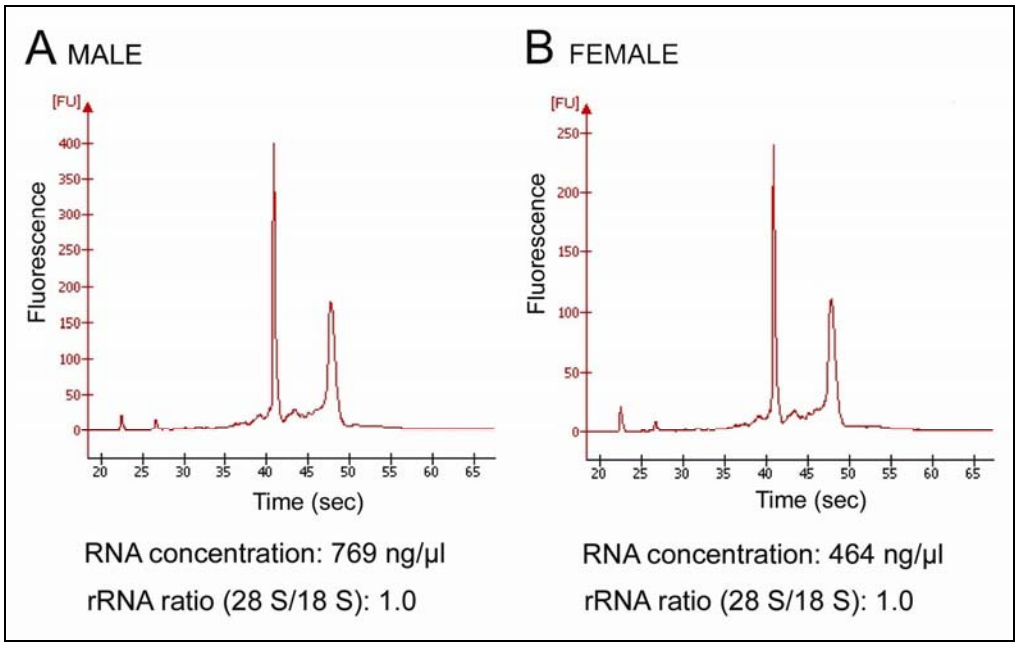


Figure 6.2 Electropherogram summary from Agilent 2100 Bioanalyzer of RNA isolated from A. male and B. female gonads

Characteristic 18 S and 28 S rRNA peaks are visible at 39 and 46 seconds with little evidence of RNA degradation. Although a 28 S:18 S of 2 is usually taken to indicate high quality RNA, RNA extracted from tissues has a lower ratio.

6.4.2 Expression Microarray and Data Analysis

The expression microarray was conducted using the Amersham CodeLink Uniset Human 20K I Bioarray, which targets 21,000 transcripts using 30-mer probes, and was performed by Dr. Feng Lin (Cancer Sciences Division, University of Southampton). Data was analysed using CodeLink Expression Analysis v4.1 (Amersham) and GeneSifter software (http://www.genesifter.net/web/). The data was normalised to remove background noise, and the detection level of genes compared between testis and ovary using the log2 values of the normalised intensity. A cut-off intensity level of 0.3 was used to filter the data, which represented an approximate four-fold increase on the average intensity of all 20K spots, so that only differences between the most differentially detected genes were compared.

Figure 6.3 depicts the scatter plot obtained for the entire data set. This graph plots the log2 intensities against one another, with each point representing a gene. The majority of genes showed similar intensities within ovary and testis. A number of candidate differentially expressed genes are visible as lying far from the diagonal line.

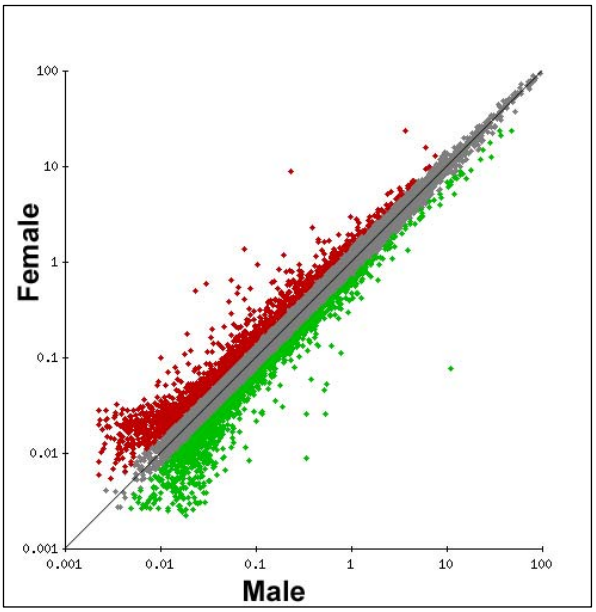


Figure 6.3 Scatterplot of the ratio of gene expression log2 intensities obtained from the Human 20K I Bioarray using RNA isolated from male and female gonads

Diagonal line indicates a ratio of 1, i.e. equal detection in both RNA samples. Outliers from the diagonal line represent genes whose detection differed in either male (green) or female (red) RNA.

The GeneSifter software was used to generate a list of the top 30 genes with the most differential intensity levels involved in cell proliferation (Figure 6.4).

6.4.3 RT-PCR Validation

The list of the top 30 genes involved in cell proliferation (Figure 6.4) and the raw data were examined and a number of genes selected for further analysis. These are listed in Table 6.1 along with the normalised intensities obtained for each gene and the fold induction in the female (F/M). In particular, cytokines were chosen, particularly interleukins, as these have been shown to promote germ cell proliferation in previous studies (Section 6.2.1). Three separate RT-PCR reactions were performed for each gene, on RNA extracted from different samples, aged 55-59 dpc (Figure 6.5).

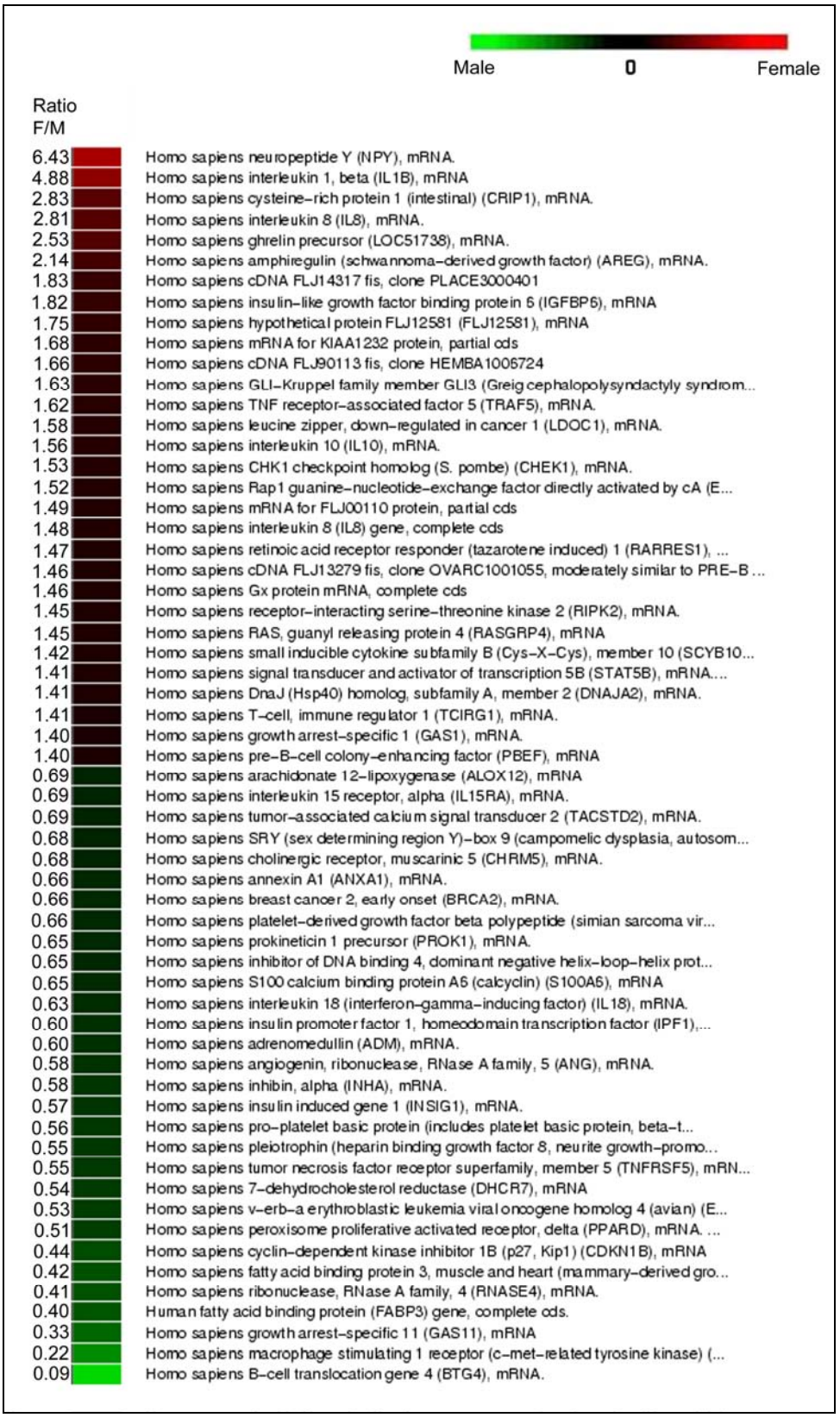


Figure 6.4 The top 30 genes involved in cell proliferation (according to GeneSifter) identified by expression microarray as being most differentially detected in male or female gonads

Genes in red were more highly detected in ovary; genes in green more highly detected in testis. Fold induction is listed on the left (female/male).

Name	ACCN #	Normalised Intensity: Female	Normalised Intensity: Male	Ratio (F/M)	Reason chosen for RT-PCR
small inducible cytokine subfamily A (Cys-Cys), member 20 (SCYA20)	NM_004591	0.02	0.09	0.18	Control for low/no detection in both
growth arrest-specific 11 (GAS11)	NM_001481	1.37	4.18	0.33	Higher detection in male; expressed in growth arrested cells
colony stimulating factor 3 (granulocyte) (CSF3)	NM_000759	2.08	3.30	0.63	Acts on the CSF3 receptor which is similar to the IL6R
cardiotrophin-like cytokine; neurotrophin-1/B-cell stimulating factor-3 (CLC)	NM_013246	0.48	0.58	0.82	Acts on the CNTFR, LIFR and IL6R, the latter two being implicated in ESC pluripotency
CNTF gene for ciliary neurotrophic factor	X60542	0.90	1.10	0.82	Acts on the CNTFR, LIFR and IL6R, the latter two implicated in ESC pluripotency
leukaemia inhibitory factor (LIF)	NM_002309	4.30	4.60	0.93	Control for equal detection in both; LIF used for ESC/EGC derivation
signal transducer and activator of transcription 5A (STAT5A)	NM_003152	0.40	0.39	1.02	Phosphorylated upon SCF activation of c-KIT
interleukin 6 (IL6)	NM_000600	1.40	1.30	1.08	Control for equal detection in both; implicated in ESC pluripotency
ciliary neurotrophic factor receptor (CNTFR)	NM_001842	13.00	12.00	1.08	Control for equal detection in both; same member of family as IL6R
interleukin 6 signal transducer (gp130, oncostatin M receptor) (IL6ST)	NM_002184	0.45	0.41	1.10	Control for equal detection in both; implicated in ESC pluripotency
bone morphogenetic protein 7 (BMP7)	NM_001719	0.54	0.44	1.23	Higher detection in female; BMPs promote PGC specification/proliferation
transcription factor 8 (TCF8)	NM_030751	21.57	17.03	1.27	Higher detection in female; cytokine repressor
interferon induced transmembrane protein 1 (IFITM1)	NM_003641	41.20	32.25	1.28	Higher detection in female; cytokine induced
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue (KIT)	NM_000222	6.66	5.20	1.28	Higher detection in female; receptor for SCF
interleukin 1 receptor, type I (IL1R1)	NM_000877	0.51	0.39	1.30	Higher detection in female; cytokine receptor
bone morphogenetic protein 2 (BMP2)	NM_001200	0.68	0.53	1.30	Higher detection in female; BMPs promote PGC specification/proliferation
FK506 binding protein 12-rapamycin associated protein 1 (FRAP1)/mTOR	NM_004958	1.98	1.44	1.37	Higher detection in female; mediates AKT signalling (involved in PGC-EGC transition)
signal transducer and activator of transcription 5B (STAT5B)	NM_012448	1.10	0.78	1.41	Higher detection in female; phosphorylated upon SCF activation of c-KIT
bone morphogenetic protein 4 (BMP4)	NM_001202	2.19	0.83	2.65	Higher detection in female; BMPs promote PGC specification/proliferation
interleukin 6 receptor (IL6R, gp80)	NM_000565	0.10	0.03	2.76	Higher detection in female; cytokine receptor; implicated in ESC pluripotency
interleukin 8 (IL8)	NM_000584	2.81	1.07	2.81	Higher detection in female; cytokine
neuropeptide Y receptor Y1 (NPY1R)	NM_000909	0.95	0.21	4.56	Higher detection in female; receptor for NPY (see below)
interleukin 1, beta (IL1B)	NM_000576	3.86	0.79	4.88	Higher detection in female; cytokine
neuropeptide Y (NPY)	NM_000905	107.73	16.76	6.43	Higher detection in female; promotes neuroprogenitor proliferation
cholecystokinin B receptor (CCKBR)	NM_000731	1.76	0.24	7.38	Higher detection in female
glutamate receptor, metabotropic 4 (GRM4)	NM_000841	2.17	0.29	7.54	Higher detection in female; expressed in colonic neoplasms with a poor prognosis
carboxypeptidase A2, pancreatic (CPA2)	NM_001869	2.92	0.25	11.70	Much higher detection in female
LIM homeobox protein 2 (LHX2)	NM_004789	2.68	0.14	19.66	Much higher detection in female; expressed in immortalised haematopoietic stem cells
XIST, coding sequence 'd' mRNA (locus DXS399E)	X56196	40.34	1.04	38.82	Positive control for high expression in female

Table 6.1 Genes selected from expression microarray data for RT-PCR verification in ovary and testis

Gene name, accession number, normalised intensity in ovary and testis, female:male ratio and a brief explanation for why the gene was selected are listed.

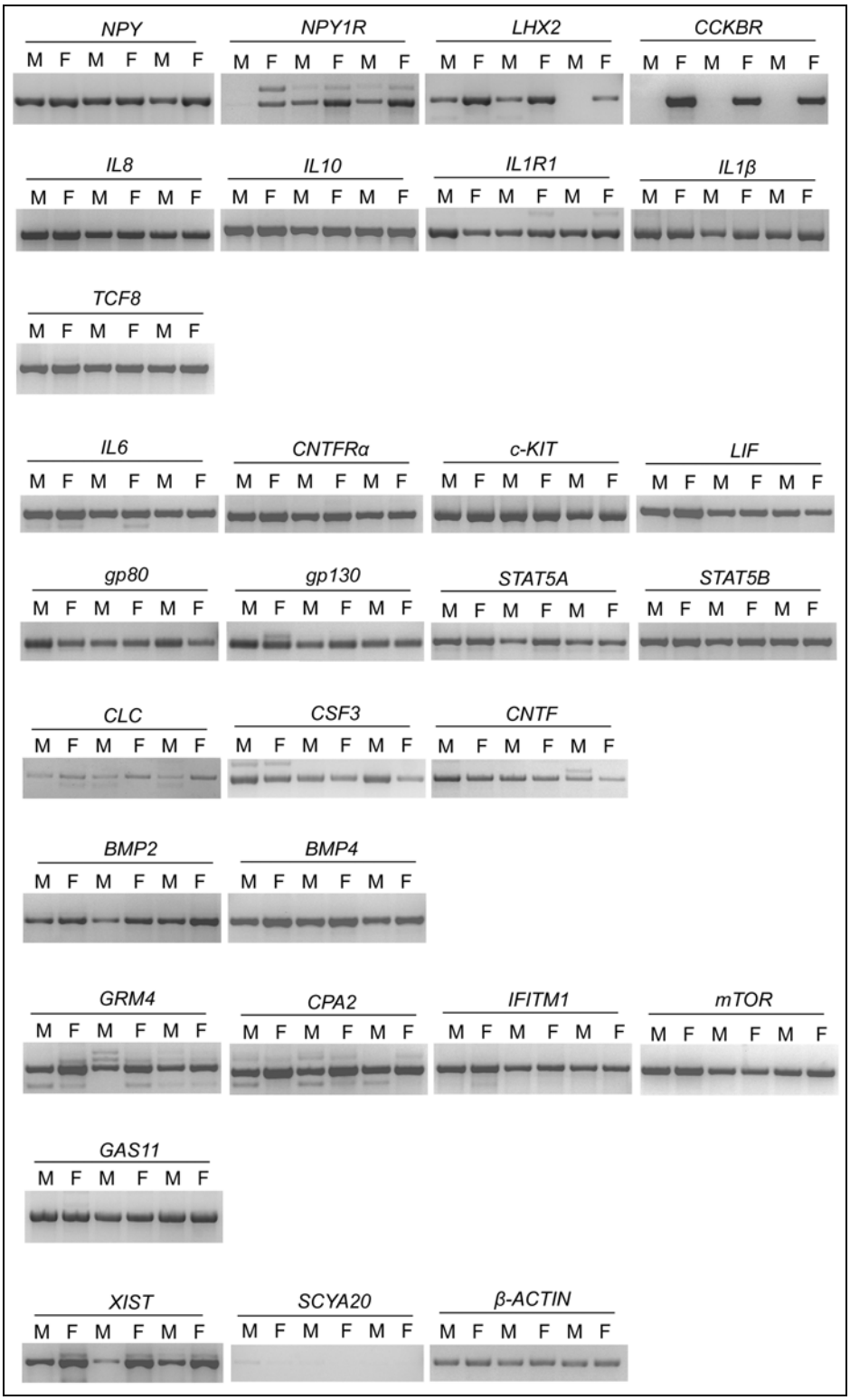


Figure 6.5 RT-PCR verification of genes selected from expression microarray data

RT-PCR for selected genes in human embryonic/early fetal ovary and testis following 35 cycles. Each repeat reaction was performed on RNA from a different gonad (55-59 dpc). Genes are shown in the same order they are mentioned in the text (below). Abbreviations: M, male (i.e. testis); F, female (i.e. ovary). For full gene names, see Table 6.1. For primer sequences see Appendix 1.

6.5 DISCUSSION

This chapter describes efforts to identify genes which may promote human germ cell proliferation. The comparison of gene expression within the fetal ovary while germ cell proliferation is still underway, with that in the testis where germ cells are entering mitotic arrest, has highlighted a number of genes which appear to be differentially expressed. Examining known functions of these genes may provide clues as to whether they may be mitogenic for germ cells.

No cell-sorting was performed, so results are based on analysis of gene expression within the entire gonad. Whilst this is somewhat unspecific, it allows for the identification of important somatic factors, as well as germ cell-specific ones.

6.5.1 Genes Potentially Involved in Germ Cell Proliferation Identified using Expression Microarray Analysis and RT-PCR

6.5.1(i) Neuropeptide Y (NPY) and Neuropeptide Y Receptor Y1 (NPY1R)

By microarray, *NPY* and one of its receptors, *NPY1R*, were 40-fold and 5-fold more highly detected in the ovary respectively. By RT-PCR, *NPY* was more strongly expressed in ovary than testis (Figure 6.5); however, this difference was only slight, likely because detection was relatively high in testis, with a normalised intensity of 17.

NPY is a 36 amino acid neuropeptide broadly expressed in the central and peripheral nervous system during development and adulthood (Allen *et al.* 1983; Danger *et al.* 1990). It promotes the proliferation of neuroprogenitors, vascular smooth muscle and endothelial cells (Hansel *et al.* 2001; Lee *et al.* 2003; Pons *et al.* 2003), and it has been linked to cell proliferation in a number of cancers, including breast, prostate and neural crest tumours (Kitlinska 2007). It appears that its effects on proliferation are mediated through the Y1 receptor subtype (Magni and Motta 2001; Reubi *et al.* 2001; Kitlinska 2007), which also showed higher expression in the ovary (Figure 6.5). It could be speculated, therefore, that NPY promotes germ cell proliferation by acting on the NPY1R within the developing ovary.

6.5.1(ii) LIM-homeobox 2 (LHX2)

LHX2 showed 20-fold increased expression in ovary compared to testis. This higher expression was verified by RT-PCR (Figure 6.5). LHX2 is expressed in mouse fetal liver at the time of active haematopoiesis (Xu *et al.* 1993). Immortalised haematopoietic stem cell lines can be generated by expressing Lhx2 in haematopoietic progenitor cells derived from mESCs (Pinto do *et al.* 1998). It maintains the growth and undifferentiated properties of mouse hair follicle progenitors (Rhee *et al.* 2006), and a similar role in germ cells within the ovary could be anticipated.

6.5.1(iii) Cholecystokinin B receptor (CCKBR)

Cholecystokinin B receptor (CCKBR) showed 7.5-fold increased expression in female compared to male gonad. By RT-PCR no transcript was detected in testis (Figure 6.5; level was 0.24 in expression microarray). CCKBR is a gastrin receptor (Kopin *et al.* 1992), which may promote the proliferation of gastric carcinoma cells (Eden and Taylor 1993). Gastrin acts via the CCKBR to promote growth of the AR42J rat acinar cell line, acting via AKT, thereby inhibiting apoptosis (Todisco *et al.* 2001). Interestingly, AKT signalling has recently been shown to promote derivation of mEGCs from PGCs (Kimura *et al.* 2008).

6.5.1(iv) Interleukins

The expression of a number of genes involved in cytokine signalling, in particular members of the interleukin family, were increased in developing ovary compared to testis, for example, *IL8* (2.7-fold), *IL10* (1.5-fold) and the *IL1R1* (1.3-fold). Despite these differences, RT-PCR for *IL8, IL10* and *IL1R1* detected approximately the same amounts of transcript in ovary and testis (Figure 6.5). It is likely that these small differences in gene expression level are not detectable by conventional RT-PCR.

IL1β expression was 4.8-fold higher in ovary (3.9 compared to 0.8 in the testis), and RT-PCR also detected more transcript in ovary compared to testis (Figure 6.5). IL1β mediates proliferation and differentiation of multipotent rat neural precursor cells (Wang *et al.* 2007), and it promotes neuroepithelial proliferation and differentiation of the chick spinal cord during development (de la Mano *et al.* 2007).

Transcription factor 8 (TCF8) encodes a zinc finger transcription factor that represses the *IL2* gene (Williams *et al.* 1991). Expression was 1.3-fold higher in ovary compared to testis; however, this slight difference in expression was not evident by RT-PCR (Figure 6.5). A role in suppressing IL2 within the ovary is contradictory, however, as IL2 promotes proliferation of mPGCs *in vitro* (Eguizabal *et al.* 2007).

6.5.1(v) LIF, SCF and related genes

A number of genes were chosen from the LIF family for further investigation. LIF is included in the culture media during mEGC derivation as it promotes their survival; however, LIFR-knockout animals have normal numbers of PGCs (Stewart *et al.* 1992; Ware *et al.* 1995). This may be explained by the fact that LIF is a member of the IL6 family of cytokines which exhibit overlapping functions (Taga and Kishimoto 1997). In the mouse there are six members of this family: IL6, IL11, LIF, OSM, CNTF and cardiotrophin 1 (CT1). Members of this family signal through receptor complexes that are dimers (or multimers) comprising high affinity growth factor-specific receptors (e.g. LIFR, OSMR, IL6R [gp80 also known as IL6Rα]) and a low affinity common receptor (gp130 also known as IL6 signal transducer [IL6ST]). Surprisingly, given the role of LIF in promoting EGC survival, gp130-mediated signalling is not required for the early stages of mPGC development, but it is required late in oogenesis (Brizzi *et al.* 1999). In addition, the receptor for SCF (required for EGC derivation), c-KIT, was chosen for RT-PCR analysis, along with STAT5A and STAT5B. SCF stimulation of c-KIT results in tyrosine phosphorylation of STAT5A and STAT5B (Molyneaux *et al.* 2003).

By expression microarray and RT-PCR, cytokines of the IL6 family were expressed at the same levels in ovary and testis (IL6, CNTFR, c-KIT, LIF, gp80, gp130, STAT5A and STAT5B), perhaps indicating that these factors are intrinsic to germ cells *per se,* rather than sex-specific. *CNTF* showed higher expression in the testis, in both the expression microarray and RT-PCR (Figure 6.5). CNTF promotes survival of mouse fetal gonocytes *in vitro* (De Miguel *et al.* 1996), and has recently been shown to enhance the formation of germ cell colonies in neonatal mouse testis culture (Kanatsu-Shinohara *et al.* 2007), therefore it may promote PGC proliferation in the testis: in 56 dpc and 64 dpc testes some PGCs were proliferative (Figure 6.1 D and F, arrows). *Cardiotrophin-like cytokine (CLC)* was detected at relatively low levels in both (0.48 and 0.58 in ovary and testis,

respectively). This cytokine acts on the IL6, CNTF and LIF receptors (Vlotides *et al.* 2004). Interestingly, slightly higher expression of *CLC* was detected in ovary by RT-PCR (Figure 6.5). Conversely, *colony stimulating factor 3 (CSF3)* showed 1.6-fold higher expression in testis by microarray, and more transcript was detected in testis than ovary (Figure 6.5). This cytokine acts on the CSF receptor, which shares significant similarity with gp130 (Taga *et al.* 1989; Kishimoto *et al.* 1995).

6.5.1(vi) Bone morphogenetic proteins 2 and 4 (BMP2 and BMP4)

BMP2 and *BMP4* showed 1.3- and 2.7-fold higher expression in ovary compared to testis, respectively, and higher expression of both in ovary was verified by RT-PCR (Figure 6.5). Signalling by endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 is involved in mPGC specification (Lawson *et al.* 1999; Ying and Zhao 2001). In *in vitro* assays, BMP2 and BMP4 enhance juvenile spermatogonial proliferation (Pellegrini *et al.* 2003; Puglisi *et al.* 2004), and BMP4 can influence spermatogonial differentiation (Pellegrini *et al.* 2003; Baleato *et al.* 2005). In organ culture, addition of BMP4 elevated PGC number in E9.5 gonads (Dudley *et al.* 2007), and, with a cocktail of other factors, BMP4 supported survival and self-renewal of mPGCs in the absence of somatic cells (Farini *et al.* 2005). BMP4 has same effect in mESCs: the absence of serum, BMP4 synergises with LIF to maintain self-renewal by inducing expression of *Id (Inhibitor of differentiation)* genes (Ying *et al.* 2003). However, in hESCs, BMP4 induces differentiation to trophoblast (Xu *et al.* 2002), and has been reported to induce germ cell differentiation from hESCs, in combination with BMP7 and BMP8b (Kee *et al.* 2006). BMP2 causes hESCs to differentiate to extra-embryonic endoderm (Pera *et al.* 2004). FGF2 represses BMP signalling in hESCs, and synergises with noggin, a BMP antagonist, to sustain undifferentiated proliferation of hESCs (Xu *et al.* 2005). In addition, BMP2, 4 and 7 induce epithelial differentiation of hECCs (Caricasole *et al.* 2000). The effect of BMPs on hPGCs/EGCs is not known.

6.5.1(vii) Glutamate receptor, metabotropic 4 (GRM4)

GRM4 showed higher expression in female gonads (Figure 6.5), consistent with the 7.5-fold higher expression in ovary detected in the expression microarray. GRM4 has been identified in the epithelial layer of a number of non-neuronal peripheral adult tissues, and

is absent from adult testis. GRM4 expression is frequently identified in colorectal carcinoma, followed by malignant melanoma, laryngeal carcinoma and breast carcinomas. It may play a role in tumour growth and progression, with expression associated with a poor prognosis in colorectal carcinoma (Chang *et al.* 2005).

6.5.1(viii) Carboxypeptidase A2, pancreatic (CPA2)

Expression of *CPA2* was 12-fold higher in ovary in the expression microarray, and higher ovarian expression was verified by RT-PCR (Figure 6.5). The significance of this higher expression is unknown as expression in gonads has not been described, nor has a role in cell proliferation.

6.5.1(ix) Interferon-induced transmembrane protein 1 (IFITM1)

IFITM1 is a 17-kDa membrane protein induced on tumour cell lines by interferon-α and interferon-γ (Deblandre *et al.* 1995). It showed 1.3-fold higher expression in ovary, with relatively high expression in both ovary and testis (32 and 41 respectively). Mouse Ifitm1 and Ifitm3 are expressed on the cell surface of PGCs. Ifitm1 activity was demonstrated to be required for PGC transit from the mesoderm into the endoderm, acting via a repulsive mechanism, such that PGCs avoided Ifitm1-expressing tissues (Tanaka *et al.* 2005). However, a recent study has suggested that *Ifitm* genes are not required for development of the germline (Lange *et al.* 2008). Higher expression in ovary may therefore be due to increased germ cell number, rather than playing any specific role in proliferation, although this difference was unverifiable by conventional RT-PCR (Figure 6.5).

6.5.1(x) Mammalian target of rapamycin (mTOR)

mTOR showed 1.4-fold higher expression in ovary. Also known as FKBP12-rapamycin complex-associated protein 1 (FRAP1), this is one of a family of proteins involved in cell cycle progression, DNA recombination and damage detection (Hay and Sonenberg 2004). mTOR activation promotes cell growth and proliferation, and it is frequently up-regulated in many cancers (Feng *et al.* 2005). PI3K, PTEN and the downstream effector Akt mediate mTOR activity, and are involved in maintaining hESC

pluripotency (Armstrong *et al.* 2006). Akt signalling promotes derivation of mEGCs from PGCs (Kimura *et al.* 2008). Given these functions, and although no difference in expression was verifiable by RT-PCR (Figure 6.5), mTOR remains a possible candidate for a role in germ cell proliferation within the ovary.

6.5.1(xi) Growth arrest-specific 11 (GAS11)

Growth arrest-specific genes are a group of genes which are up-regulated in the murine 3T3 cell line by serum starvation and contact inhibition. It is speculated that the gene products are involved directly in reversible growth arrest, and they may be tumour suppressor genes (Schneider *et al.* 1988). The expression microarray detected three-fold higher expression of *GAS11* in testis; however, no difference was apparent by RT-PCR (Figure 6.5). *GAS11* is also known as *Gas8* in mouse, and is expressed in testes during postmeiotic development of male germ cells, with its gene product proposed to play a role in sperm motility. However, only low levels of expression are detected before puberty (Yeh *et al.* 2002). Expression and function in human testes have not yet been examined. Another member of the growth arrest-specific gene family, Gas6, promotes SCF-induced growth and/or survival of mPGCs (Matsubara *et al.* 1996).

6.5.1(xii) Controls

β-actin expression was approximately equal in both samples by RT-PCR (Figure 6.5), indicating equal amounts of RNA for each reaction. *XIST* served as a control for high expression in ovary, where 40-fold higher expression was detected by microarray. Low levels were detected in male by expression microarray and RT-PCR (Figure 6.5). *SCYA20 (small inducible cytokine subfamily A, member 20*) was used as a control for low level expression in both (0.02 in ovary compared to 0.09 in testis), with consistent RT-PCR verification of absent or very weak expression.

6.5.2 Accounting for Increased Germ Cell Number

It is unclear what cells in the gonad the genes identified here are expressed in, and, if expressed by germ cells, higher expression may merely reflect the increased number of germ cells within the ovary. *POU5F1*, the gene for OCT4 which is expressed exclusively

by germ cells within the gonad, showed 1.6-fold higher expression in ovary: a difference, therefore, likely due to increased germ cell number. Consistently, *IFITM1*, expressed on PGC surface, showed 1.3-fold higher expression in ovary compared to testis. This approximate figure could be used as a reference ratio; i.e. genes with a fold-induction value above 1.6 could be taken as having a 'real' increase in expression and be worthy of investigating further, for example, BMP7 (2.7) rather than BMP2 (1.3).

6.5.3 Summary

This chapter aimed to identify genes which could promote hPGC - and therefore hEGC - proliferation. Comparing gene expression levels by expression microarray and RT-PCR in developing ovary and testis has highlighted a number of genes which are more highly expressed in ovary, and which also promote proliferation in other tissues, including *NPY* (and its receptor *NPY1R*), *CCKBR*, *GRM4*, *CPA2* and *LHX2*. NPY promotes neuroprogenitor proliferation acting through the NPY1R; CCKBR promotes proliferation of gastric carcinoma cells; GRM4 signalling may promote tumour growth, and LHX2 maintains the growth of hair follicle progenitors.

To progress this study, further investigations could use real-time PCR to quantify expression levels of candidate genes. This would provide a higher level of sensitivity than that provided by the initial RT-PCR screen. Protein expression and localisation could be confirmed by immunohistochemistry, and any candidate mitogenic cytokines could be added to the media during hPGC culture. For example, NPY could be added, and any effect on hEGC derivation monitored by, for example, using a BrdU assay or Ki67 immunohistochemistry.

CHAPTER 7: Summary and Future Work/Directions

7.1 The Starting Point - Objectives and Strategies

Embryonic stem cell research has progressed markedly since their first description in the mouse in 1981 and the human in 1998. While no ESCs have been used in human therapy, the group of Yamanaka has demonstrated that induced pluripotent stem cells can be generated by transducing somatic cells with Oct4, Sox2 and Klf4; allowing for the theoretical generation of ESCs, and thus tissues, which would circumvent immune rejection and limited source cells. In fact, a mouse model for sickle cell anaemia has been cured using such a technique.

This work has only been made possible by research into molecules governing pluripotency in ESCs. Embryonic germ cells (EGCs), another pluripotent stem cell, provide a further avenue for research into pluripotency. Insights into the germ cell lineage give clues into the origins and development of testicular germ cell tumours. In addition, further investigations might provide details of the developmental pathway which should be mimicked during the *in vitro* generation of gametes from ESCs. For these reasons, this thesis investigated the human germ cell lineage and associated aspects of pluripotency.

7.2 The Human Germ Cell Lineage: *in vivo* and *in vitro*

hEGCs are notoriously difficult to derive and culture, compared with the relative ease of that for their mouse equivalents. mEGCs in many respects resemble mESCs, in terms of growth characteristics and gene expression. Conditions used for hEGC derivation and culture currently mimic those used for mEGCs. However, hEGCs do not remain pluripotent, indicating that these cells may have different requirements. Chapter 3 sought to determine whether the difference in behaviour between hEGCs and hESCs/mEGCs could be due to a difference in gene expression. Whilst all pluripotent cell types tested expressed OCT4 and NANOG, the early human germ cell lineage was found not to express SOX2, either *in vivo* as hPGCs, or upon conversion to hEGCs in culture, in contrast with hESCs and mPGCs/EGCs. SOX2 is proposed to act with OCT4 and NANOG in initiating and maintaining the expression of genes which promote pluripotency. Interestingly, many of these genes were expressed by hPGCs, despite the absence of SOX2. Of all genes tested, only the presence of FGF4 correlated with presence/absence of SOX2: being absent in the hPGCs but expressed in hESCs. Unlike ESCs and mEGCs, hEGCs have not been reported to lead to teratocarcinoma formation in immunocompromised mice. Whether this is due to

the lack of SOX2 is an interesting question, and one which was pursued. Transfection of a SOX2 expression vector into gonad-derived cells and subsequent transplantation into mice did not generate teratomas; however, proliferation would have been expected, given that the SOX2-negative germ cells were proliferative *in situ*. Further attempts at this experiment, and attempting transfection of hPGCs with a SOX2 expression vector *in vitro*, are warranted.

7.3 Expression of Pluripotency Determinants in Testicular Germ Cell Neoplasia

Chapter 4 sought to investigate the expression profile of the three major determinants of pluripotency in the transformed human germ cell lineage, *in vitro* and *in vivo*. Germ cell tumours were the first source of pluripotent stem cells: once isolated some cell lines have retained pluripotency and some have become nullipotent. Regardless of differentiation capability, ECCs expressed OCT4, SOX2 and NANOG *in vitro*. However, *in vivo*, expression of SOX2 correlated with pluripotency status of the TGCT: the pluripotent compartments of EC tumours expressed OCT4, SOX2 and NANOG, whereas seminomas, which resemble germ cells, only expressed OCT4 and NANOG. The role of SOX2 in pluripotency is contentious, therefore, given that SOX2-negative hEGCs are considered pluripotent, but fail to remain so indefinitely and have not been shown to generate teratomas *in vivo*. Nuclear SOX2 was detected in a subset of CIS cells, the precursors of TGCTs, where expression may indicate progression towards a malignant phenotype. It was also expressed in infantile and adult spermatogonia, localised to the cytoplasm and nucleus respectively; here it may maintain progenitor cell characteristics, a role fulfilled in other cell types. SOX2 was found to be expressed by some Sertoli cells in CIS tubules, but not in Sertoli cells of normal tubules, indicating perhaps a soluble factor may induce SOX2 expression. If this were true it may prove advantageous if such a factor could be identified, and could do so in hEGC cultures.

The results of this study conflicted reports of SOX2-negative CIS cells (Korkola *et al.* 2006; Biermann *et al.* 2007), and gonocytes and normal adult testis (de Jong *et al.* 2008), using a monoclonal SOX2 antibody. It will be important to perform immunoblotting to ensure SOX2 is being recognised by the polyclonal antibody used here, and also to perform stainings using the monoclonal SOX2 antibody used in these other studies, to see if the same staining pattern is observed. In addition, as there is a heterogeneous population

of germ cells in both the testis and ovary during development, it would be interesting to determine whether SOX2 expression in early specimens co-localises with OCT4 (first trimester) or VASA (second trimester) (Anderson *et al.* 2007). This work is currently being conducted at the University of Copenhagen, Denmark.

7.4 Investigating the Association of the OCT4-SOX2-NANOG Transcription Factor Complex with Pluripotency

The expression of OCT4, SOX2 and NANOG was not sufficient to reprogram a cell to pluripotency in this study, as shown by transfection of the two transcription factors into SOX2-positive neuroprogenitors and stomach epithelial cells as described in Chapter 5. Cells retained expression of differentiation markers and did not induce expression of *FGF4*, a gene whose expression is activated by OCT4 and SOX2. Surprisingly the $OCT4^-$, $NANOG^-$, $SOX2^+$ neuroprogenitors and stomach epithelial cells expressed genes which are proposed to be targets of the transcription factor complex in hESCs. In addition, neuroprogenitors and stomach epithelial cells expressed cell surface markers commonly used to denote pluripotency, namely SSEA3, SSEA4, TRA-1-60 and TRA-1-81. This indicates that the expression of such markers should be interpreted with some caution when being used to assess pluripotency. It would be interesting to determine whether other differentiated cells express these 'pluripotent' genes and markers.

7.5 Identification of Candidate Genes for Germ Cell Proliferation

In Chapter 6, a number of factors which were more highly expressed in female compared to male gonads were identified by expression microarray as candidate genes involved in promoting germ cell proliferation. This differential expression was verified by RT-PCR for selected genes. Of particular interest are *Neuropeptide Y* and its receptor *NPY1R*, the *Cholecystokinin B receptor* and *LIM-homeobox 2*, which all have proven roles in promoting cell proliferation in other cell types. Further verification will need to be performed, including quantification of gene expression by real-time PCR. Immunohistochemistry could be used to identify which particular cells in the gonad express these factors, and soluble factors, for example NPY itself, could be added to the culture medium of germ cells to determine whether they promote derivation and improve duration in culture. Preliminary immunohistochemistry results indicate that the

Cholecystokinin B receptor is expressed by germ cells specifically in both ovary and testis, however, no difference in protein abundance was detected (Martin McDonnell, Human Genetics Division, University of Southampton; pers. comm.). In addition, preliminary culture experiments did not identify NPY as having any effect on hEGC derivation when added to the culture medium (Dr. Marie Best, Human Genetics Division, University of Southampton; pers. comm.), however, this will be re-investigated using a 96-well counting method to quantify any effect on hPGC survival or proliferation.

It is hoped that the information provided here, and future studies which emanate from it, will prove instructive for improving human germ cell culture *in vitro* and also provide insights into cancer cell biology. The differences between hESCs and hEGCs in terms of gene expression indicate that there may be different mechanisms governing pluripotency in these cell types.

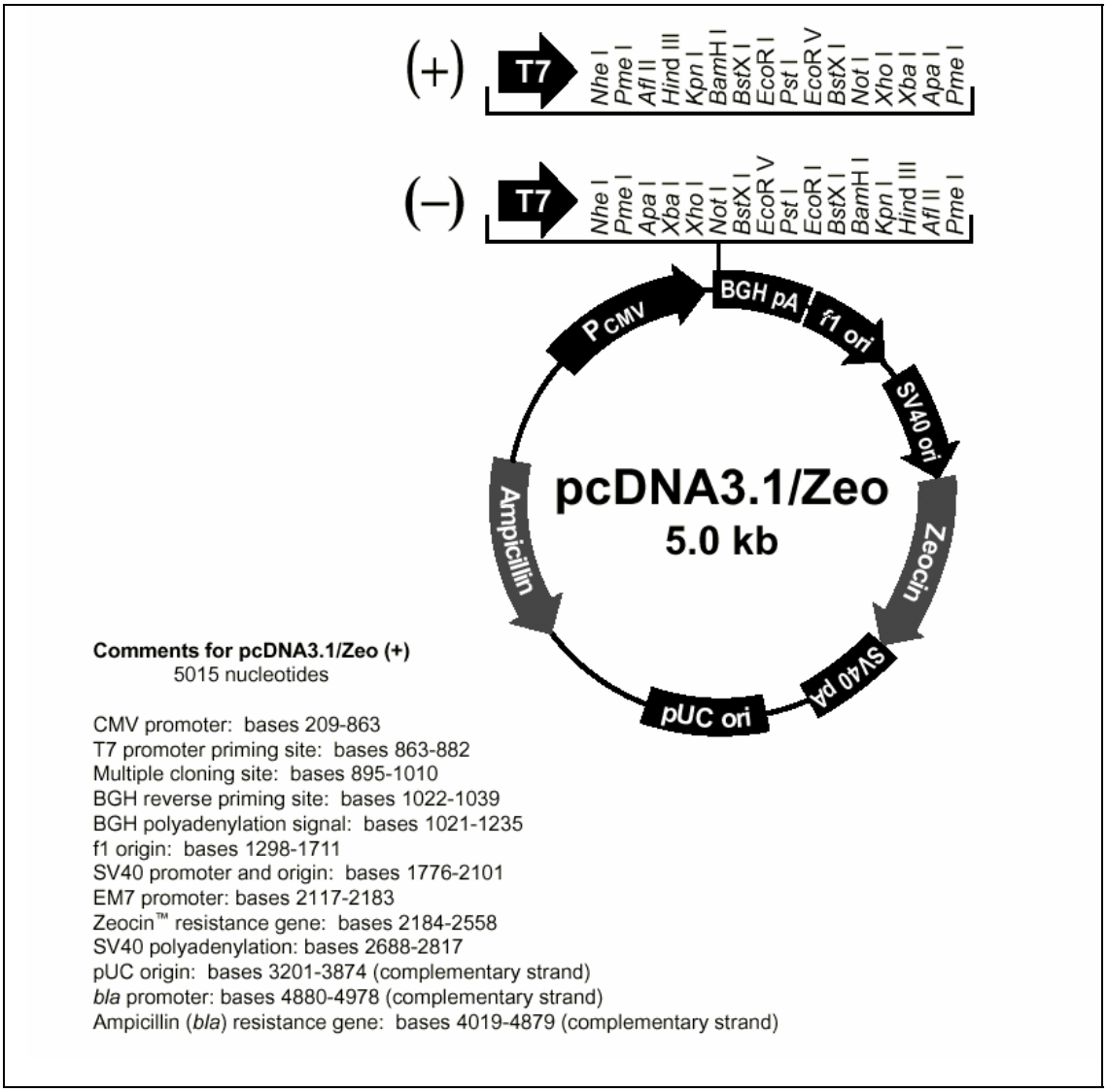
Appendix 1. RT-PCR Primer Sequences

Gene	Primer Sequence		Annealing Temperature (°C)	Product Size (bp)
	Forward (5'-3')	Reverse (5'-3')		
β-ACTIN	TTCTACAATGAGCTGCGTGTGGCT	TCTCCTTAATGTCACGCACGA	60	376
BMP2	CTCCCCCTGCTCGCTGTTGTTGTG	TGCTGGGGGTGGGTCTCTGTTTCA	64	573
BMP4	AGACGCCGCTGCTGCTC	CTCCCGCGTGGCCCTGAATCTC	59	435
CCKBR	CCGCCGGGTCGAGCTGAGTAAG	TGACGGTGCCAAAGATGAATGTGC	63	419
CKIT	GGACACCGGGCCAGTATCTA	ACGGCCACAGTTCTCTAAATG	57	551
CLC	TGGGCCTGGCGGATGGGATTATTA	TGGGTCAGCCGCAGTTTGTCATTG	64	423
CMYC	GCCCACCACCAGCAGCGACTCT	CCTTGGGGGCCTTTTCATTGTTTT	60	442
CNTF	CCGGGGCTGGCGTAGTAGTAGGAC	AGCGAGGATGGGCAAGGACAGTTC	58	533
CNTFRα	AAGGGCTTCTACTGCAGCT	TCACTCCATGTCCCAATCTCA	55	508
CPA2	CAAAAATCGTATGTGGCGGAAGAC	TGACGGGCTGGCAAGAGGA	58	492
CSF3	AGCGGCCTTTTCCTCTACCAG	ACAGCGGCTCATCCCAGTGC	60	561
DKK1	ACGGTCATTTTCTCTTTCTTTCTC	AGTAATTCCCGGGGCAGCACATAG	60	439
DPPA4	CGAGGCTCCGCTTCTTCTAC	ATTTTGGCCTCTTTTGCTGTGCTA	55	416
FBX15	TTGGGCAATTATACTGAAAGAAAA	TACTCGGGGCTATCATCCAAAAAG	54	456
FGF4	CCTCGGGCCGGGATGTCG	AGGCGTTGTAGTTGTTGGGAAGGA	64	517
GAS11	CCCGCAAGCTGGAGGATGTT	GAGAAGGTGCCGCCGACTGTGGT	61	514
GJA1	CGCCCCACGGAGAAAACCAT	ATCGGGGAAATCAAAAGGCTGTG	55	413
gp80	GAGCACCCCATCCCTGACGA	CGCCTTGCCCGAACTCCTC	60	493
gp130	GCTGTATGAAGGAAGATGGTAAGG	GAAAGTCACAGGCAGGGATAGTTA	60	366
GRM4	CTGGGCGCCCGCATTCTGG	CCTTGGGCTCCCGTGGTATCTTCA	63	484
HESX1	CGCTCAGCTCGGGGAAAAC	TCGCCATTAGAAACTGTGATT	53	503
HPRT	CCTGGCGTCGTGATTAGTGATGAT	AGCTTGCGACCTTGACCA	60	472
IFITM1	CGCTCCACGCAGAAAACCACACT	AGGGGGCAGGGGCAACAGC	60	522
IL1β	CAGGCCGCGTCAGTTGTTGT	TTATATCCTGGCCGCCTTTGGTC	57	592
IL1R1	ACCGGCCAGTTGAGTGACA	GGTAGACCCTTCCCCAACAGT	53	450
IL6	CCCAGTACCCCCAGGAGAAGATT	GTTGGGTCAGGGGTGGTTATTG	60	425
IL8	ACCGGAAGGAACCATCTCACTG	GCATCTGGCAACCCTACAACA	55	445
IL10	AAGGCCGTGGAGCAGGTGAAGAAT	GAAGCCCCAAGCCCAGAGACAAGA	57	571
LHX2	CGCGCTCGGGACTTGGTTTATCAC	ATGCGCTTGGTCTTCTGGCTGCTC	57	418
LIF	ATGTCACAACAACCTCATGAA	GATCTGCTTATACTTCCCCAG	60	465
mTOR	GCCTCCCGGATCATTCACCCTATT	GTCTCAGCCATTCCAGCCAGTCAT	59	403
MYST3	GATCCGCCACTCACCCGTATG	TCCTGTTGCCCCTCTTTCTGATTC	56	456
NANOG	GCGCGGTCTTGGCTCACTGC	GCCTCCCAATCCCAAACAATACGA	63	426
NPY	TGCTAGGTAACAAGCGACTG	CTGCATGCATTGGTAGGATG	55	386
NPY1R	TTTGGTGAGGCGATGTGTAAG	GTAAGAGGGAGCCAGCAGACT	54	518
OCT4A	GATCGGATCCATGGCGGGACACCTGGCT	CCTTCCCAAATAGAACCC	60	532
REST	AGGGCCCCATTCGCTGTGAC	AAGAGGTTTAGGCCCATTGTGA	58	445
SCYA20	GTGCGCAAATCCAAAACAGAC	CAAAACAATATAAACAAAAGAATG	49	465
SET	AACTCCGCCAACCATTTTT	CCATATCGGGAACCAAGTAGTA	55	491
SKIL	GCCGCTTCATCTCCGCTTCT	TCTTGCTTCCCGTTCCTGTCTG	54	452

SMARCAD1	AATAACCGTTTGCTGCTCAC	TTGGCGATGTAATAAAGGATG	52	429
SOX1	CCGGGGAATGGGAGGACAGGAT	ACGCGGGGGAGGGTGATGG	60	526
SOX2	GGCACCCCTGGCATGGCTCTTG	TTCTTGTCGGCATCGCGGTTTTTG	60	484
SOX3	GACCGCGCCGCAAGACCAAGACG	GGCGGCCGCGGCTGCTGTG	68	413
SOX14	GCGCAAGATGGCCCAGGAAAAC	CAGAGCGCCGGTAGCCAAGGTGTG	63	424
SOX21	GGGGGCCCGGTTTGTATGTA	AGATTCGAGCCGGTCACTGGTC	59	561
STAT3	CCCCCGCACTTTAGATTCATTG	AGGCACCAGGAGGCACTTGTCTAA	57	510
STAT5A	CCCCGGAACGCAACCTGTGGAACC	GGGGGCGAGAGGCGGGAGTCAAG	63	442
STAT5B	CCCCGGCCCAGTGGAGGAGAT	CAGTGGCCGTCGCATTGTTGTC	61	530
TCF8	CGCAGTCTGGGTGTAATCGTAAAT	CATGCCCTGAGGAGAACTGG	55	476
TDGF1	ACGATGTGCGCAAAGAGAAC	GAGGGCAGGGCAAAGAAGTAAGAA	58	434
UTF1	CGCGCTGGGGGAACTCG	AGCGGGGTGGCGTCTGG	66	432
XIST	AAGAAGATTGCAGTAAAACGAT	AGCCTAAGGAGACATGACTACTAA	51	548
ZIC3	TCAGCTGAGCCGGCCCAAGAAGA	AGGGAGCTCGGGTGCGTGTAGGAC	60	415

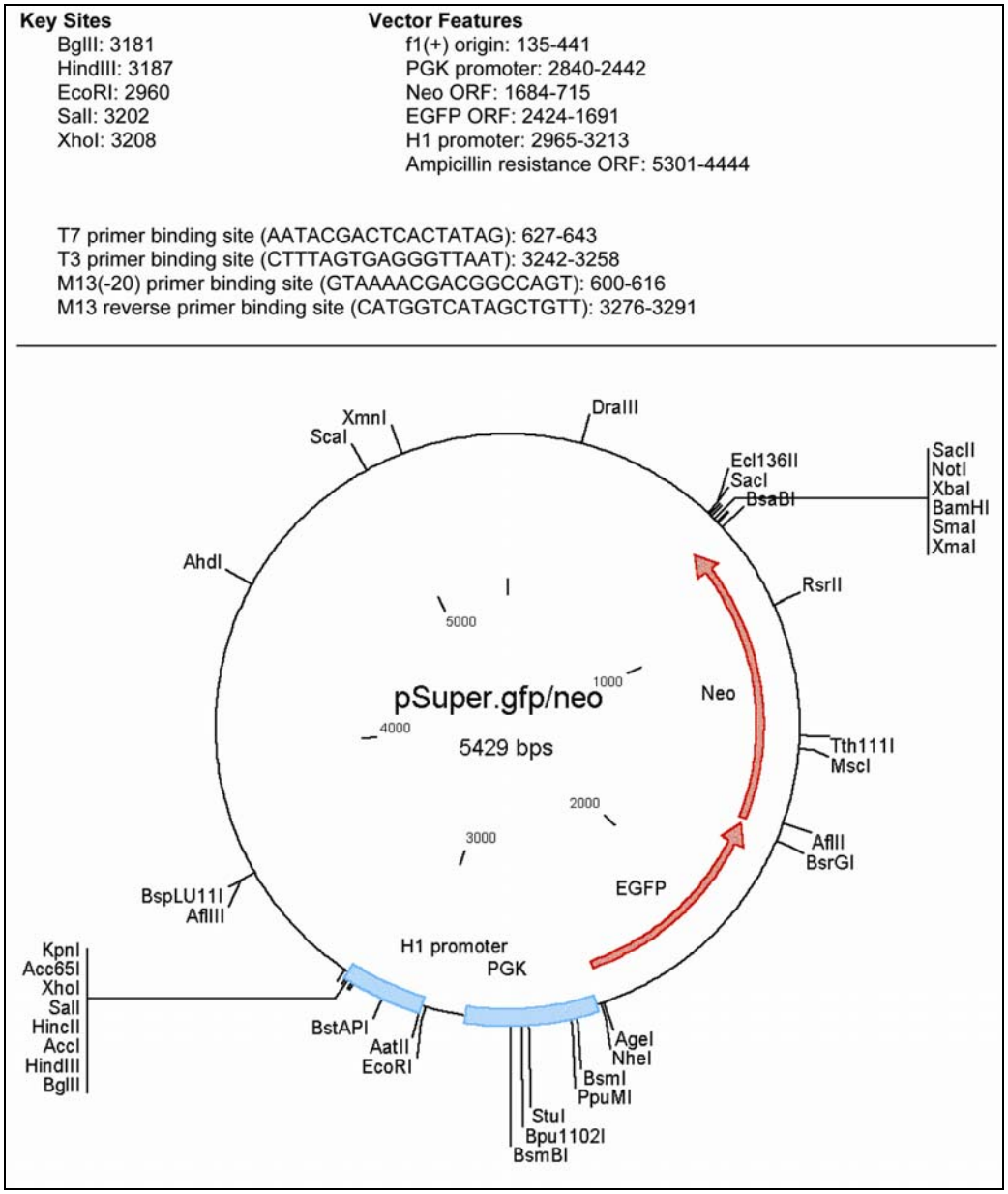
Appendix 2. Commercial Vector Maps

(i) pcDNA3.1Zeo+ (Invitrogen, Carlsbad, CA, US)



From http://www.invitrogen.com/manuals.html.

(ii) pSuper.gfp/neo (Oligoengine, Inc., Seattle, WA, US)



From http://www.oligoengine.com/products/psuper.html#maps.

Appendix 3. Ethics

(i)-(iii) Ethical approval for the use of human embryonic and fetal material from the Southampton & South West Hampshire Local Research Ethics Committee

(i)

Our Ref: CPW/sta

09 May 2005

Professor I.T. Cameron
MNFP Group
Developmental Origins of Health and Disease
Level F (815) Princess Anne Hospital
Southampton SO16 5YA

SOUTHAMPTON & SOUTH WEST HAMPSHIRE RESEARCH ETHICS COMMITTEES
1ST Floor, Regents Park Surgery
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023 8036 3462
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clair.wright@nhs.net
General Enquiries: sharon.atwill@nhs.net
Application Submission: submissions@gp-j82203.nhs.uk

Dear Professor Cameron,

Study title: **The collection of human embryonic and fetal tissue at termination of pregnancy.**
REC reference: **296/00**
Protocol number:
EudraCT number:

Amendment number: **1**
Amendment date: **7th April 2005**

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on Wednesday 27th April 2005.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Notice of Substantial Amendment Form dated 7th April 2005
Annual Progress Report Form dated 7th April 2005

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Management approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 296/00	**Please quote this number on all correspondence**

Yours sincerely,

Catherine Gordon

Mrs Clair Wright
REC Manager

E-mail: clair.wright@nhs.net

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

Enclosure 1

List of names and professions of members who were present at the meeting and those who submitted written comments

Dr R Patel (Chairman)

Dr H McCarthy (SPR in Haematology)

(ii)

NHS

Central Office for Research Ethics Committees (COREC)

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.

To be completed in typescript by the Chief Investigator and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available in section 5 of our Standard Operating Procedures available at www.corec.org.uk/applicants/help/docs/SOPs.doc.

Details of Chief Investigator:

Name:	Professor I.T. Cameron
Address:	MNFP Group, Developmental Origins of Health and Disease Division (University of Southampton) Level F (815), Princess Anne Hospital, Southampton SO16 5YA
Telephone:	023-8079-6044
E-mail:	itc@soton.ac.uk
Fax:	023-8078-6933

Full title of study:	The collection of human embryonic and fetal tissue at termination of pregnancy
Name of main REC:	Southampton & South West Hampshire Research Ethics Committee
REC reference number:	296/00
Date study commenced:	24th October 2000
Protocol reference *(if applicable)*, current version and date:	
Amendment number and date:	

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC application form

Yes *xx*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

xxx ***No***

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

xxx ***No***

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold

Summary of changes

Briefly summarise the main changes proposed in this amendment. Explain the purpose of the changes and their significance for the study.

Supporting scientific information should be given (or enclosed separately) where the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study.

The original application was to establish a tissue resource which therefore did not have a fixed or predetermined end-point but the proposed duration of the project was requested as a "minimum of 5 years", which period will have elapsed towards the end of this year. This tissue bank is proving to be a valuable resource. The obtaining of informed consent has not been problematic (584 to date) and no patients have subsequently withdrawn consent. Approval is therefore sought to extend the project by a further 5 years, to October 2010.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

List of enclosed documents

None

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: ...Iain T. Cameron.....

Date of submission: .....

(iii)

SOUTHAMPTON & SOUTH WEST HANTS
LOCAL RESEARCH ETHICS COMMITTEE

Chairman: Dr Audrey Kermode

Manager: Mrs Clair Wright
Trust Management Offices
Mailpoint 18
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Tremona Road
Southampton
SO16 6YD

Tel: (023) 8079 4912
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Ref: CPW/DBL

15 June 2001

18 JUN 2001

RECEIVE

Professor I Cameron
Obstetrics & Gynaecology
P A H

Dear Professor Cameron

Submission No:296/00 – The collection of human embryonic and fetal tissue at termination of pregnancy.

In response to your letter dated 22nd May 2001, I am pleased to confirm ethical approval for the amended Patient Information Sheet and Consent Form

This approval has been granted under Chairman's action by the Vice-Chairman Dr Mary Carroll and Rev. Peter Speck, and will be recorded at the Committee meeting in June.

Yours sincerely,

C Wright.

Mrs Clair Wright
Research Ethics Manager

Appendix 4. Published Articles

(i) The following original manuscript details work described in this thesis.

RM Perrett, L Turnpenny, JJ Eckert, M O'Shea, SB Sonne, IT Cameron, DI Wilson, E Rajpert-De Meyts, NA Hanley, 2008. The early human germ cell lineage does not express SOX2 during *in vivo* development or upon *in vitro* culture. *Biol Reprod*, 78 (852-8). [Enclosed]

(ii) The following original manuscript is from additional work carried out during this research period.

EA Hoivik, L Aumo, R Aesoy, H Lillefosse, AE Lewis, **RM Perrett**, NA Hanley, M Bakke, 2008. DNA methylation controls cell type specific expression of steroidogenic factor 1. *Endocrinology*. [Epub ahead of print] [Enclosed]

(iii) The following review articles were published during this research period.

L Turnpenny, CM Spalluto, **RM Perrett**, M O'Shea, KP Hanley, IT Cameron, DI Wilson, NA Hanley, 2006. Evaluating human embryonic germ cells: Concord and conflict as pluripotent stem cells. *Stem Cells*, 24 (212-220). [Enclosed]

DM Kristensen, SB Sonne, AM Ottesen, **RM Perrett**, JE Nielsen, K Almstrup, NE Skakkebaek, H Leffers, E Rajpert-De Meyts, 2008. Origin of pluripotent germ cell tumours: The role of microenvironment during embryonic development. *Mol Cell Endocrinol*, 288 (111-8). [Enclosed]

(iv) The following original manuscript has been submitted during this research period.

RM Perrett, SB Sonne, JE Nielsen, DM Kristensen, H Leffers, NA Hanley, E Rajpert-De Meyts, 2008. Divergent roles of SOX2 in human testis during development: Lessons from the expression pattern in germ cell neoplasia. [Manuscript in revision for re-submission to *Int J Dev Biol*]

BIOLOGY OF REPRODUCTION **78,** 852–858 (2008)
Published online before print 16 January 2008.
DOI 10.1095/biolreprod.107.066175

The Early Human Germ Cell Lineage Does Not Express SOX2 During In Vivo Development or upon In Vitro Culture[1]

Rebecca M. Perrett,[3,4] Lee Turnpenny,[3,4] Judith J. Eckert,[3,5] Marie O'Shea,[3,4] Si Brask Sonne,[6] Iain T. Cameron,[3,5] David I. Wilson,[3,4] Ewa Rajpert-De Meyts,[6] and Neil A. Hanley[2,34]

Centre for Human Development, Stem Cells and Regeneration,[3] Human Genetics Division,[4] and Developmental Origins of Health and Disease Division,[5] University of Southampton, Southampton SO16 6YD, United Kingdom
Department of Growth and Reproduction,[6] Copenhagen University Hospital, Rigshospitalet, DK-2100 Copenhagen, Denmark

ABSTRACT

NANOG, POU5F1, and SOX2 are required by the inner cell mass of the blastocyst and act cooperatively to maintain pluripotency in both mouse and human embryonic stem cells. Inadequacy of any one of them causes loss of the undifferentiated state. Mouse primordial germ cells (PGCs), from which pluripotent embryonic germ cells (EGCs) are derived, also express POU5F1, NANOG, and SOX2. Thus, a similar expression profile has been predicted for human PGCs. Here we show by RT-PCR, immunoblotting, and immunohistochemistry that human PGCs express POU5F1 and NANOG but not SOX2, with no evidence of redundancy within the group B family of human *SOX* genes. Although lacking SOX2, proliferative human germ cells can still be identified in situ during early development and are capable of culture in vitro. Surprisingly, with the exception of *FGF4*, many stem cell-restricted SOX2 target genes remained detected within the human SOX2-negative germ cell lineage. These studies demonstrate an unexpected difference in gene expression between human and mouse. The human PGC is the first primary cell type described to express POU5F1 and NANOG but not SOX2. The data also provide a new reference point for studies attempting to turn human stem cells into gametes by normal developmental pathways for the treatment of infertility.

embryonic, gamete biology, gene regulation, human, human development, human stem cell biology, primordial germ cells, SOX2

INTRODUCTION

From original experiments in mice, embryonic stem cells (ESCs) and embryonic germ cells (EGCs) are, unequivocally, two types of pluripotent stem cell [1]. The former are derived from the inner cell mass (ICM) taken into laboratory culture; the latter are presumed to represent an analogous in vitro conversion of the primordial germ cell (PGC), the diploid precursor of the male and female gametes. A third pluripotent stem cell, the embryonal carcinoma cell (ECC), arises from in vivo malignant transformation of the PGC. Comparative studies of these different cell types and their respective parent cells offer a collective view on the derivation and maintenance of pluripotent stem cells. In addition, improved knowledge of the germ cell lineage can benefit research into the different types of germ cell tumor (GCT), particularly those affecting the testis, which are of increasing frequency in the Western world [2]. Information will also provide a reference point for studies aiming to replicate normal development for the in vitro generation of gametes from human stem cell populations for future fertility treatment.

Studying the development of human germ cells is hampered by restricted access to appropriately aged material and by their intractability in culture [3]. Human embryonic material is inaccessible from 14 days (UK legal limit for in vitro culture of the preimplantation embryo) until potential access to material with appropriate consent at social/voluntary termination of pregnancy (rarely earlier than 4 wk postconception) [4]. Thus, researchers have extrapolated heavily from other species, most notably the mouse, with implicit and, where analysis has permitted, mostly correct assumption of high interspecies conservation [5, 6]. Mouse PGCs become apparent during gastrulation and over the following days migrate via the gut mesentery to the gonadal ridge [7]. This same path seems to be followed in human embryos, as first-trimester material, stained for alkaline phosphatase (AP) activity, identifies germ cells in the gut mesentery and gonad [1]. A few groups, including our own, have taken these cells into culture to generate cells that have been termed human EGCs [3, 8, 9]. In our experience, derivation is defined by acquiring highly proliferative cells positive for AP activity and cell surface antigens, such as SSEA4, TRA1–60, and TRA1–81, commonly used as markers of pluripotent cell types [3]. However, contrary to the equivalent mouse cell type, common experience has indicated a difficulty maintaining this cell state over numerous passages in prolonged culture, suggesting value in further characterizing the human germ cell lineage.

Three of the main transcription factors identified in the ICM and ESC are POU5F1 (previously known as OCT4), NANOG, and SOX2 [10]. They have been suggested to act collaboratively in the promoter region of genes encoding two sets of transcription factors: activating expression of those that maintain pluripotency, while repressing transcription from those that enact differentiation [11]. In this study, we have analyzed the expression of POU5F1, NANOG, and SOX2

[1]Supported by a UK Department of Health Clinician Scientist award (to N.A.H), Wellcome Trust project grant GR074320MA (to N.A.H.), International Research Mobility Award from the Worldwide Universities Network (to R.M.P.), and the Danish Cancer Society (to E.R.M.). R.M.P. is the recipient of an MRC Ph.D. studentship in stem cell research. L.T. is an MRC/Juvenile Diabetes Research Foundation (JDRF) Career Development Fellow in stem cell research. The authors declare no conflicts of interest.
[2]Correspondence: Neil A. Hanley, Human Genetics Division, Duthie Building, Mailpoint 808, Southampton General Hospital, Tremona Rd., Southampton SO16 6YD, U.K. FAX: 44 0 23 8079 4264; e-mail: N.A.Hanley@soton.ac.uk

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First decision: 20 November 2007.
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TABLE 1. Numbers and ages of fetal material used in this study.

Experiment	Age[a]
Immunohistochemistry or immunocytochemistry (see Figs. 1 and 2)	Male: 48, 55, 56, 59, 63, and 73 dpc Female: 48, 53, 54, 59, 63, and 73 dpc
Immunoblotting (see Fig. 3)	Pooled sample of 3 male gonads 52, 56, and 63 dpc Pooled sample of 3 female gonads 52, 52, and 55 dpc Pooled sample of 3 mouse male gonads E14 Pooled sample of 6 mouse female gonads E14
RT-PCR (see Figs. 4 and 7)	Pooled sample of male gonads 51, 53, 59, and 61 dpc Pooled sample of female gonads 51, 54, 55, 57, and 61 dpc
Germ cell culture (see Fig. 6)	Cultures initiated from 11 male gonads 50–72 dpc (average age 58 dpc) Cultures initiated from 14 female gonads 53–65 dpc (average age 57 dpc)

[a] All samples are derived from human material unless otherwise stated.

during early human germ cell development and compared the data to those acquired from human ESCs and various ECCs. We have also studied the expression in the fetal gonad of many genes described as stem cell restricted [11] and that have been reported to require POU5F1 and SOX2 [12–14] or POU5F1, NANOG, and SOX2 [11] for their expression.

MATERIALS AND METHODS

Collection of Human Embryonic and Fetal Material and Germ Cell Tumor Samples

Ethical approval, collection, and staging of human embryonic and fetal material was carried out as described previously, using the Carnegie classification and fetal foot length to provide a direct assessment of gestational age as days or weeks postconception (dpc or wpc) [15–17]. Human preimplantation embryos were obtained with ethical permission and informed consent under a licence from the UK Human Fertilisation and Embryology Authority (RO142). Table 1 details the numbers and ages of human fetal material used in this study. The Regional Committee for Medical Research Ethics in Denmark approved the use of human germ cell tumor material. The tissue samples from adults with testicular neoplasms were obtained directly after orchidectomy and macroscopic pathological evaluation.

Immunohistochemistry/Immunocytochemistry and Alkaline Phosphatase Activity Staining

Tissue processing of human embryonic and fetal samples, immunohistochemistry (IHC), immunocytochemistry (ICC), and alkaline phosphatase (AP) activity staining were performed as described previously [3, 17, 18]; primary antibodies used are detailed in Supplemental Table 1 available online at www.biolreprod.org. Adult testicular samples were fixed overnight at 4°C in 4% paraformaldehyde or formalin and subsequently embedded in paraffin. A series of 12 testicular tumors were analyzed by IHC, including classical seminomas and various nonseminomatous tumor components. Either biotin- or fluorescently labeled secondary antibodies were used according to the manufacturer's instructions. Anti-rabbit (1:800), anti-goat (1:300), and anti-mouse (1:100) biotinylated antibodies were from Vector Laboratories. Fluorescently labeled secondary antibodies were fluorescein isothiocyanate (FITC) anti-mouse (1:64) or anti-goat (1:64) (both from Sigma-Aldrich). For biotinylated secondary antibodies, either streptavidin horseradish peroxidase (SA-HRP; 1:200; Vector Laboratories), SA-FITC (1:150; Sigma-Aldrich), or SA-Texas Red (1:200; Vector Laboratories) conjugates were used according to the manufacturer's instructions. Controls omitted primary or secondary antibody. For bright-field immunohistochemistry, the color reaction was developed using diaminobenzidine (Merck) containing 0.1% hydrogen peroxidase (Sigma-Aldrich) with toluidine blue counterstaining or, for the GCT samples, using aminoethyl carbazole substrate (Zymed) counterstained with Mayer hematoxylin.

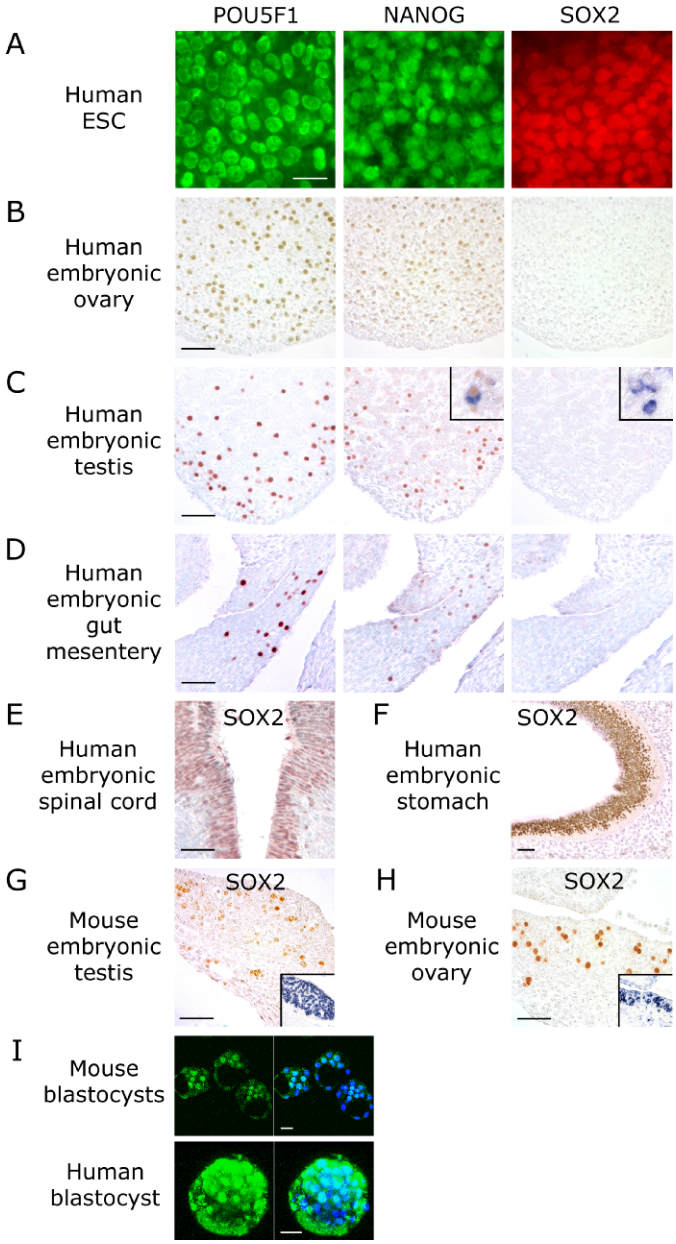

FIG. 1. Expression of POU5F1, NANOG, and SOX2. ICC and IHC panels are shown for human ESCs (**A**) and the following human embryonic sites: ovary (**B**), testis (**C**), gut mesentery (**D**), spinal cord (**E**), and stomach (**F**). The example shown in **B** is at 54 dpc, in **C**–**F** at 48 dpc (insets in **C** also include AP staining). SOX2 staining is shown in mouse embryonic testis (E13.5; **G**) and ovary (E12.5; **H**) (insets show AP staining). **I**) Human and mouse blastocysts counterstained with DAPI. Bars = 40 μm (**A** and **I**) and 300 μm (**B**–**H**).

Protein Preparation, SDS-PAGE Electrophoresis, and Western Blotting

Tissues and cells were rinsed with PBS and treated with ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM sodium chloride, 0.5% Triton X-100 [Sigma-Aldrich]) containing a set of protease inhibitors (Complete; Roche Diagnostics) for 30 min with gentle trituration. Lysates were stored at −80°C. Single-dimension SDS-PAGE was carried out vertically in buffer (25 mM Tris-HCl [pH 8.3], 250 mM glycine, 0.1% SDS). Cell lysates containing 15 μg of protein were combined with an equal volume of 2× SDS gel-loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% bromophenol blue) and heated at 95°C for 5 min before gel loading. Proteins were electrotransferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) in transfer buffer (48 mM Tris-HCl [pH 8.3], 39 mM

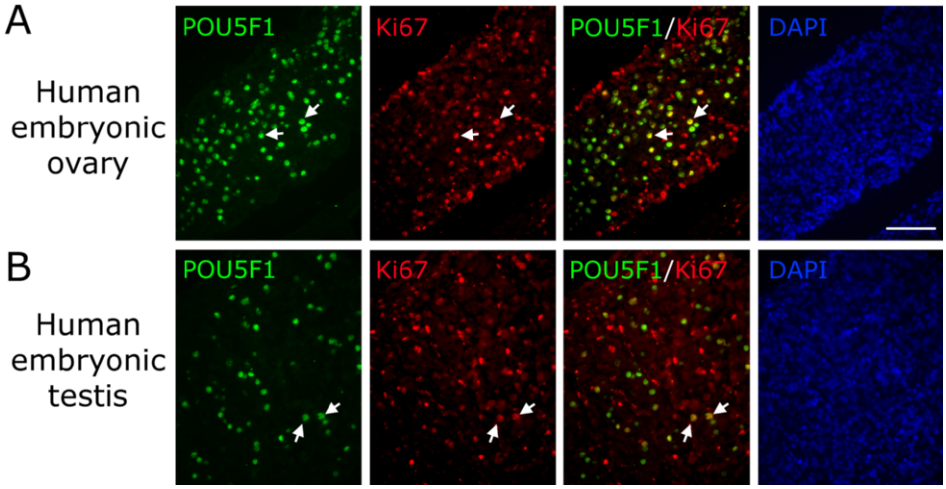

FIG. 2. PGCs are proliferative in situ during human development. Dual IHC for POU5F1 (green) and the proliferation marker MK167, identified using the Ki67 antibody (red), shown for the human embryonic ovary (**A**) and testis (**B**) at 54–56 dpc. Arrows point to examples of PGCs that are dual stained. Bar = 300 μm.

glycine, 20% methanol). Blocking of nonspecific binding sites was carried out by immersion in PBS containing 5% nonfat powdered milk and 0.1% Tween-20 (Sigma-Aldrich) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated with the membrane overnight at 4°C. Membranes were washed three times in PBS containing 0.1% Tween-20 and then incubated with peroxidase labeled anti-rabbit antibody for SOX2 (Amersham Biosciences; 1:50 000) or anti-goat antibody for POU5F1 (Sigma-Aldrich; 1:200 000) in blocking buffer for 1 h at room temperature. β-actin was detected by 1-h incubation with mouse anti-β-actin peroxidase conjugated antibody (Sigma-Aldrich; 1:50 000) at room temperature. Membranes were washed three times in PBS/0.1% Tween-20 and once in PBS, and developed using advanced enhanced chemiluminescence reagents (Amersham Biosciences) according to the manufacturer's instructions.

Reverse Transcriptase-PCR

Total RNA was isolated from tissues using Tri-Reagent (Sigma-Aldrich) and cDNA synthesized from 1 μg per sample with Superscript III (Invitrogen). Wherever possible, intron-spanning primer pairs were designed (Supplemental Table 2 available online at www.biolreprod.org). Negative (water and –RT) and positive (genomic) control reactions were performed concurrently.

Human Germ Cell, ESC, and ECC Culture

Human gonad-derived cells were processed, plated, and cultured as described previously [3]; in total, 25 cultures were initiated, as detailed in Table 1. The hECC lines NTERA-2 (clone D1 [ECCAC], TERA-1, and 2102Ep [both gifts from Peter Andrews, University of Sheffield] were cultured in Dulbecco modified Eagle medium (DMEM; PAA Laboratories) containing 10% fetal calf serum (PAA Laboratories) and 1% penicillin/streptomycin (Invitrogen) in 5% CO_2 at 37°C. Human ECCs were passaged using glass beads (VWR International) and maintained at high density. The hESC lines HUES1 and HUES7 were obtained under MTA from Harvard University [19] and maintained in KO-DMEM containing 1% penicillin/streptomycin, 1% GlutaMAX, 1% nonssential amino acids, 10% KO-Serum Replacement (all from Invitrogen), 10 ng/ml FGF2 (Peprotech), and 0.1% 2-mercaptoethanol (Sigma-Aldrich) in 5% CO_2 at 37°C. Human ESCs were cultured on mitotically inactivated mouse embryonic fibroblasts and passaged using 0.05% Trypsin/EDTA (PAA Laboratories).

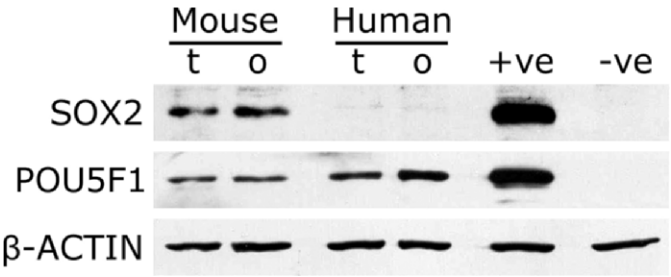

FIG. 3. SOX2 expression in human and mouse embryonic gonads. Immunoblotting for the detection of SOX2, POU5F1, and β-actin. Positive control, NTERA-2 D1 ECCs; negative control, PANC1 cells. Ovarian (o) and testicular (t) samples were prepared from specimens listed in Table 1.

RESULTS

Germ Cell Expression of SOX2 Differs Between Human and Mouse

The human fetal gonad is apparent as a distinct structure from 32 dpc with sex determination marked by the expression of *SRY* and *SOX9* at ~41–44 dpc in the male [18]. Thereafter, sex cords comprised of Sertoli cells and germ cells become increasingly apparent within the testis from 48 dpc during late embryonic (up to 56 dpc) and early fetal (thereafter) development [18]. POU5F1, NANOG, and SOX2 are known as three critical transcription factors in human ESCs [11] (Fig. 1A). Nuclear POU5F1 and NANOG proteins were clearly detected within germ cells in the gonad of the embryonic ovary and testis at Carnegie stages 19–22 (48–54 dpc; Fig. 1, B and C). In sharp contrast, SOX2 was not detected within the human female or male gonad either prior to sex determination or later during the first trimester (ranging from 48 to 73 dpc for both sexes; Table 1, Fig 1, B and C; data not shown). The expression of some genes, such as *Ddx4*, is altered on the arrival of the PGC to the gonadal ridge in mice [20]. Therefore, we also studied PGCs within the gut mesentery at 48 dpc, a location consistent with their migration from yolk sac wall to gonadal ridge. Nuclear POU5F1 and NANOG were again clearly detected; however, SOX2 was not (Fig. 1D). The validity of the SOX2 antibody within the same human embryonic sections was confirmed by clear staining of the neuroprogenitors in the spinal cord and stomach epithelial cells (Fig. 1, E and F). *Sox2* transcripts have been demonstrated in the gonadal ridge of mouse embryos [6]. Consistent with this finding, nuclear SOX2 protein was present in primordial germ cells within the mouse embryonic testis and ovary (E13.5 and E12.5, respectively; Fig. 1, G and H). Nuclear SOX2 was also clearly detected within the ICM of mouse blastocysts (Fig. 1I) [21]. In human blastocysts, SOX2 detection was more diffuse but included nuclear localization within the ICM.

The SOX2-negative human germ cells included ones that were proliferative in situ as marked by dual immunoreactivity for POU5F1 and the proliferative marker MK167 (recognized by the Ki67 antibody; Fig. 2). There were more of these double-stained cells in the embryonic ovary at 54–56 dpc than in the embryonic testis of the same age, consistent with male

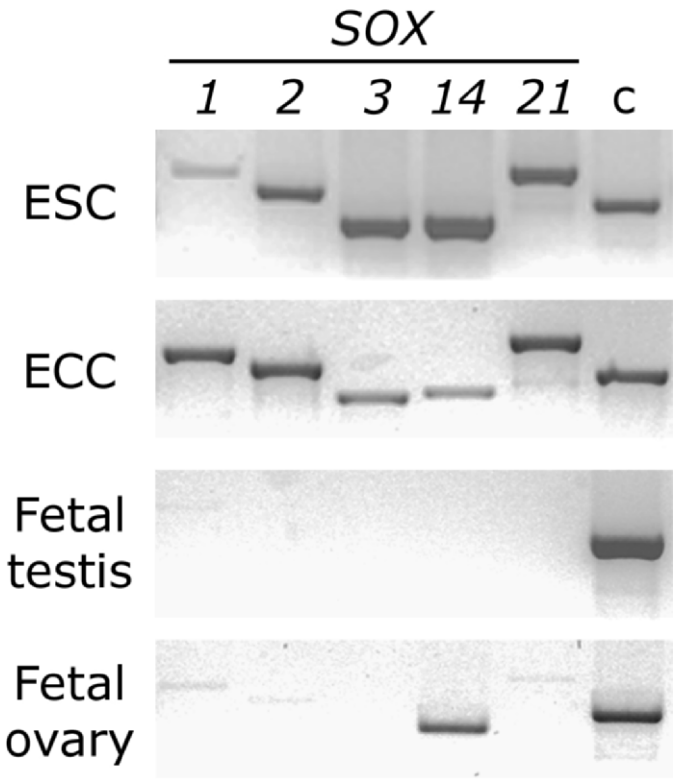

FIG. 4. The potential for SOX2 redundancy in the human germ cell lineage. RT-PCR panel is shown for members of the group B *SOX* gene family in human ESCs, ECCs (NTERA-2 D1 cells), and the fetal gonad (samples are listed in Table 1).

PGCs entering mitotic arrest following testicular cord formation.

These data, demonstrating an interspecies difference in PGC SOX2 expression, were further validated by immunoblotting of protein isolated from fetal gonads (Table 1, Fig. 3). SOX2 was present as a 35-kDa band in protein isolates from mouse gonads at E14 but not from the corresponding human organs. POU5F1 detection is shown as a positive control for the presence of PGCs.

Redundancy Is Unlikely to Compensate for Lack of SOX2 in Human Germ Cells

One potential explanation for this species difference is redundancy for SOX2 within group B of the *SOX* gene family, which comprises *SOX1*, *SOX2*, *SOX3*, *SOX14*, and *SOX21*. By RT-PCR for 35 cycles, *SOX2*, *SOX3*, *SOX14*, and *SOX21* transcripts were detected in both human ESCs and NTERA-2 D1 ECCs (Fig. 4). The weak identification of *SOX1* in ESCs may represent low-level expression per se or, feasibly, minor spontaneous ectodermal differentiation as is commonly encountered in human ESC culture. However, transcripts for all *SOX* group B genes were undetected in four samples of human testes collected during the late embryonic and early fetal periods (51–61 dpc; Fig. 4, Table 1). In five ovarian samples ranging from 51 to 61 dpc, a very faint band was just discernible for *SOX1* and *SOX21* (Fig. 4, Table 1). Only *SOX14*, encoding a putative repressor of SOX2 function [22], was clearly detected within the ovary after amplification for 35 cycles (Fig. 4). The failure to detect *SOX2* transcripts convincingly within these testicular and ovarian samples following extensive PCR cycles corroborates the protein data from IHC and immunoblotting (Figs. 1 and 3).

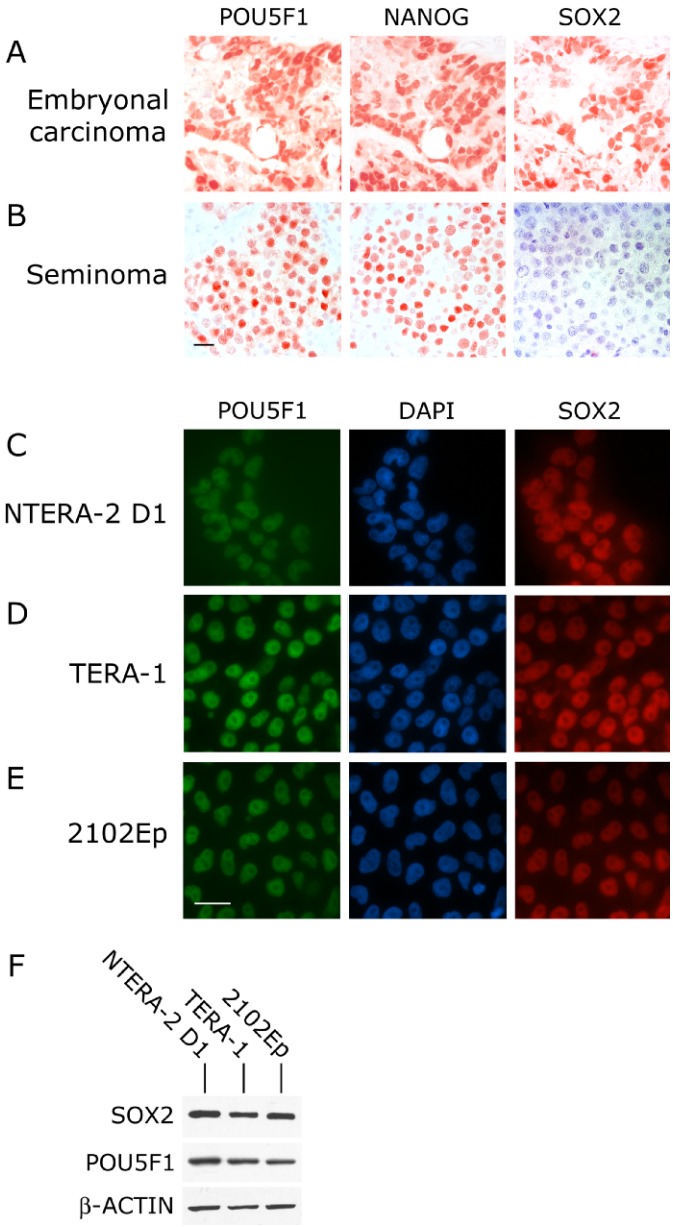

FIG. 5. Expression of POU5F1, NANOG, and SOX2 in germ cell tumor cell types. Fixed sections of pluripotent embryonal carcinoma (**A**) and fixed sections of nullipotent seminoma (**B**). **C**–**E**) Cells fixed after culture on fibronectin-coated glass slide: pluripotent ECC line, NTERA-2 D1 (**C**) and nullipotent ECC lines TERA-1 (**D**), and 2102Ep (**E**). **F**) Immunoblotting is shown with detection for SOX2, POU5F1, and β-actin. Bars = 40 μm.

SOX2 in Germ Cell Tumors and Embryonal Carcinoma Cell Lines

The ECC represents malignant transformation of the PGC. Our findings led us to interrogate SOX2 expression in different GCTs in vivo and in vitro. Previously, *SOX2* transcripts have been reported in a "germ cell carcinoma" sample [6]. In agreement with data from others [23–25], we did not detect SOX2 in samples of seminoma, a nullipotent human GCT; however, it was clearly visualized as nuclear protein in pluripotent nonseminomatous embryonal carcinoma. Both seminomas and pluripotent GCTs expressed nuclear POU5F1

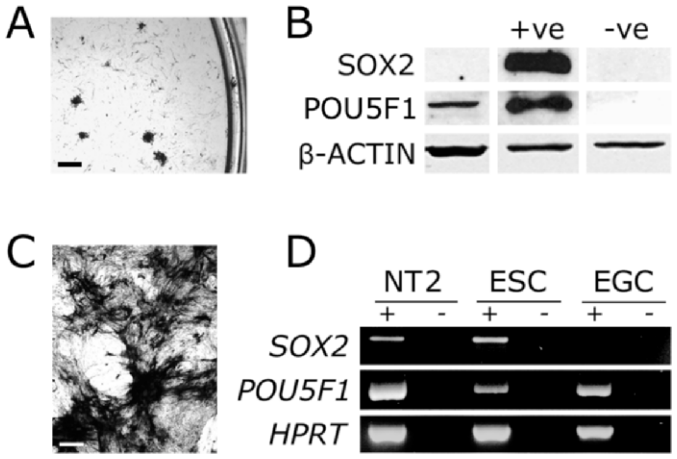

FIG. 6. SOX2 is not detected in human germ cell cultures. **A**) Image of germ cell-derived culture showing colonies after 1 wk that were positive for AP activity. Bar = 2 mm. **B**) Immunoblotting for the detection of SOX2, POU5F1, and β-actin. Left column, germ cell culture; positive control, NTERA-2 D1 cells; negative control, PANC1 cells. Four POU5F1-positive/AP-positive cultures were analyzed after 1 wk, and SOX2 was not detected in any of them. **C**) Image of a passaged germ cell culture fulfilling VP/EGC criteria. Bar = 250 μm. **D**) RT (+) of mRNA isolated from the EGC culture shown in **C** after 3.5 wk and PCR for 32 cycles. NTERA-2 D1 cells (NT2) and ESCs shown as positive controls. Omission of RT (–) is shown as negative control. The same results were obtained from another EGC culture.

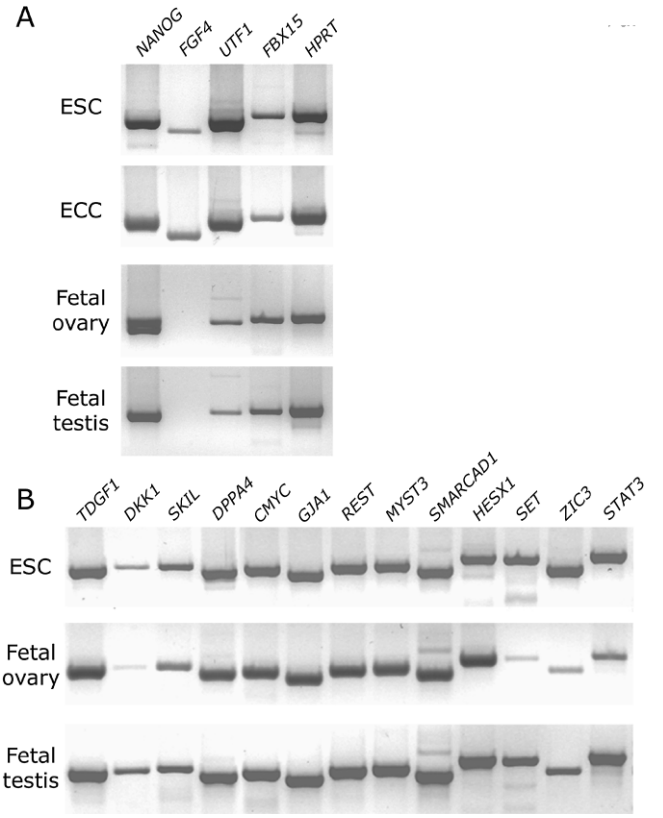

FIG. 7. RT-PCR analysis of genes regulated by OCT-SOX enhancers and those identified by bioinformatic analysis as targets of POU5F1, SOX2, and NANOG. **A**) Genes previously recognized as targets of POU5F1 and SOX2 [12–14]. **B**) Genes previously described to be expressed in human ESCs when POU5F1, NANOG, and SOX2 are bound to the respective promoter regions [11]. *NANOG* and *HPRT* are shown in **A** as controls. Samples analyzed are shown in Table 1.

and NANOG (Fig. 5, A and B). In contrast to NTERA-2 D1 cells, once taken into culture some embryonal carcinomas have yielded nullipotent cell lines that are no longer capable of differentiation to derivatives of all three germ layers (e.g., TERA-1 and 2102Ep cells). SOX2 was clearly expressed equivalently in both pluripotent and nullipotent hECCs localizing indistinguishably to the nucleus in all lines tested (Fig. 5, C–F).

Human Germ Cells Persisting in Culture In Vitro Remain SOX2 Negative

We have previously assigned germ cell cultures as "poorly proliferative" (PP) or "vigorously proliferative" (VP), according to their growth characteristics, with the latter taken as indicative of conversion to the EGC state [3]. Akin to ESCs, human EGCs demonstrate expression of the nuclear transcription factor POU5F1 and AP activity [1]. However, SOX2, required for the maintenance of pluripotent ESCs [21], was not expressed in the human germ line in situ. These findings led us to question whether SOX2 expression was induced in culture. Numerous human PGC cultures were established, and samples of both PP and VP/EGC cultures were analyzed. POU5F1 protein and *POU5F1* transcripts and AP activity served as positive controls for the presence of the germ cell lineage (Fig. 6). Neither SOX2 protein nor *SOX2* transcripts were detected by immunoblotting or RT-PCR, respectively, in either early, PP, or VP/EGC cultures.

SOX2 Appears Dispensable for the Expression of Genes Previously Identified to Require OCT-SOX Interaction or Cooperative POU5F1-SOX2-NANOG Function

Given the lack of SOX2 in human germ cells yet the presence of both POU5F1 and NANOG, we investigated a selection of genes recognized as targets of these critical transcription factors. POU5F1 and SOX2 are known to act cooperatively in up-regulating *FGF4* [12], *UTF1* [13], and *FBX15* [14]. However, despite the absence of SOX2, *UTF1*, and *FBX15* transcripts were detected in the human fetal ovary and testis. In contrast, *FGF4* was not detected (Fig. 7A, Table 1). A further set of 13 genes were analyzed that are expressed in ESCs when the respective promoter regions are bound by POU5F1, NANOG, and SOX2 [11]. All 13 stem-cell-restricted transcripts were detected in fetal gonads after RT-PCR for 35 cycles albeit for *DKK1*, *SET*, *ZIC*, and *STAT3* more weakly in the human fetal ovary (Fig. 7B, Table 1).

DISCUSSION

Previous studies have revealed highly concordant gene expression profiles across different pluripotent stem cells, the cells of the ICM and PGCs [5, 24, 26]. Here, our analysis of the human germ cell lineage was borne of recognized difficulties in the long-term maintenance of human germ cell cultures [1]. We hypothesized that the starting human PGCs lack a critical factor(s) or fail to induce one in in vitro culture that is required for maintained self-renewal and pluripotency. Contrary to predictions from other studies [6] or previously presumed conservation [5], we have identified that *SOX2*/SOX2 expression, identified as fundamental to stem cell pluripotency [10], was not detected in human PGCs as either transcripts or protein. In contrast, SOX2 was present in the human ICM, ESCs, and various ECCs. Amounts of SOX2 and POU5F1 are known to alter in cells derived from different stages of mouse development; mouse germ-line stem cells, which are cultured

spermatogonial stem cells of highly restricted developmental potential, express abundant *Sox2* and *Pou5f1* transcripts but limited corresponding protein [27]. However, mouse PGCs clearly express SOX2 [6 and herein]. At equivalent stages of human development, human PGCs are distinguished by expressing POU5F1 and NANOG but lacking SOX2.

Genome sequencing projects have revealed remarkable conservation across seemingly diverse species. Therefore, a likely explanation underlying cross-species differences in phenotype is altered regulation of gene expression. Thus, the regulation of *SOX2* in human PGCs must differ from that in ESCs (and presumably the epiblast) where an upstream enhancer is bound by POU5F1 and a further downstream composite element by both POU5F1 and SOX2 [28, 29]. The high interspecies sequence conservation of these elements and the wider *SOX2* locus suggest that alternative factors, such as epigenetic mechanisms, may be responsible for divergent *SOX2* expression [30, 31]. Although the earliest germ cell lineage was inaccessible in our human specimens (i.e.. prior to those cells in the gut mesentery at 6–7 wk postconception), our data imply that SOX2 expression must cease either during germ cell specification from cells of the SOX2-positive epiblast or soon afterward. As reports unfold of human ESCs differentiated toward functional germ cells [32], it will be interesting to observe whether SOX2 disappears in mimicry of normal human development. Redundancy among group B SOX family members would limit functional consequences of undetectable SOX2 for the human PGC. This appears unlikely from our data. Only *SOX14* transcripts were detected clearly in fetal ovary samples and only after relatively high numbers of PCR cycles. Furthermore, to date, SOX14 has been recognized to counteract rather than substitute for the role of SOX2 [22].

The collective data infer that, alongside POU5F1 and NANOG, SOX2 is necessary but not sufficient for pluripotency. The epiblast of SOX2 null mice fails to progress in vivo, and SOX2 null blastocyst outgrowths fail to generate ESCs in vitro [21]; however, whereas mouse PGCs give rise to robust pluripotent EGC lines, the SOX2-positive PGCs themselves are nullipotent [33]. Similarly, although previous data on nullipotent human ECC lines were limited largely to analysis of transcripts [24], our data reveal relatively equivalent quantities of correctly localized nuclear SOX2 protein regardless of a capacity for broad differentiation. SOX2 expression, as demonstrated here and by others [23–25], identifies patients with a pluripotent GCT phenotype rather than a nullipotent seminoma. Discovery of ways to induce SOX2 expression in cultured human germ cells might allow improved models of human GCTs. For instance, with the initiation of neoplastic transformation of human germ cells proposed to occur in utero [34], the lack of SOX2 in proliferative PGCs may explain the predilection for SOX2-negative testicular seminomas in humans compared to mice.

Identifying the consequences of missing SOX2 offers comparative insight into its role when present in other cell types. Where coexpressed, SOX2 is considered to act cooperatively with POU5F1 and NANOG in regulating critical cohorts of target genes. From bioinformatic studies, their collective association with gene promoters is proposed to induce the expression of genes that determine self-renewal, while repressing those associated with differentiation [11]. Our data suggest that SOX2 is dispensable in the former function, as, without its detection, all 13 of our arbitrarily selected "ESC-restricted" target genes from Boyer and colleagues, as well as *UTF1* and *FBX15*, were expressed in the fetal ovary and testis. Indeed, most recently, evidence has been published that the major role for SOX2 in ES cells is in maintaining *POU5F1* expression [35]. This implies that the regulation of both *POU5F1* and *SOX2* is different in human germ cells—*SOX2* because of its apparent absence and *POU5F1* because of its independence from SOX2 protein. In contrast, our data do support a pivotal role for SOX2 in promoting *FGF4* expression [12, 36]; *FGF4* transcripts were undetected in the SOX2-negative germ cell lineage. The difference between *FBX15* (detected) and *FGF4* (not detected) is interesting, as both have been shown hypermethylated and correspondingly absent in mouse germ-line stem cells [27].

Finally, it has been discussed whether, on the basis of gene expression profiles, the closest equivalent of the human ESC is an early germ cell and, indeed, whether ESC derivation arises from an early germ cell phenotype [37]. On the basis that the earliest human germ cells detected are SOX2 negative, this hypothesis appears unlikely. In conclusion, we have discovered a fundamental difference in gene expression between human and mouse, demonstrating the limitations of interspecies extrapolation. The data highlight SOX2 as an important transcription factor for further investigation in attempts to understand the relationship between human PGCs, GCTs, and the derivation, self-renewal, and pluripotency of human EGCs. The information is also instructive in attempts to generate human gametes from stem cell sources for ambitious fertility treatments.

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Deoxyribonucleic Acid Methylation Controls Cell Type-Specific Expression of Steroidogenic Factor 1

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Steroidogenic factor 1 (SF1) is expressed in a time- and cell-specific manner in the endocrine system. In this study we present evidence to support that methylation of CpG sites located in the proximal promoter of the gene encoding SF1 contributes to the restricted expression pattern of this nuclear receptor. DNA methylation analyses revealed a nearly perfect correlation between the methylation status of the proximal promoter and protein expression, such that it was hypomethylated in cells that express SF1 but hypermethylated in nonexpressing cells. Moreover, *in vitro* methylation of this region completely repressed reporter gene activity in transfected steroidogenic cells. Bisulfite sequencing of DNA from embryonic tissue demonstrated that the proximal promoter was unmethylated in the developing testis and ovary, whereas it was hypermethylated in tissues that do not express SF1. Together these results indicate that the DNA methylation pattern is established early in the embryo and stably inherited thereafter throughout development to confine SF1 expression to the appropriate tissues. Chromatin immunoprecipitation analyses revealed that the transcriptional activator upstream stimulatory factor 2 and RNA polymerase II were specifically recruited to this DNA region in cells in which the proximal promoter is hypomethylated, providing functional support for the fact that lack of methylation corresponds to a transcriptionally active gene. In conclusion, we identified a region within the *SF1/Sf1* gene that epigenetically directs cell-specific expression of SF1. (*Endocrinology* 149: 5599–5609, 2008)

DNA METHYLATION IS a major epigenetic mechanism that control developmental gene expression. Coordinated waves of demethylation and *de novo* methylation establish the genome-wide methylation pattern during embryogenesis, and function to maintain cellular phenotypes through clonal inheritance of spatiotemporal expression of key developmental genes (1). Mammalian cytosine DNA methyl transferases (Dnmts) are divided into two groups based on their preferred DNA substrate. Dnmt1 copies the methylation pattern during DNA replication. In concordance with this, Dnmt1 is expressed constitutively in proliferating cells and is located at the replicating foci during S-phase (2). *De novo* methylation is carried out by the DNA methyl transferases, Dnmt3a and Dnmt3b. These factors are highly expressed in the developing mouse embryo in which they carry out *de novo* methylation after implantation (3). Genetic manipulation of the *Dnmt* genes has demonstrated that appropriate DNA methylation is required for normal mammalian development (4).

Steroidogenic factor 1 (SF1; also called adrenal-4 binding protein and officially designated NR5A1) is a nuclear receptor encoded by the *Fushi tarazu factor-1 gene* (*Ftz-F1*; for simplicity we refer to the gene as *SF1/Sf1*). SF1 plays fundamental roles in the development and function of steroidogenic organs, and targeted deletion of the *Sf1* gene causes adrenal and gonadal agenesis and nearly immediate postnatal death due to respiratory distress caused by glucocorticoid deficiency (5). During mouse embryogenesis, SF1 is expressed from embryonic day (E) 9.0 in the adrenogonadal primordium, a population of cells that arises from the coelemic epithelium of the urogenital ridge (6). The adrenal and gonadal anlagen progressively individualize and are separate structures at E12.5. The adrenal cells form the outer cortex with invasion by migratory neural crest cells that form the inner adrenal medulla by E16–E16.5. SF1 expression is confined to the cortical region of the adrenal gland (7). In this location, it is maintained postnatally in which it transactivates the genes encoding cytochrome P450 steroid hydroxylases, the melanocortin type 2 receptor, which binds ACTH, cholesterol transporters, and other genes responsible for the steroidogenic phenotype (8). SF1 is expressed in the bipotential gonad. In the developing testis, it is maintained in the

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Abbreviations: A2780, Human ovarian carcinoma cells; ChIP, chromatin immunoprecipitation; Dnmt, DNA methyl transferase; E, embryonic day; HeLa, human cervix epitheloid carcinoma; Hepa, hepatoma liver cells; H295R, human adrenocortical carcinoma cells; HEK-293, human embryonic kidney cells; HRP, horseradish peroxidase; Luc, luciferase; MA-10, mouse Leydig tumor cells; MeCP2, methyl-CpG-binding domain protein 2; MSC-1, mouse Sertoli cells; NIH-3T3, mouse embryonic fibroblast cells; polII, RNA polymerase II; SDS, sodium dodecyl sulfate; SF1, steroidogenic factor 1; SMAT-1, mouse prepubertal Sertoli cells; USF, upstream stimulatory factor; VMH, ventromedial hypothalamic nucleus.

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Leydig cells, in which it is essential for male sexual differentiation due to its role, as in the adrenal cortex, in regulating expression of the cytochrome P450 steroid hydroxylases among other genes responsible for testosterone synthesis. In Sertoli cells, SF1 regulates expression of the *Müllerian inhibiting substance* (*MIS*) and *insulin-like factor 3* (*Insl-3*) genes. After sex determination, SF1 is gradually down-regulated in the ovary from E12.5 and reappears postnatally in theca and granulosa cells and in the corpus luteum once folliculogenesis commences (9). SF1 is also expressed in the gonadotrophs of the pituitary, ventromedial hypothalamic nucleus (VMH), and spleen (8). A corresponding expression pattern of SF1 is observed in human fetal and adult tissues (10, 11).

Several transcription factors have been implicated in the regulation of the *SF1/Sf1* gene: Sp1 (stimulatory protein-1) (12, 13), GATA4 (14), Sox9 (SRY-type high-mobility-group-box protein 9) (15), WT-1 (Wilms tumor suppressor) (16), Lhx-9 (lim homeobox gene-9) (17) and Cited2 (CBP/p300 interacting transactivator with ED-rich tail-2) (18). In transient transfection experiments, expression also depends on a conserved E-box motif in the proximal promoter (13, 19, 20), bound with different consequences by two basic helix-loop-helix factors. Upstream stimulatory factor (USF) family members activate transcription (21–23), whereas Pod-1 seemingly represses SF1 expression (24). In *Pod-1* knockout mice, SF1 is expressed ectopically in cells that would normally have expressed Pod-1, supporting a repressive role of this factor (25). In the last few years, two studies using transgenic mouse models have identified enhancer elements regulating SF1 expression in a tissue-specific fashion. One of these studies identified a fetal adrenal-specific enhancer in intron 4 (26). Transcription from this intronic enhancer is initiated by the binding of heterodimeric complexes of Pbx1/Prep1 and Pbx1/Hox. Subsequently when the expression of SF1 is established, SF1 acts back on fetal adrenal-specific enhancer in a positive autoregulatory manner (26). Furthermore, the same group has also detected a VMH-specific enhancer in intron 6 (27). Despite these advances, we are only starting to reveal the mechanisms underlying tissue-specific expression of SF1, and little attention has focused on potential epigenetic regulation of this gene.

In this study we demonstrate that the proximal promoter of the *SF1/Sf1* gene is subject to DNA methylation. It is hypomethylated in cells and tissues that express SF1 but hypermethylated in nonexpressing cells and tissues, indicating that epigenetic mechanisms contribute to the restricted expression of this factor. Analyses of fetal tissues indicate that the DNA methylation pattern is established at an early embryonic stage in structures that are determined to express SF1 and faithfully conserved during development.

Materials and Methods

In vitro methylation of reporter plasmids

A fragment spanning −185/+141 bp (*Apa*I/*Eco*RI) of the mouse *Sf1* gene was inserted upstream of luciferase (lacking a minimal heterologous promoter) to create the reporter plasmid *Sf1* (−185/+141)/luciferase (Luc). *Sf1* (−185/+141)/Luc was methylated *in vitro* by using the *Sss*I CpG methylase (New England Biolabs Inc., Beverly, MA).

Cell culture

Mouse adrenocortical tumor cells (Y1), human cervix epitheloid carcinoma cells (HeLa), mouse Sertoli cells (MSC-1), and mouse hepatoma liver cells (Hepa-c1c6) were cultured in DMEM (high glucose) supplemented with 10% fetal calf serum. Human ovarian carcinoma cells (A2780) were maintained in DMEM (low glucose) supplemented with 10% fetal calf serum, and L-glutamine. Human adrenocortical carcinoma cells (H295R) were cultured in a 1:1 mixture of DMEM (high glucose): HAM F12 supplemented with 2% ITS+ (Collaborative Research, Bedford, MA) and 2% Nu-Serum (Collaborative Research). Mouse prepubertal Sertoli cells (SMAT-1) were grown in DMEM (high glucose), 10% fetal calf serum, and amino acids. Human embryonic kidney cells (HEK-293 EBNA) were cultured in DMEM (high glucose), L-glutamine, 10% fetal calf serum, and 25 mM HEPES. Mouse embryonic fibroblast cells (NIH-3T3) were maintained in DMEM (high glucose), 10% fetal calf serum, and 25 mM HEPES. Mouse Leydig tumor cells (MA-10) were cultured in a 1:1 mixture of DMEM (high glucose):HAM F12, 15% horse serum, 20 mm HEPES, and 40 μg/ml gentamicin. All cells were maintained in 5% CO_2 humidified atmosphere at 37 C, and growth medium also contained penicillin (100 U/ml) and streptomycin (100 μg/ml) (except for MA-10 cells).

Transfection of cells and luciferase assay

Cells were plated in 12-well plates and transiently transfected the following day using Superfect (QIAGEN, Valencia, Sweden). Cells were transfected with reporter plasmid (*Sf1*(−185/+141)/Luc, 300 ng/well) and a plasmid encoding β-galactosidase (pCMV5/LacZ, 100 ng/well) to control for transfection efficiency. Twenty-four hours later, the cells were lysed in luciferase buffer [10 mM Tris-HCl (pH 8), 4 mM EDTA, 150 mM NaCl, 0.65% Nonidet P-40], and total cell extracts were analyzed for luciferase and β-gal activity on a LUCY-3 luminometer (Anthos, Salzburg, Austria) using the luciferase assay kit from BIO Thema AB (Dalarö, Sweden).

Immunoblotting

Total cell extracts were separated by SDS-PAGE and blotted to nitrocellulose membranes. The membranes were incubated with 6% milk/primary antibodies/secondary antibodies, each incubation step performed for 1 h at room temperature. Antibody dilutions were 1:1000 for the anti-SF1 antibody [06-431; Upstate Biotechnology, Lake Placid, NY; 28740 (H-60) from Santa Cruz Biotechnology (Santa Cruz, CA) for verification of SF1 in HeLa cells], whereas anti-β-actin (ab-6276; Abcam, Cambridge, UK) and secondary horseradish peroxidase (HRP)-conjugated antibodies were used at 1:10000 [antimouse conjugated to HRP was from Santa Cruz Biotechnology; antirabbit HRP conjugated antibody was from Pierce (Rockford, IL)]. Chemiluminescence signals were developed by using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology).

Genomic DNA preparation

Genomic DNA from cell lines, paraffin sections, and human or mouse tissue was isolated using the QIAGEN DNeasy kit or with a standard protocol using proteinase K. Mouse embryonic tissues were pooled before DNA preparation. Mice used in this study were of C57/BL6 background. Ethical approval, collection, and staging of human fetal material were carried out as described previously, using the Carnegie classification and fetal foot length to provide a direct assessment of gestational age as weeks after conception (28).

DNA methylation analysis by HpaII/MspI digestion

Genomic DNA (250 ng) from human and murine cell lines were digested with *Hpa*II (methylation sensitive restriction enzyme, recognizing 5′-CCGG-3′) or its isoschizomer *Msp*I (methylation insensitive). All samples were codigested with *Nco*I (digests outside SF1 and control PCR targets) to aid digestion and PCR. Digested samples were subjected to PCR amplification using appropriate primers (see Table 1) and resolved on a 2 or 3% agarose gel. Selected regions with no *Hpa*II/*Msp*I restriction sites of human IGF-II exon 9 or mouse β-globin genes were used as internal controls in the PCRs.

TABLE 1. Overview of the PCR primers used in this study

Species	Application	Target region		Primer sequence (5′)
Human	*Hpa*II-dig PCR	SF1	F	CCACCCTCAGCCCCCAGATA
			R	GGGGGTAGCATGGTGGGAGG
		IGF-II	F	CTTGGACTTTGAGTCAAATTGGCC
			R	GAGGGTCGTGCCAATTACATTTCA
	Bisulfite PCR (nested)	SF1 outer	F	AAAAAAAACTAACCATTAAAAACCTAAAC
			R	TTTTTATTTTTAGTTTTTAGATAGATAGGGGTATT
		SF1 inner	F	GTAAATGAAGAGAAATATTAATAAAGAAGG
			R	AAAAATAACAATAAACACCAAAAATCC
Mouse	*Hpa*II-dig PCR	SF1	F	CCACCCTCAGCCCCCAGATA
			R	GGGGGTAGCATGGTGGGAGG
		β-Globin	F	ACACAGGATAGAGAGGGCAGGAG
			R	GTCTGTTTCTGGGGTTGTGAGTC
	Bisulfite PCR (nested)	SF1 outer	F	TAGTGTTTTGGTTTTTGTTTTTATTTAGG
			R	AATAAAAAAAATAAATCTAAAAAAACCCCTAAC
		SF1 inner	F	TGAAGAGAAATATTAATAAAGGAGGAGAAAG
			R	AAAAATAAACACTAAACACCAAAATCCTAAT
	qChIP	SF1 promoter	F	CCAAATGAAGAGAAACACCAACAAAG
			R	GCTAGCGGGCTCTCAGAAACTT
		SF1 upstream	F	CTTGGGATTGGTACAGGTAGTGAGG
			R	AGCCTTCTCTCAAACTTCAAACTCCT

F, Forward; R, reverse; dig, digestion; qChIP, quantitative ChIP.

Bisulfite sequencing

Genomic DNA (100–500 ng) was subjected to sodium bisulfite modification with the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. PCR amplification was carried out using primers specific for bisulfite-converted DNA sequence of the selected gene area to target either mouse or human origin (see Table 1). The amplified PCR product was subcloned into the pGEM-T-easy vector (Promega, Madison, WI) and subsequently sequenced and analyzed. Only PCR clones with at least 95% C-to-T conversion outside CpG sites were analyzed further.

Laser dissection from paraffin embedded tissues

Laser dissections were performed with a system comprised by an inverted microscope (Axiovert 200; Zeiss, New York, NY) and an air-cooled nitrogen laser (model VSL-337 ND-S) from P.A.L.M. Microlaser Technologies GmbH (Munich, Germany). Microdissection was performed on 7-μm-thick paraffin sections. Paraffin embedding and sectioning were performed according to standard protocols.

Chromatin immunoprecipitation (ChIP) assay

Cells were grown to 95% confluence on a 500-cm^2 plate. The cells were rinsed with PBS and fixed in 1% formaldehyde at room temperature for 10 min, rinsed again in PBS, and collected into 100 mM Tris-HCl (pH 8.7), 10 mM dithiothreitol by scraping. Cells were collected by centrifugation and sequentially washed in ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)]. Nuclei were resuspended in lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1× protease inhibitor cocktail (Roche, Indianapolis, IN)] and sonicated to an average length of 400–800 bp. The lysates were centrifuged at 14,000 rpm for 10 min and the supernatant diluted 1:10 in dilution buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), 1× protease inhibitor cocktail (Roche)]. One milliliter of the chromatin solution was precleared with the addition of recombinant protein G agarose (Invitrogen, Carlsbad, CA), sheared herring sperm DNA (2 μg), and normal rabbit IgG (sc-2027; Santa Cruz). Precleared chromatin was then incubated with 5 μg of specific antibody or normal rabbit IgG and rotated at 4 C overnight. Polyclonal antibodies for the ChIP experiment were against USF2 (N-18; Santa Cruz), methyl-CpG-binding domain protein 2 (MeCP2; ab2828; Abcam), Dnmt3a (H-295; Santa Cruz), and RNA polymerase II (ab5131, Abcam). The beads were harvested by centrifugation at 3000 rpm for 15 sec and washed sequentially for 20 min with the following buffers: low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1)], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl (pH 8.1)] and buffer III [1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 0.25 M LiCl, 10 mM Tris-HCl (pH 8.1)] and four times with Tris/EDTA buffer. Immunoprecipitates were eluted three times with 100 μl 1% SDS and 0.1 M $NaHCO_3$, and the eluates were pooled. After dilution, 100 μl of the supernatant were saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Reverse cross-linking of samples was performed by incubation at 65 C overnight. After 1 h proteinase K digestion, samples were purified using QIAquick PCR purification kit (QIAGEN), resuspended in 40 μl of elution buffer [10 mM Tris-Cl (pH 8.5)]. Subsequently quantitative PCRs were performed with 1 μl of immunoprecipitate and serial dilution of input material by using the iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA) with primers spanning the SF1 proximal promoter area (−128/+70) or an upstream region (−8500/−8305; see Table 1 for primers). The quantitative PCR was performed on an iQ5 real-time PCR detection system (Bio-Rad).

Results

The proximal promoter contains CpG sites

The sequences analyzed in this study correspond to the regions spanning −129/+122 of the human *SF1* gene and −123/+125 of the mouse *Sf1* gene containing the proximal promoter region and part of exon 1 (Fig. 1, A and B; for simplicity, these regions will hereafter be referred to as the proximal promoter). The proximal promoter is conserved among species, contains binding sites for several transcription factors, and can promote reporter gene activity *in vitro* (12, 13). Sequence analyses of the selected region revealed 14 putative CpG sites in the human sequence and 10 in the corresponding mouse sequence. Many of the sites were conserved between the two species including one that coincided with the E-box motif (Fig. 1, A and B). Notably, according to the *cpgplot* program (www.ebi.ac.uk/emboss/cpgplot), the mouse and human proximal promoter does not reside within CpG islands. [A CpG island is frequently defined as a region of DNA of greater than 500 bp with a G+C equal to or greater than 55% and observed CpG/expected CpG of 0.65 (29)].

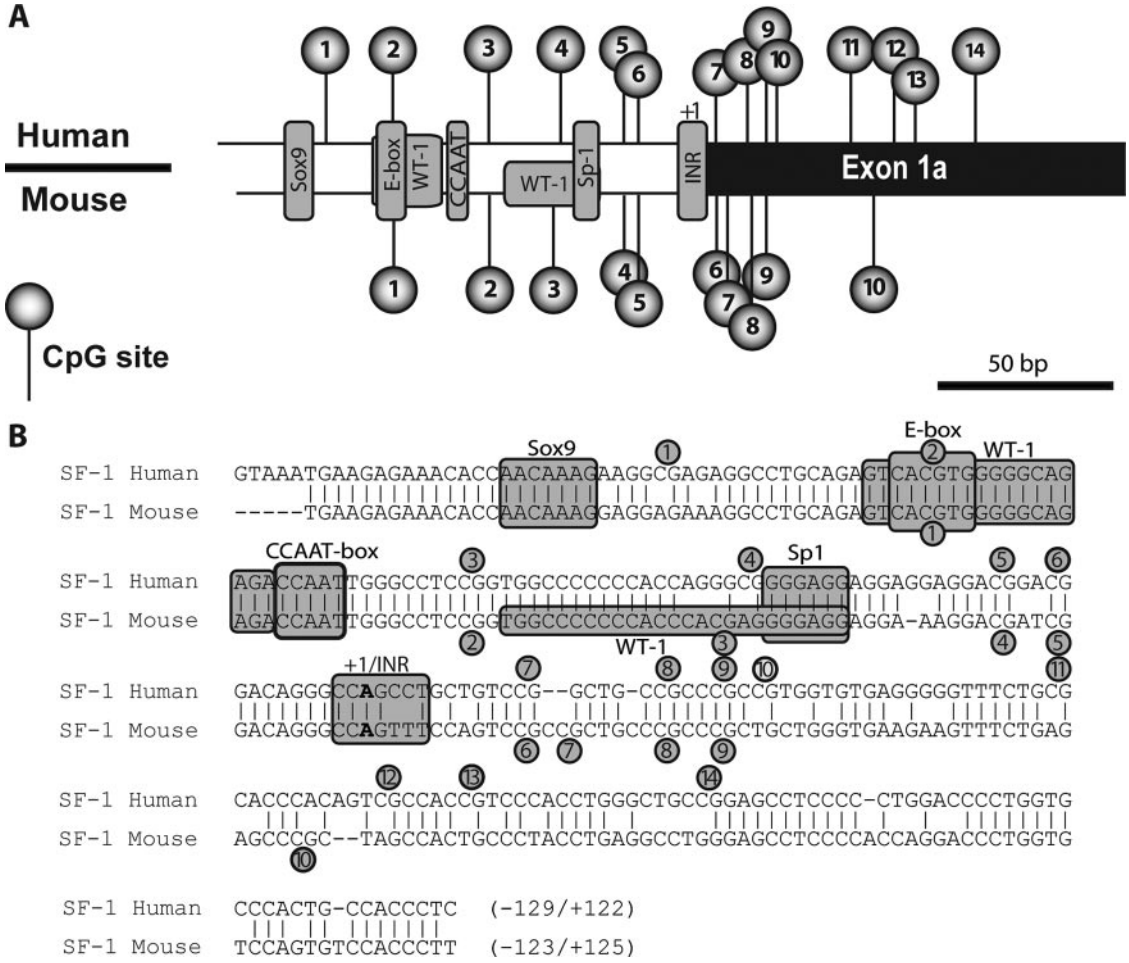

FIG. 1. Overview of the CpG sites in the proximal promoter. A, Overview of CpG sites (indicated as *lollipops*) in the proximal promoters of the human (*top*) and mouse (*bottom*) *SF1/Sf1* genes. The regions shown correspond to the region spanning −129/+122 in the human gene and the region spanning −123/+125 in the mouse gene. The positions of DNA regulatory elements present in the proximal promoters are indicated (12–16, 19, 20). A putative INR element (46) is present at the start site of transcription (+1) (47, 48). B, Alignment of the human and mouse *SF1/Sf1* genes over the region selected for bisulfite sequencing. The CpG sites are indicated as *circles*, and the *numbering* corresponds to that in A. As also shown in A, the positions of functional DNA-regulatory elements are indicated.

The proximal promoter is inactive when methylated

To investigate whether the activity of the proximal promoter is dependent on methylation status, we constructed a reporter gene plasmid in which the fragment spanning −185/+141 of the mouse *Sf1* gene was inserted upstream of luciferase [*Sf1*(−185/+141)/Luc]. The plasmid was propagated in the *Escherichia coli* strain DH5α (which does not contain CpG methylases) and subjected to *in vitro* methylation using the *Sss*I CpG methylase. To verify that the original reporter plasmid was indeed unmethylated and that *in vitro* methylation had occurred, the plasmids were digested with *Hpa*II, which is sensitive to methylation, and with its isoschizomer *Msp*I, which is methylation insensitive (see Fig. 2A for *Hpa*II/*Msp*I sites). As demonstrated in Fig. 2B, *Hpa*II readily digested unmethylated *Sf1* (−185/+141)/Luc (Fig. 2B, *left panel*), whereas it did not digest the methylated plasmid (Fig. 2B, *right panel*). As expected, *Msp*I acted similarly on both methylated and unmethylated plasmid (Fig. 2B). To correlate methylation status with reporter gene activity, unmethylated and *in vitro* methylated *Sf1* (−185/+141)/Luc were transfected into the steroidogenic cell lines H295R (human adrenocortical tumor cell line) and Y1 (mouse adrenocortical tumor cell line). As expected, based on the fact that these cell lines express high levels of SF1, *Sf1* (−185/+141)/Luc gave rise to relatively high luciferase activity when untreated with *Sss*I CpG methylase (Fig. 2C). *In vitro* methylation resulted in a 95.4 and 99.5% repression of luciferase activity in H295R and Y1 cells, respectively (Fig. 2C). These experiments thus suggest that the proximal promoter of the *Sf1* gene is inactive when methylated.

The proximal promoter is hypomethylated in cells and tissues that express SF1

To determine whether the methylation status of the proximal promoter corresponded to the expression profiles of SF1 in various cell lines of mouse and human origin, genomic DNA was isolated and digested with *Hpa*II/*Mse*I followed by PCR (see Fig. 2A for *Hpa*II/*Msp*I sites and positions of PCR primers). The presence of a band on gel electrophoresis after PCR indicates that *Hpa*II is unable to digest the template

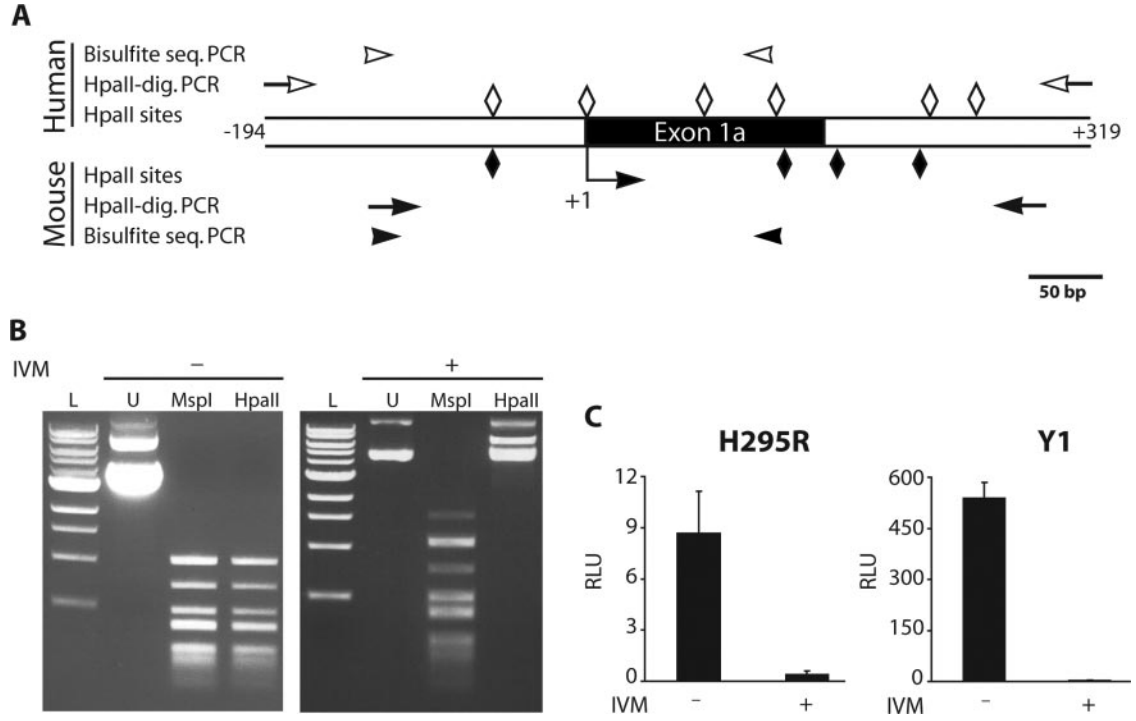

FIG. 2. *In vitro* methylation inhibits transcription from the proximal promoter. A and C, Overview of the positions of the primers used for the *Hpa*II/*Msp*I digestion-based methylation analyses (*arrows*) and the bisulfite sequencing (*arrowheads*). The *Hpa*II sites are indicated as *diamonds* (six sites in the human promoter, four sites in the mouse promoter). *Open symbols* (top of gene diagram) indicate positions of primers and *Hpa*II sites in the human gene, and *closed symbols* (below gene diagram) denote *Hpa*II sites and primers for the mouse gene. The transcriptional start site is indicated as +1 (*bent arrow*). B, *Sf1* (−185/+141)/Luc was left untreated or methylated *in vitro* by the methyltransferase *Sss*I and the methylation status confirmed by *Hpa*II/*Msp*I digestion. IVM, *In vitro* methylated; L, 1-kb DNA ladder; U, undigested *Sf1* (−185/+141)/Luc; *Msp*I, *Msp*I-digested *Sf1* (−185/+141)/Luc; *Hpa*II, *Hpa*II-digested *Sf1* (−185/+141)/Luc). C, H295R and Y1 cells were transfected with *Sf1* (−185/+141)/Luc (300 ng) or the same plasmid that had been methylated *in vitro* (IVM) (300 ng). Luciferase activity was determined 24 h after transfection and normalized against β-gal activity from LacZ/pCMV (100 ng) that was included in all transfection experiments (n = 6). The experiment was repeated with different batches of *in vitro* methylated *Sf1* (−185/+141)/Luc, all giving the same result.

DNA and hence that it is methylated. As demonstrated in Fig. 3A, the proximal promoter was unmethylated (*i.e.* no PCR product was generated after *Hpa*II digestion) in human (Fig. 3A, *left panel*) and murine (Fig. 3A, *right panel*) cells of steroidogenic origin (*i.e.* adrenocortical H295R and Y1 cells and the mouse Leydig cell line, MA10). Furthermore, it was unmethylated in the murine Sertoli-derived cell lines SMAT-1 and MSC-1 (Fig. 3A, *right panel*). In contrast, PCR on

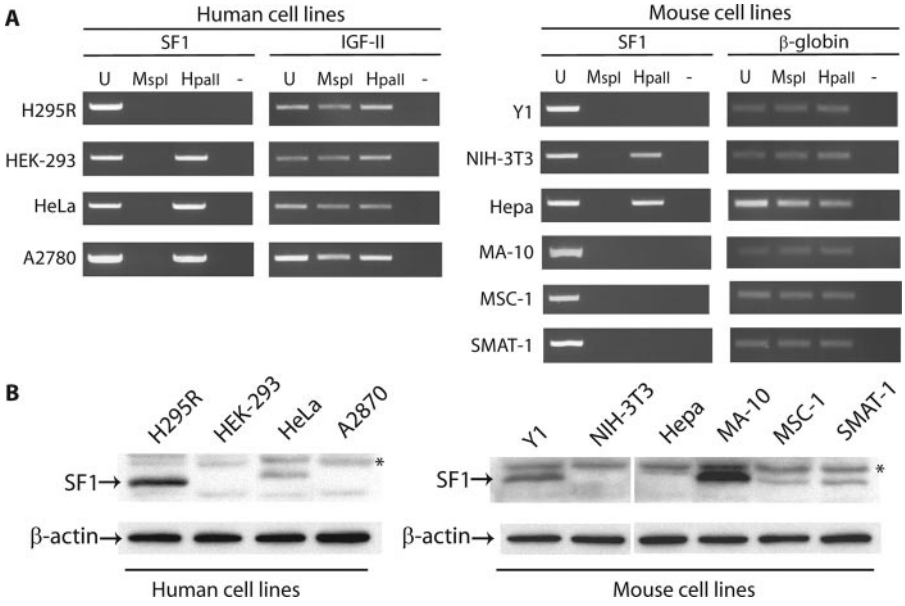

FIG. 3. DNA methylation analyses of the proximal promoter by *Hpa*II digestion. A, Genomic DNA from cell lines of human (*left panel*) and murine (*right panel*) origin was submitted to *Hpa*II/*Msp*I digestion followed by PCR. (Please see *Materials and Methods* for origin of cell lines.) Selected regions of the human IGF-II and mouse β-globin genes (containing no *Hpa*II sites) were used as controls. U, Undigested DNA; MspI, *Msp*I digested DNA; *Hpa*II, *Hpa*II digested DNA; −, no DNA. Representative data from two or three independent sample preparations for each cell line are shown. PCR analyses were performed three times for each sample, and full reproducibility was observed. B, Total cell lysates was prepared from the human (*left panel*) and mouse (*right panel*) cell lines and subjected to Western blot analyses using an anti-SF1 antibody. Equal protein loading was verified using an anti-β-actin antibody. The experiments were performed three times with similar results and representative blots are shown. *, Unspecific band.

*Hpa*II-treated DNA isolated from HEK-293 cells, HeLa cells (human cervix epithelial carcinoma), A2780 cells (human epithelial ovarian carcinoma), NIH-3T3 cells (mouse embryonic fibroblasts), and Hepa cells (mouse hepatoma cells) generated PCR products indistinguishable from undigested DNA. Thus, the proximal promoter was methylated in these cell lines (Fig. 3A, *left* and *right panels*). By immunoblotting, SF1 was detected in cells with hypomethylated proximal promoter (Fig. 3B). In contrast, a hypermethylated proximal promoter correlated with lack of SF1 expression with one exception: HeLa cells expressed low levels of SF1 (Fig. 3B, *left panel*). The presence of SF1 in HeLa cells was confirmed by an antibody raised against a different epitope (data not shown). The band corresponding to SF1 migrated at a slightly higher molecular weight than observed for H295R cells. The reason for this is unknown. Searching the literature, we have not found other reports describing the expression of SF1 in HeLa cells. However, SF1 is expressed in the uterus (30) that, like the cervix, originates from the Müllerian ducts.

To investigate the precise methylation status of each individual putative CpG site, we performed bisulfite sequencing on genomic DNA isolated from the different cell lines. In concordance with the results in Fig. 3, practically all potential sites in the human (Fig. 4A) and mouse (Fig. 4B) proximal

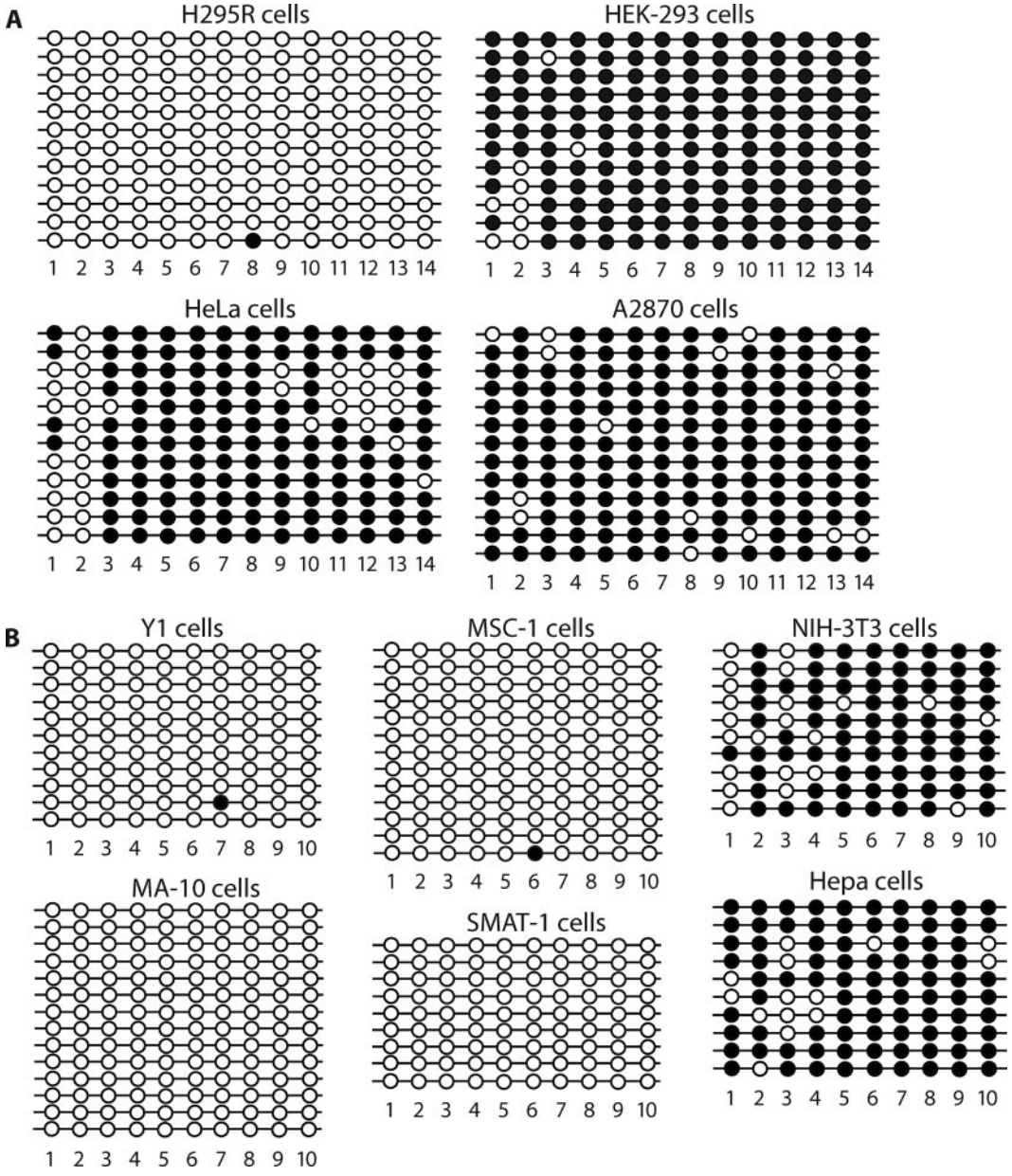

FIG. 4. Site-specific CpG methylation analyses of the proximal promoter in cell lines. Bisulfite sequencing was performed on DNA isolated from the human (A) and murine (B) cell lines used in Fig. 3. The analysis covered 14 CpG sites (in the region spanning −129/+122) in the human gene and 10 CpG sites (in the region spanning −123/+125) in the mouse gene. *Filled circles* indicate methylated CpG sites, and *open circles* represent unmethylated CpG-sites. *Horizontal lines* represent individual analyzed clones. The CpG sites are *numbered* corresponding to the overview in Fig. 1.

promoter were unmethylated in cell lines that were sensitive to *Hpa*II digestion and that expressed SF1 (*i.e.* H295R, Y1, MA-10, SMAT-1 and MSC-1 cells). Similarly, the proximal promoter was hypermethylated in cells that did not express SF1 (*i.e.* HEK-293, A2870, NIH-3T3, and HeLa cells). Despite the apparent presence of SF1 protein in Hela cells, the proximal promoter was heavily methylated (Fig. 4A), in agreement with the *Hpa*II digestion analysis in Fig. 3A. However, interestingly, all clones examined from this cell line were demethylated at the CpG site at position 2 (Fig. 4A), located within the E-box of the human proximal promoter (Fig. 1B).

Cell lines, especially those of tumorigenic origin, can exhibit aberrant methylation patterns that are unrepresentative of the normal tissues from which they originate. Therefore, we examined the methylation status of the proximal promoter in adult mouse tissues. The adrenal gland consists of a medulla and cortex, with only the cortical cells expressing SF1. Thus, genomic DNA isolated from the whole gland yielded clones that were either completely unmethylated or completely methylated (Fig. 5A). Laser capture microdissection separated the cortex and medulla and established that genomic DNA isolated from the cortex was responsible for the unmethylated clones, whereas DNA from the medulla was completely methylated (Fig. 5A). The capsule and subcapsular areas were avoided in the laser capture microdissection. Thus, capsular cells that are devoid of SF1 (31) and cells in the subcapsular area that have been suggested to lack SF1 protein (7) were not included in our analyses. In the adult mouse ovary, SF1 is expressed in both granulosa and theca cells as well as the corpus luteum (32). In agreement with this expression pattern, we found 11 of 13 clones generated from the ovary to be almost completely demethylated across the proximal promoter (Fig. 5B, *left panel*). In the mouse adult testis, SF1 expression is confined to Leydig and Sertoli cells and is not expressed in germ cells or peritubular myoid cells (31). In the adult rodent testis, Leydig and Sertoli cells constitute approximately 10 and 8% of the total cell number, respectively, whereas spermatogonia, spermatocytes, and spermatids account for approximately 70% (33). When bisulfite sequencing was performed on adult mouse testis, it became evident that approximately 24% of the CpG sites were methylated (Fig. 5B, *middle panel*), which would suggest that

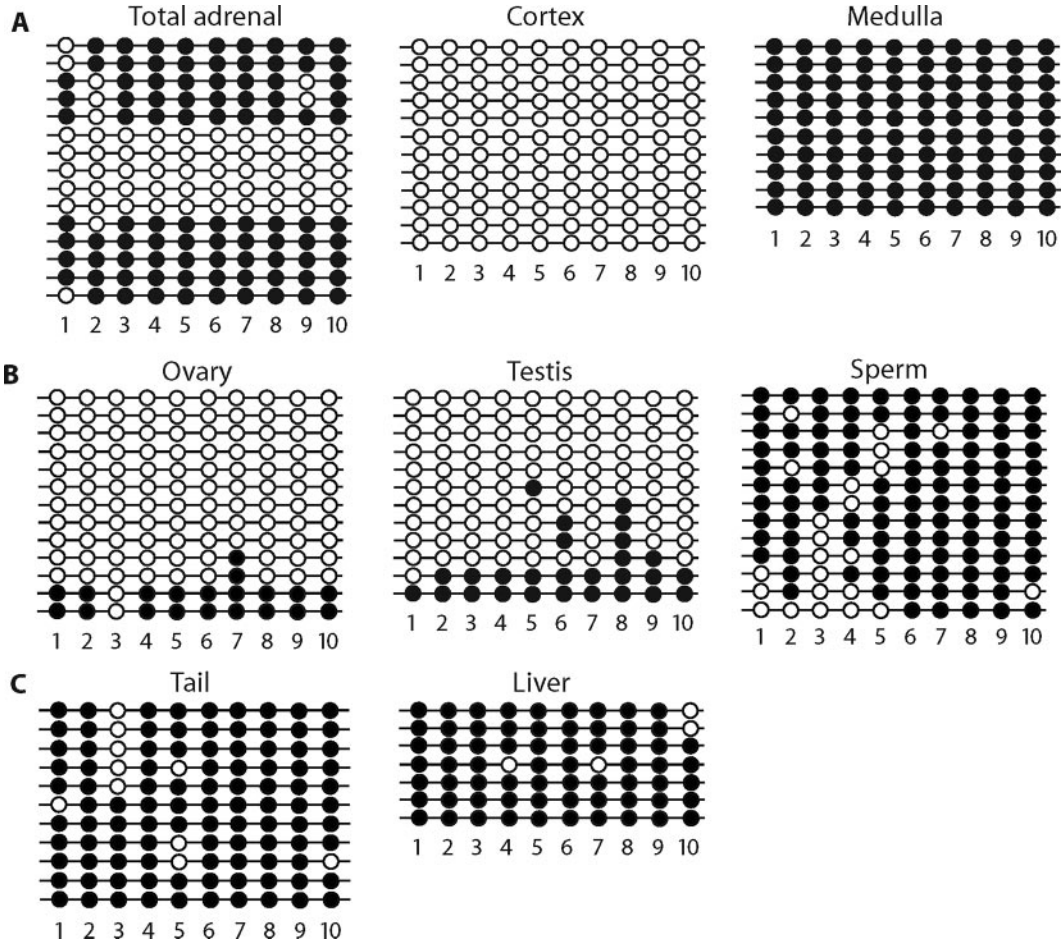

FIG. 5. Site-specific CpG methylation analyses of the proximal promoter in adult mouse tissue. A, B, and C, The analysis covered 10 CpG sites (in the region spanning −123/+125) in the mouse *Sf1* gene. CpG sites are numbered corresponding to overview in Fig. 1. *Filled circles* indicate methylated CpG-sites, and *open circles* represent unmethylated CpG sites. A, Paraffin sections of mouse adrenal glands were subjected to laser capture microdissection to isolate cortical and medullary cells. Bisulfite sequencing was performed on whole sections (total adrenal; *left panel*) or isolated cortical cells (cortex; *middle panel*) and medullary cells (medulla; *right panel*). B, Bisulfite sequencing on DNA isolated from mouse ovary (*left panel*), testis (*middle panel*), and mature sperm (*right panel*). C, Bisulfite sequencing on DNA isolated from tail (*left panel*) and liver (*right panel*). *Horizontal lines* represent individual analyzed clones. CpG sites are *numbered* corresponding to the overview in Fig. 1.

the proximal promoter is demethylated in a greater number of cells than could be accounted for by the Leydig and Sertoli cell lineages. (In total 56 PCR clones, generated from four testes, were analyzed; mean 24 ± 8%; one representative experiment is shown in Fig. 5B, *middle panel*.) Probably, demethylated promoter is present in cells of the germ cell lineage because primordial germ cells become demethylated around E10.5 and E13.5 and is not completely remethylated until the pachytene stage (34). As expected, nearly complete methylation was apparent in mature sperm (Fig. 5B, *right panel*). The proximal promoter was hypermethylated in genomic clones derived from the tail and liver (Fig. 5C). Taken together, these results indicate that the methylation status of the proximal promoter strongly correlates with the expression of the protein in both adult mouse tissues and cell lines.

Methylation of the proximal promoter during development

As sexual differentiation occurs, SF1 expression is maintained in the developing testis but declines to undetectable levels in the ovary (9). To determine whether this differential expression is reflected in the methylation status, we performed bisulfite sequencing on DNA isolated from murine gonads at E13.5 and 16.5 (Fig. 6A). The proximal promoter was virtually completely demethylated in both sexes at both stages (Fig. 6A). The lack of methylated clones in the developing testis is in agreement with the fact that SF1-expressing cells constitute the majority of the testicular cells and that the germ cells contain mainly demethylated DNA at these developmental stages (34, 35). Because SF1 expression is undetectable in the ovary at these time points, our results would indicate that the *Sf1* gene is demethylated in the proximal promoter early in development and that it remains in this state in cells programmed to express SF1 at later stages. As expected, the proximal promoter was heavily methylated in the developing liver (Fig. 6B). A similar pattern was observed in the kidney at E13.5. However, at E16.5, a number of putative CpG sites were demethylated in the kidney, particularly in the more distal part of the proximal promoter region (Fig. 6B). Neither the developing nor adult kidney expresses SF1 (Refs. 30, 36 and Hanley, N., unpublished data). The observed level of demethylation is therefore unlikely to allow SF1 expression in this tissue.

During human development, as in the mouse, SF1 is expressed in the bipotential gonad and throughout testicular development (10). At 9 wk after conception, when sex cords have formed, the proximal promoter was essentially demethylated in the developing testis (Fig. 7). Interestingly, similarly to the mouse, DNA from human kidney also revealed relative low levels of methylation. SF1 is not expressed in the human kidney (Hanley, N., unpublished data), and at present it is not clear why the proximal promoter is partially demethylated in this organ during development.

The unmethylated proximal promoter recruits transcriptional activators. ChIP experiments were performed to determine whether the methylation status affected the recruitment of transcription factors to the proximal promoter. Occupation of three factors was analyzed: USF2, which has previously been demonstrated to interact with the unmethylated active proximal promoter (23); MeCP2, which interacts with methylated DNA (37); and RNA polymerase II (polII). As evident

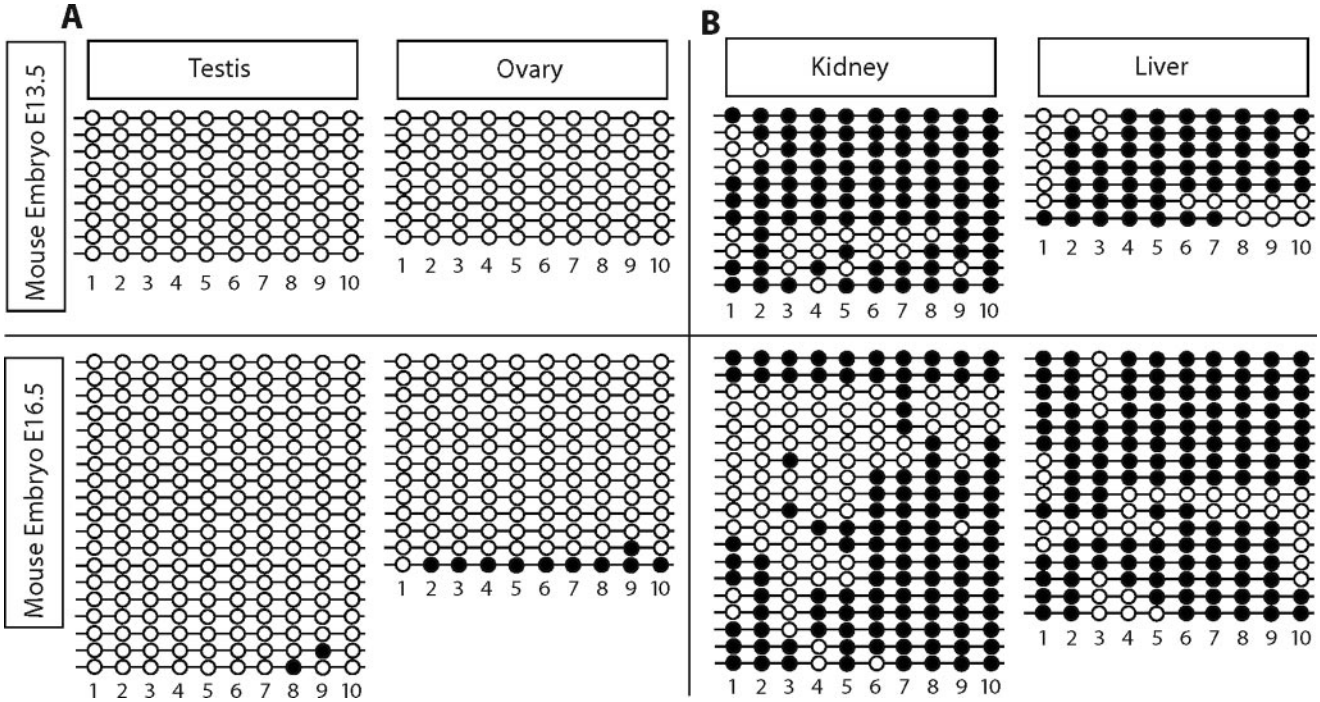

FIG. 6. Site-specific CpG methylation analyses of the proximal promoter in embryonic tissue. Bisulfite sequencing was performed on DNA isolated from mouse testes, ovaries, kidney, and liver at E13.5 and E16.5 as indicated. The analysis covered 10 CpG sites (in the region spanning −123/+125) of the *Sf1* gene. *Filled circles* indicate methylated CpG sites, and *open circles* represent unmethylated CpG sites. *Horizontal lines* represent individual analyzed clones. CpG sites are *numbered* corresponding to the overview in Fig. 1.

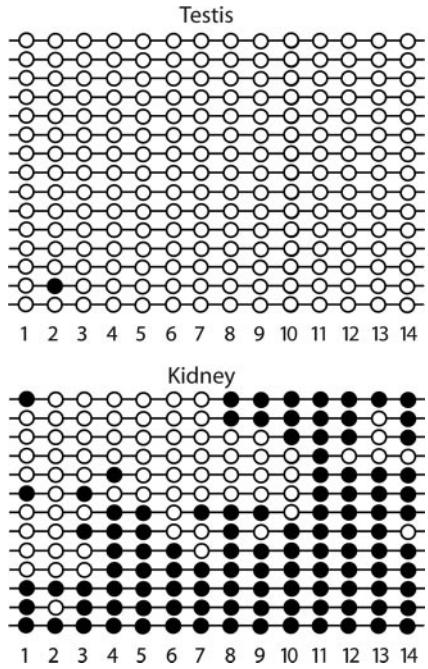

FIG. 7. Site-specific CpG methylation analyses of the proximal promoter in fetal human tissue. Bisulfite sequencing was performed on DNA isolated from the testis and kidney from a human embryo (8 mm foot length, around 9 wk of development). *Filled circles* indicate methylated CpG sites, and *open circles* represent unmethylated CpG sites. *Horizontal lines* represent individual analyzed clones. CpG sites are *numbered* corresponding to the overview in Fig. 1.

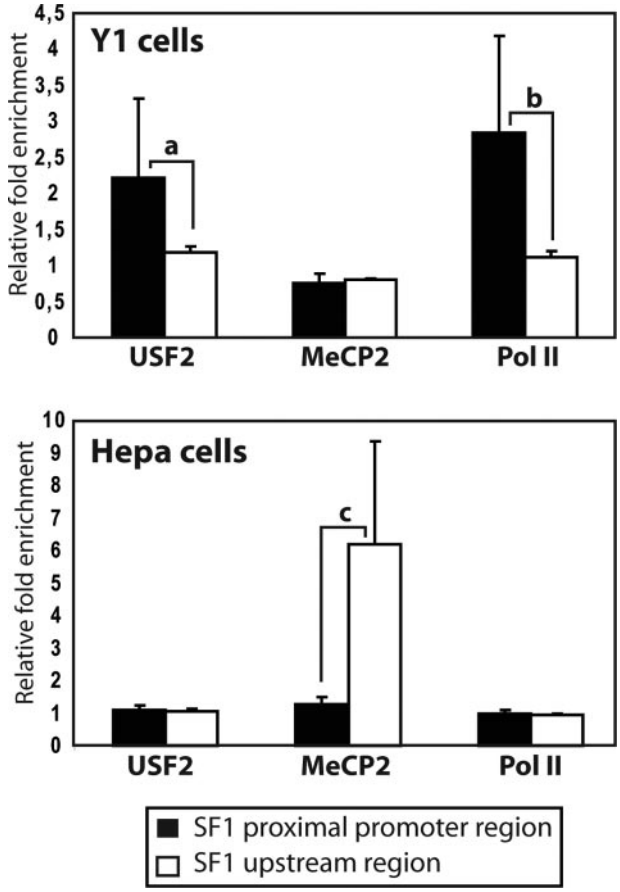

FIG. 8. Differential recruitment of transcriptional regulators to the proximal promoter, depending on methylation status. ChIP experiments were performed on Y1 and Hepa cell extracts using antibodies against USF2, MeCP2, and RNA polII as indicated. The quantitative PCR values were related to preimmune IgG control values and are presented as relative fold enrichment in relation to 0.1% input material. Statistical analyses were *t* tests with pooled variances. a, $P = 0.015$; b, $P = 0.005$; c, $P = 0.012$ (Y1: n = 9; Hepa: n = 6).

from the results presented in Fig. 8, the transcriptional activator USF2 was specifically located at the proximal promoter in Y1 cells, whereas no interaction above background levels were detected in Hepa cells. Moreover, antibodies against polII also specifically precipitated the proximal promoter from Y1 cells (Fig. 8), further supporting that the unmethylated status correlates with a transcriptionally active promoter. As expected, MeCP2 interacted only with *Sf1* in Hepa cells (Fig. 8, *lower panel*). We found that MeCP2 was recruited to a region upstream of the proximal promoter. This is partly in conflict with the study by Xue *et al.* (38), who suggested that MeCP2 interacts with the proximal promoter in SF1-negative endometrial stroma cells. The recruitment of MeCP2 might differ between nonexpressing cells, but we note that Xue *at al.* did not use upstream or downstream primer pairs, hindering a direct comparison of the two studies.

Discussion

Several studies have established that DNA methylation plays regulatory roles in developmental gene expression (39–43). We demonstrate a striking correlation between SF1 protein expression and methylation status of the proximal promoter, indicating that DNA methylation is a major mechanism to regulate cell and tissue-specific expression of SF1. Whereas our work was ongoing, Xue *et al.* (38) published a study that links methylation and SF1 protein expression in endometrial and endometriotic cells. In normal endometrial cells that do not express SF1, the proximal promoter was found to be hypermethylated, whereas DNA from endometriotic cells, which do express SF1, was hypomethylated over the same region (38). This study also revealed that MeCP2, which contributes to the repressed state of methylated DNA, is recruited to the proximal promoter in endometrial cells (38). Thus, together, these studies demonstrate that methylation of the proximal promoter directs SF1 expression in both normal tissues and diseased tissue exhibiting aberrant levels of SF1 protein.

We observed low, but consistent, levels of SF1 in HeLa cells (Fig. 3B). Interestingly, all PCR-generated clones from HeLa cells were unmethylated at the CpG site located within the E-box (Fig. 4A). Because this DNA regulatory element has repeatedly been identified as crucial for SF1 expression, we speculate that the specific lack of methylation allows binding of a factor that can direct low levels of SF1 expression in HeLa cells, regardless of the overall hypermethylation of the proximal promoter. Of specific interest in this regard is a recent study suggesting that abnormal expression of USF2 and its interaction with the E-box partly accounts for the aberrant expression of SF1 in endometriosis (23). The demethylation/remethylation processes that occur in germ cells complicated

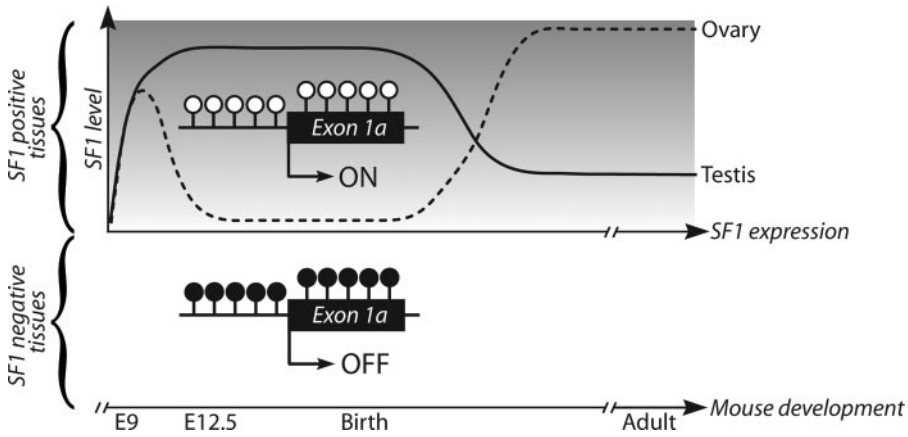

FIG. 9. Proposed mechanism for tissue-specific expression of SF1 by DNA methylation. In SF1-negative tissues, DNA methylation of the proximal promoter prevents expression of SF1. In contrast, the proximal promoter is hypomethylated in SF1-expressing tissues. We hypothesize that the methylation pattern is established at the earliest time point of SF1 expression (E9 in the mouse) and maintained thereafter in a clonal manner. Both cell lineages that express SF1 continuously and those that express SF1 periodically maintain a demethylated pattern. Various transcription factors presumably interact with the hypomethylated promoter, controlling the level of SF1 expression. Unmethylated CpG sites are indicated by *open lollipops*, whereas methylated CpG sites are shown as *filled lollipops*. Key time points are indicated. Expression profiles are based on a published report (49).

the establishment of a correlation between DNA methylation and SF1 expression in the testis. DNA methylation patterns are generally erased during gametogenesis, and complete remethylation in the male is not evident until the pachytene stage of meiosis (34). In line with this, our analyses of embryonic testis demonstrated complete demethylation of nearly all clones examined (Fig. 6), and furthermore, a far greater number of clones than would be accounted for by the Leydig and Sertoli lineages were demethylated in adult testes (Fig. 5B). To determine the methylation status of the proximal promoter in SF1 expressing *vs.* nonexpressing cells in the ovary and testis, we performed bisulfite sequencing on cells sorted from mice expressing enhanced green fluorescent protein under the control of 50 kb of the 5′ *Sf1* promoter region (44). However, these analyses demonstrated that the methylation pattern of the transgenic construct differs from that of the *Sf1* gene, and the presence of multiple copies of the transgenic construct in the SF1/enhanced green fluorescent protein mice impeded these analyses (Hoivik, E. A., M. Bakke, and K. L. Parker, unpublished results).

Although we did not analyze the methylation status at the earliest time point of SF1 expression in the adrenogonadal primordium (at E9.0 in mice), we hypothesize that the methylation pattern of the proximal promoter is established at this stage and that it is maintained thereafter. Thus, as depicted in Fig. 9, the proximal promoter will be demethylated in cells that are programed to express SF1 continuously or in an interrupted manner and methylated in other cells. In agreement with this idea, the down-regulation of SF1 levels that occurs in the ovary at the time of sexual differentiation is not associated with a change in methylation status (Fig. 6). Instead, the change in SF1 expression during ovarian development is more likely to be caused by the action of transcription factors (although we cannot exclude epigenetic modifications elsewhere in the gene). Genetic studies have identified several transcription factors that appear to affect SF1 expression in the developing mouse embryo. SF1 expression is reduced to a minimum in mice carrying a targeted deletion of *Lhx9* (17). Similarly, the knockout models for WT-1 (16) and the Pbx1 (45) exhibit undetectable levels of SF1, clearly indicating that these factors act upstream of SF1. Moreover, Pod1 most likely acts as a repressor of SF1 expression because loss of this factor leads to enhanced SF1 expression in the developing testis, followed by ectopic expression of SF1 and aberrant commitment of precursor cells to the steroidogenic lineage (25). Thus, a number of transcription factors, in addition to tissue-specific promoters (26, 27), and epigenetic regulatory mechanisms seemingly work side by side to control the time and cell-specific expression of SF1. Interestingly, *in silico* analyses suggest the presence of CpG islands within the human and mouse *SF1* genes. Typically, CpG islands are associated with regions that are involved in transcriptional regulation (1), and future experiments should answer whether these regions confer epigenetic regulation. According to our preliminary analyses, the previously identified VMH-specific intronic enhancer (26, 27) resides within a CpG island.

In conclusion, our results clearly indicate that epigenetic mechanisms control the expression of SF1 during embryonic development, in adult tissues, and in cell lines. In this study we have not investigated the mechanisms that direct the cell-specific methylation pattern of the proximal promoter. But because SF1 is a transcription factor associated with embryonal lineage commitment, the *SF1/Sf1* gene provides an interesting candidate for future studies that aim to reveal the mechanisms whereby CpG methylation contributes to cell type-specific gene expression during development.

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STEM CELLS®

Embryonic Stem Cells–Characterization Series: Concise Review

Evaluating Human Embryonic Germ Cells: Concord and Conflict as Pluripotent Stem Cells

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Key Words. Human • Embryonic germ cell • Primordial germ cell • Stem cell • Embryo • Gonad

ABSTRACT

The realization of cell replacement therapy derived from human pluripotent stem cells requires full knowledge of the starting cell types as well as their differentiated progeny. Alongside embryonic stem cells, embryonic germ cells (EGCs) are an alternative source of pluripotent stem cell. Since 1998, four groups have described the derivation of human EGCs. This review analyzes the progress on derivation, culture, and differentiation, drawing comparison with other pluripotent stem cell populations. STEM CELLS *2006; 24:212–220*

INTRODUCTION

In mammalian biology, two clear sources of untransformed pluripotent stem cell have been described (Fig. 1). The inner cell mass (ICM) of the early embryo gives rise to the derivatives of all three germ layers in the developing embryo. Taking the ICM into in vitro culture offers the opportunity to derive embryonic stem cells (ESCs). These cells, first attained from mouse embryos by Evans and Kaufman [1] and, independently, by Martin in 1981 [2], retain the ability for broad differentiation but also undergo self-renewal. As such, they are the pluripotent stem cell that has been the focus of most research. An alternative source of pluripotent cells arises later in development. Germ cells are the sole means of transmitting genetic information to the next generation in their ultimate form as haploid gametes, spermatozoa, and ova. However, before meiosis, these cells exist as diploid primordial germ cells (PGCs). PGCs share significant similarities to the cells of the ICM and, once taken into in vitro culture, can lead to the generation of embryonic germ cells (EGCs) (Fig. 1), the parallel of ESCs. The derivation of human EGCs (hEGCs) has been reported now by several groups worldwide [3–6]. This review brings together these experiences and compares their emerging biology with that of human ESCs (hESCs), beginning from the historical starting point of PGC-EGC studies in mice.

Origin, Migration, and Proliferation of PGCs

To understand EGCs, it is first necessary to comprehend how PGCs arise and proliferate [7, 8]. The mammalian germ cell lineage is specified significantly later than in many other species (e.g., *Drosophila*, *Caenorhabditis*, and *Xenopus*). Only after blastocyst formation are the origins of mouse PGCs (mPGCs) detected as a choice of cell fate during gastrulation among the daughter cells of the proximal epiblast. Signaling from bone morphogenetic proteins (Bmps), particularly Bmp4 and Bmp8b acting via the type 2 activin receptor-like kinase [9–11], generates a population of cells that are maintained by Oct4 [12] and distinguished from the surrounding somatic cells by a longer cell cycle (16 versus 7 hours).

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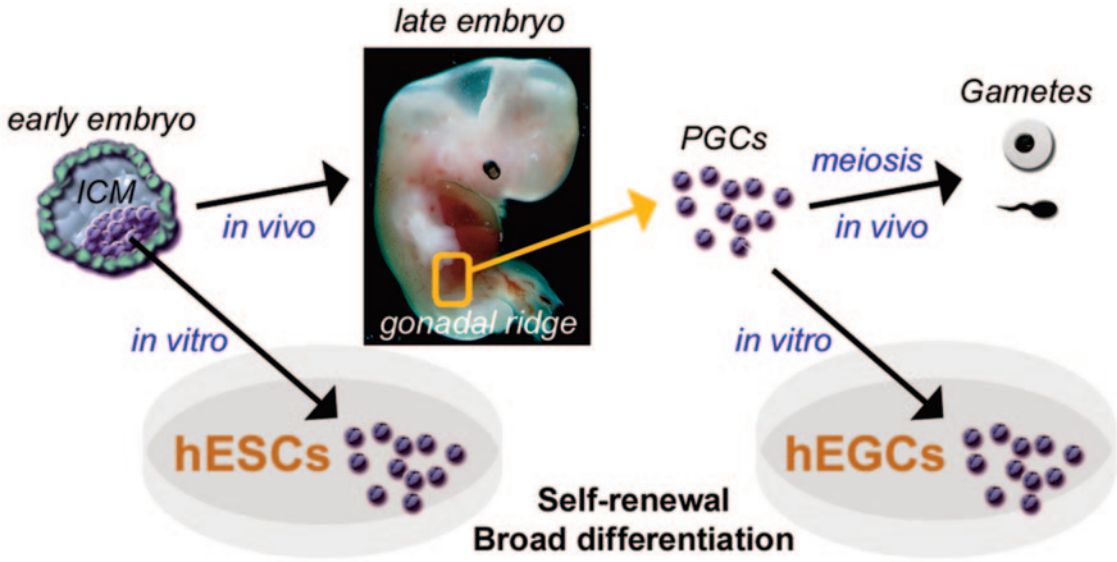

Figure 1. Cartoon of human embryonic stem cell and embryonic germ cell derivation. Abbreviations: hEGC, human embryonic germ cell; hESC, human embryonic stem cell; ICM, inner cell mass; PGC, primordial germ cell.

Understanding the biology and proliferation of mammalian PGCs requires appreciation of a fascinating journey from their origin in the epiblast, adjacent to the extraembryonic ectoderm, through the gut mesentery, to their final destination in the developing gonad (Fig. 2) [13]. Although the mechanisms regulating early mobilization are unclear, or even random, later migration relies in part on the chemokine Sdf-1 released from somatic cells. In mice, these signals act on the PGC surface receptor Cxcr4b to activate phosphatidylinositol 3 kinase and G-protein–coupled pathways that are responsible for PGC motility and direction, respectively [14–16]. In addition to chemoattraction, migrating germ cells require other stimuli for their maintenance, such as stem cell factor (SCF) (also known as steel factor, mast cell growth factor, or Kit ligand), acting via its receptor c-Kit. Disruption of this signaling pathway leads to germ cell apoptosis and a failure of PGCs to reach the gonadal ridge [17].

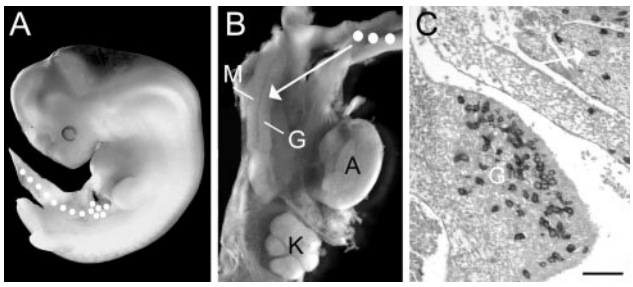

Figure 2. Migration of human primordial germ cells. Representation of human primordial germ cell (PGC) migration from the allantois to the gonadal ridge in the intact embryo **(A)** and through the gut mesentery within the dissected abdomen **(B)** at approximately 6 weeks after conception. The gonadal ridge (G) has developed on the medial surface of the mesonephros (M) adjacent to the adrenal gland (A) and superior to the kidney (K). **(C):** Human embryo section corresponding to **(B)** showing PGCs darkly stained for alkaline phosphatase activity in the gonad (G) and throughout the folds of the gut mesentery (arrow). Bar = 250 μm.

Between 10.5 and 12.5 days postconception (dpc) in mice, PGCs arrive at the gonadal ridge, and those that fail regress [18]. The environment of the gonad and the opportunity for different somatic cell interactions induce several changes, amongst which cell shape condenses and the dead-box transcription factor, Ddx4, is expressed. This homologue of the *Drosophila vasa* gene is essential for male germ cell proliferation and subsequent meiosis [19]. The early gonadal events coincide with sex determination, the process when chromosomal sex is translated into either testis or ovary. Similar dimorphism becomes apparent within the germ cell lineage [20]. Female germ cells reactivate the second X chromosome before entering meiotic prophase at ~13.5 dpc. The male germ cells enter mitotic arrest as prospermatogonia. Whereas the former mechanism seems largely intrinsic, the somatic signal from the testis is ill defined. It most likely arises from the Sertoli cells that surround the germ cells within the sex cords; a signal before 12.5 dpc can override the meiotic tendency of female germ cells [21]. In both sexes, arrival in the gonadal ridge also heralds the final erasure of methylation patterns.

Knowledge of human PGCs (hPGCs) is more difficult because they are less amenable to study. Germ cells are apparent in the gonadal ridge during the fifth and sixth week of development, with further PGCs detected in the gut mesentery, most likely in transit (Fig. 2). By 41 to 44 dpc (Carnegie stages 17 and 18), Sertoli cell differentiation and testicular cord formation is associated with decreased numbers of PGCs in male compared with female embryos, presumably due to mitotic arrest [22]. In contrast, proliferation continues in the developing fetal ovary during the remainder of the first trimester. This ability for continued mitosis in female fetuses carries potential significance. Female menopause results from an exhausted supply of gametes and is considered premature if prior to 40 years. One hypothesis to explain this untimely ovarian demise is inadequate provision of germ cell number before meiosis. It becomes plausible, therefore, that genes associated with premature ovarian

failure are candidate regulators of PGC proliferation and vice versa.

Mouse EGC Derivation

The identification of mPGCs fostered attempts to isolate and study their properties in vitro. Survival depended on age: PGCs from 13.5 dpc onwards could be cultured for days, with female cells entering meiosis. In contrast, cells at 11.5 to 12.5 dpc did not survive unless temperature was reduced to 30°C, favoring continued mitosis rather than meiosis of female germ cells [23]. Even so, cells survived little more than 1 week, either succumbing to apoptosis or differentiating. Problematic to these cultures was the apparent aversion of germ cells to standard cell culture substrates (e.g., plastic, glass, and gelatin). In contrast, gonad explant cultures demonstrated greater survival of PGCs when supported by outgrowing, adherent somatic cells. Hence, growth-arrested, embryo-derived feeder cell monolayers were adopted as an effective adherent substratum for PGCs [24]. This more reliable culture support system allowed distinction between pregonadal migratory and gonadal postmigratory PGCs. Culture behavior mirrored events in vivo: later stages yielded fewer motile PGCs, especially in male cells [25]. However, it was evident that some migratory cells retained mitotic activity in culture, albeit for a limited period.

Survival and proliferation could be promoted by the synergistic action of SCF and leukemia inhibitory factor (LIF), although proliferation did not progress beyond that of their in vivo counterparts [26–28]. Critically, extra supplementation with fibroblast growth factor 2 (Fgf2) effected their continued proliferation beyond normal in vivo cessation, a criterion in defining conversion or derivation from PGCs to EGCs [29, 30]. Remarkably, these more persistent cells possessed properties redolent of the mouse ESC (mESC) lines first established 10 years earlier, satisfying all criteria for pluripotency, both in vitro (giving rise to embryoid bodies [EBs] containing derivatives of all three germ layers) and in vivo (contribution to all tissues and the germ line of chimeric animals). Subsequently, derivation has been proven most successful from PGCs before and during migration but remains attainable from PGCs within the gonadal ridge until 12.5 dpc [31]. After this time, presumably mitotic arrest in male PGCs and the first stage of meiosis in female cells renders them refractory to EGC derivation. This contrasts to a population of multipotent germline stem (MGS) cells that has been isolated recently from the mouse neonatal testis, most probably derived from the diploid spermatogonial stem cell population [32].

Human EGC Derivation

The knowledge acquired over the years from mPGC-mEGC culture was a sensible starting point for attempts to derive hEGCs. However, in contrast to the plethora of laboratories that have derived hESC lines, reports of hEGC derivation remain limited. The first report (soon after the initial description of hESCs in 1998 [3, 33]) has been reinforced by three additional groups (ourselves in 2003 and two other groups in 2004, the latest group reporting twice with slightly varied methodology [4–6, 34]). This restriction may be due in part to the complications in acquiring ethically approved, continued access to the required starting material but also doubtless reflects the difficulties in the derivation and culture management of hEGCs, as acknowledged by some groups [4, 5, 35]. Nevertheless, this progress demonstrates that the process is practically, rather than just theoretically, possible.

The Starting Human PGC Population

The nature of acquiring human material from first-trimester voluntary/social termination has restricted the age of the PGCs available. This material spans 5 to 9 weeks after conception, with no bias evident between groups from the use of the antiprogestogen mifepristone/RU486 versus surgical termination of pregnancy (Table 1). The earliest specimens would contain PGCs shortly after arrival in the gonad, which might be expected to maintain proliferation better in culture; however, there has been little evidence to support this. Whereas there is an apparent upper age limit for mEGC derivation of 12.5 dpc in both males and females, it is remarkable that an upper limit related to sex cord formation cannot be applied to human male PGCs, as all reports include derivation from the fetal testis after this event. There are several potential explanations for this: male PGCs might resume proliferative activity once freed from Sertoli cell influence; cord formation may not arrest all male PGCs (i.e., a small cohort of proliferative PGCs might persist); or use of the entire urogenital ridge, as reported by most groups [3, 5, 6, 34], might include a population of extragonadal PGCs. Our continuing investigation considers both the retention of mesonephros, which contains some alkaline phosphatase (AP)-positive cells [4], and its removal from the fetal gonad. To date, no significant difference is apparent.

Preparation of Human PGCs for Culture

Liu et al. [5] described a comparison of gonad cell dissociation and plating combinations, confirming the optimal protocol as essentially that used by the other groups—mechanical disaggregation, enzymatic dissociation, and gentle trituration—before plating on a monolayer of growth-arrested feeder cells (Table 1). We have compared enzymatic dissociation with puncturing the intact organ, as developed initially for the isolation of mPGCs [36]. Equally effective at generating hEGC cultures, cells are released directly into culture media, avoiding protracted washing and resuspension. At least theoretically, this avoidance of proteolytic enzymes minimizes damage to cell-surface markers and receptors during initial preparation and plating.

Basic In Vitro Culture Media

In reports to date, the basic composition of culture media has shared many similarities to mEGC derivation methodology, comprising a mix of Dulbecco's modified Eagle's medium (DMEM) or knockout DMEM (KO-DMEM) with nonessential amino acids, beta-mercaptoethanol, and L-glutamine. Choice of serum varied between 10%–15% fetal bovine serum (FBS), ESC-tested FBS, or knockout serum replacement (KO-SR) (Table 1).

Whereas serum provides essential nutrients, its use is far from ideal, being the primary source of unknown factors with the potential to affect derivation or induce differentiation. Alternatively, the serum-free supplement KO-SR can (in conjunction with KO-DMEM) decrease the propensity for spontaneous differentiation of mESCs [37] and can improve the derivation efficiency of mEGC, although growth is reduced [38]. Its effects

Table 1. Methodology in published reports of human embryonic germ cell derivation

Report	Collection	Age[a]	Material	Dissociation	Feedeis	Medium[b]	Additives[c]	Efficiency	Duration
Shamblott et al. [3, 77]	Surgical	5–9	Gonadal ridges and mesenteries	Mechanical and enzymatic (trypsin or collagenase IV, hyaluronidase V, DNase I)	STO (irradiated)	DMEM + 15% FBS	hrLIF, forskolin, 1–2 ng/ml hrFGF2	Variable	20–25 passages
Turnpenny et al. [4, 70]	Surgical	7–9	Gonads	EDTA treatment; mechanical and enzymatic (collagenase IV, DNase I)	STO (irradiated)	KO-DMEM + 15% KO-SR/ES-FBS	hrLIF, forskolin, 4 ng/ml hrFGF2	8/56 = 14%	10–12 passages
Park et al. [6]	Surgical	9	Gonadal ridges and mesenteries	Mechanical and enzymatic (collagenase IV, DNase I)	STO (mitomycin C)	DMEM + 15% FBS	hrLIF, forskolin, 1 ng/ml hrFGF2	Unstated; 1 line	1 year
Liu et al. [5]	RU486	6–8	Gonadal ridges and mesenteries	Mechanical or mechanical and enzymatic (trypsin)	pMEFs (mitomycin C)	DMEM + 15% FBS	hrLIF, forskolin, 1 ng/ml hrFGF2	Unstated; multiple specimens pooled	6 months
Pan et al. [34]	RU486	6–9	Gonadal ridges	Mechanical and enzymatic (trypsin)	STO/pMEFs (mitomycin C)	KO-DMEM + 15% FBS/KO-SR	hrLIF, forskolin, 1 ng/ml hrFGF2	2/40 = 5%	15 passages

[a]Weeks after conception.
[b]All formulations included L-glutamine, β-mercaptoethanol, nonessential amino acids, and sodium pyruvate.
[c]All added hrLIF at 1,000 U/ml and forskolin at 10 μM.
Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hrFGF2, human recombinant fibroblast growth factor 2; hrLIF, human recombinant leukemia inhibitory factor; KO, knockout; pMEF, primary mouse embryonic fibroblast; SR, serum replacement.

on the efficiency of hEGC derivation remain conjecture due to the relatively unpredictable nature of these cells in culture, with or without serum.

Feeder Layers

The reporting groups have predominantly used either mouse STO fibroblasts or primary mouse embryonic fibroblasts (pMEFs) as feeder layers (Table 1). Shamblott et al. [3] are the only researchers to date who have reported the comparison of feeder layers. Although in their experience STO cells were preferable to either pMEFs, human fetal fibroblasts, or gelatin-coated plates, further formal analyses of feeder layer characteristics, such as via proteomic approaches [39], are required to discern between the factor cohorts. This is particularly important given the complexity of in vivo PGC-somatic cell interaction within the fetal gonad and the consequences of alien interactions, such as the in vitro meiotic influence of the fetal lung on mPGCs [40]. Similar to this experience, it is conceivable that different cell types, providing differing factor combinations, will improve the sequential derivation and maintenance of hEGCs.

Media Additives and Critical Factors

In addition to the influence of the basic media and feeder layers, all groups reporting hEGC derivation and culture have included other additives. Their use originates from mouse pluripotent stem cell derivation and culture. Definitive requirements for any in equivalent hEGC cultures have yet to be established conclusively.

Stem Cell Factor. Perhaps the role of SCF is the most understandable. The sterile mouse mutants *Sl* (*Steel*) and *W* (*Dominant White Spotting*) arose from mutations affecting SCF and its receptor, c-Kit, respectively, and revealed a direct role in the proliferation and maintenance of PGCs en route to the gonad [41]. c-Kit is present on the cell surface of PGCs. Its ligand, SCF, exists as two isoforms: a membrane-bound (mbSCF) factor and a soluble form (sSCF), produced by proteolytic cleavage. mbSCF is critical for the proliferation of PGCs in vivo [42]. In vitro, it promotes mPGC survival by inhibition of apoptosis for longer than sSCF [17, 26, 43, 44]. The latter isoform, however, increases telomerase activity in vitro [45]. It is therefore easy to envisage the benefit of both sSCF supplementation combined with a feeder layer, such as STO, supplying mbSCF [46]. As in the mouse, c-KIT and SCF are also present in human fetal gonads [47] (our unpublished data).

Leukemia Inhibitory Factor. The role of LIF is intriguing. PGC development in LIF-deficient mice is normal, suggesting either no role or redundancy between related cytokines. Indeed, oncostatin M can substitute for LIF in affecting survival and/or proliferation of mPGCs in culture [46, 48, 49], although neither can substitute for feeder support [46]. In contrast, LIF, originally isolated from buffalo rat liver (BRL) cells for its differentiation inhibiting activity [50], can obviate the requirement for feeder layers in mESC culture, where signaling via the LIF receptor (LIFR), gp130, and intracellular Stat3b has a proven role in maintaining mESC pluripotency [51].

There are differences in pluripotent stem cells between mice and humans. Despite activation of the LIFR/gp130-STAT3B pathway, LIF (administered in human recombinant form) does not maintain self-renewal of hESCs, which require feeder cells or their conditioned media with an extracellular matrix [52, 53]. However, this highly pleiotropic cytokine may have earlier effects. In LIF-deficient mice, blastocysts fail to implant [54]. The receptors LIFR and gp130, although dispensable for normal early embryogenesis, are necessary for diapause, the suspension of mouse development at the blastocyst stage in the event of unfavorable conditions. Under these circumstances, the ICM can maintain its undifferentiated state for months [55]. Although

human embryos do not enter such a phase, LIF increases the number and quality of human blastocysts in serum-free culture [56], and medium conditioned by BRL cells potentially improves hESC derivation [57].

In PGCs, however, the effects of LIF are debatable. LIFR/gp130/Stat3b signaling inhibits the progression to meiosis of cultured female mPGCs [58], but this has not been reported in human cells. Similarly, although all groups have included LIF during hEGC derivation and culture, none have systematically proven its necessity. Certainly LIF, LIFR, gp130, and associated signaling components are expressed in human fetal gonads [59] (our unpublished data). Perhaps active LIFR/gp130/Stat3b signaling in vivo sustains hPGCs in an undifferentiated suspended state (akin to diapause). If this is the case, once removed and exposed to myriad other, as-yet-undefined influences in vitro, this effect may be overridden, resulting in the loss of hPGCs by cell death and/or differentiation (including meiosis).

Fibroblast Growth Factor 2. As described earlier, Fgf2 was the key addition that enabled mPGCs to continue proliferation in cultures already containing LIF and SCF [60]. It is unclear specifically which Fgf receptors transduce its effects, but the receptivity of mPGCs is important. Potentially, only transient (approximately 12-hour) exposure to Fgf2 is necessary for mEGC derivation [41], after which its supplementation, along with SCF, can be discontinued so long as cells are maintained on feeders in the presence of serum [29]. However, after 11.5 dpc, reduced Fgf receptor expression also correlates to diminished derivation efficiency. This effect is more pronounced in females, perhaps due to the approach of meiosis [60, 61].

Fgf2 functions as a potent mitogen in many cell types and induces telomerase activity in cultured mouse neural precursor cells [62]. This suggests overlap or synergism with SCF in maintaining telomerase activity of mPGCs when taken into culture.

Forskolin. All groups deriving hEGCs have included forskolin, which raises intracellular cAMP levels and stimulates mitosis in cultured mPGCs [63]. Whether this action compensates for an endogenous hormone is not known; however, in mEGCs it does not substitute for the survival mediated by LIF and SCF [64].

Although included by the groups reporting hEGC derivation, systematic definition of optimal concentrations and time exposures of all these and other growth factors remains a significant challenge for hEGC researchers. Furthermore, the recent description of mouse MGS cells arose from cultures that primarily maintained spermatogonial stem cells, i.e., the self-renewing cells that give rise to spermatozoa [32]. These cultures included glial-derived neurotrophic factor (GDNF) and epidermal growth factor (EGF). Whereas MGS cells did not develop in standard mEGC derivation media (LIF, SCF, and Fgf2) [32], the converse effect of GDNF and EGF on mouse or human EGC derivation and maintenance is unknown.

Growth Characteristics of Human PGCs and EGCs

The essence of hPGC-EGC basic biology can be summarized as overcoming the in vitro sensitivity of PGCs that have been removed from their specialized in vivo niche, the continuation (or reacquisition) of proliferation, and the maintenance of self-renewal and pluripotent properties in prolonged culture.

As with mouse, culture as gonadal explants mimics the supportive in vivo environment for hPGCs, but outgrowth is limited (Fig. 3). Dissociation and replating isolates the germ cells, but the consequences are not all beneficial. The fetal gonad is heterogeneous. In vivo, germ cells develop supportive intercellular contacts with specialized somatic cells, such as Sertoli (nurse) or granulosa cells. The loss of supportive in vivo paracrine factors is detrimental, yet other somatic influences (e.g., from the Sertoli cell) inhibit proliferation [20]. In stark contrast, culturing the ICM benefits from maintained intercellular contact with the same cell type.

mESCs and mEGCs are derived from inbred strains, such as 129/Sv mice, which display a high incidence (1%) of spontaneous teratocarcinoma and yield mEGCs with relative efficiency [41, 65]. Conversely, hESCs and hEGCs are derived from genetically heterogeneous sources, which prohibit such standardization and may restrict the frequency of derivation. In our experience, hPGC-EGC cultures acquire one of two differing characteristics, designated either PP (poorly proliferating) or VP (vigorously proliferating) [4]. The latter arise from approximately 15% of starting cultures and comprise colonies of varying morphology and/or networks of proliferating migratory-like cells (the term *migratory* is taken from the description by Shamblott et al. [3]; Fig. 3). Neither resembles colonies of hESCs. Although PP cultures have survived with AP^+ cells beyond 50 days, these cultures have never converted to intense proliferation, despite factors such as Fgf2. The 15% of cultures that rapidly proliferate tend to do so early—typically within the first 2 weeks in culture. This suggests that the problem of survival is less important than that of conversion to a cell with

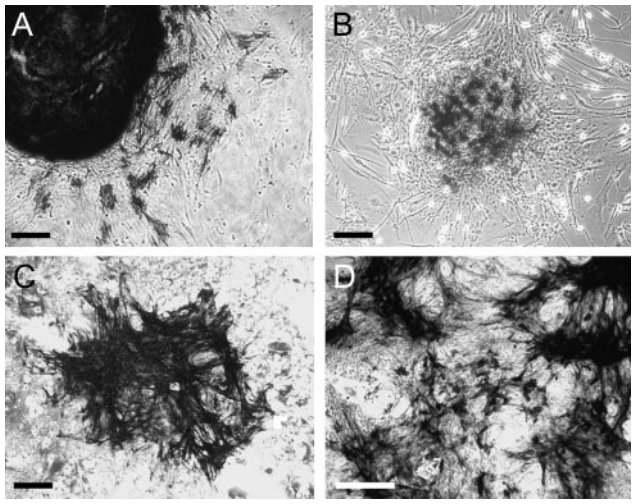

Figure 3. Behavior of human primordial germ cells (PGCs) and embryonic germ cells (EGCs) in culture. **(A):** Cultured gonadal explant with limited outgrowth of alkaline phosphatase (AP)-positive PGCs. Strong AP activity is preserved within the explant where contacts with supporting somatic cells are maintained. **(B):** Colony containing AP^+ cells with a more tightly packed morphology, in contrast to **(C),** where the cells have adopted a more open, migratory-like morphology, and **(D)**, an open network of vigorously proliferative AP^+ cells that lack obvious colony formation. Bar = 500 μm.

VP/hEGC characteristics. Information on the molecular differences between preconversion and postconversion germ cells within the same and across different species would be instructive.

Several groups, including ourselves, have noted difficulty in maintaining hEGCs undifferentiated long-term [4, 5, 35]. This problem of undifferentiated status contrasts with other pluripotent stem cell types: hESCs and human embryonal carcinoma cells (hECCs) and mESCs and mEGCs, all of which have been more extensively characterized. In our experience, VP hEGC cultures have proliferated extensively; however, the proportion of cells expressing pluripotent markers (e.g., OCT4 and stage-specific embryonic antigen [SSEA] family members; see below) declines over time, variably from 2 to 3 months onwards, and is exacerbated by freeze-thaw routines [4]. In striking contrast, a single report from Park et al. [6] described continuous, undifferentiated culture of hEGCs for 12 months, despite similar methodology and starting material (Table 1). All human pluripotent cell types are continuously prone to spontaneous differentiation, and their properties, including karyotype, can alter with high passage number [66]. This group's experience needs to be shared by other researchers, including characterization of whether the properties of this line can be retained through freeze-thaw cycles.

In Vitro and In Vivo Characterization of hEGCs

hEGCs have been subject to the same tests of stem cell status as hESCs and hECCs. In addition, loss of pluripotent markers merits greater consideration of meiotic progression as well as normal somatic differentiation [58].

Gene Expression Analyses

The self-renewal of karyotypically normal hEGCs has been assessed by the expression of characteristic markers (Table 2 and references therein). All groups report AP activity. SSEA1 and SSEA4 are present; however, groups either report variable SSEA3 expression or its absence. It remains unclear what significance should be attached to the expression of SSEA family members. In hESCs and hECCs, SSEA1 is absent until the onset of differentiation, whereas SSEA3 is readily detected. In contrast, hEGCs start out SSEA1-positive, with, at best, only weak SSEA3 immunoreactivity (Table 2). Although this profile might suggest early differentiation, the strongly SSEA1$^+$/EMA-1$^+$ starting population of PGCs within the gonad argues against this [4]. It is also difficult to be certain of the significance of these differences when the genes under discussion perhaps mark, but do not regulate, cell phenotype. Conversely, genes and proteins with known function in pluripotent cells serve as more informative markers. Particular salient examples are the nuclear transcription factors OCT4 and NANOG, which have key roles in the maintenance of pluripotency [67–69] and are expressed in hPGCs [4, 70]. All groups have identified OCT4 in hEGC by reverse transcription–polymerase chain reaction and/or immunocytochemistry. Some groups have identified telomerase activity either by telomerase repeat-amplification protocol assay or the expression of hTERT. Additional genes, such as *SOX2, FGF4, STELLAR*, *FRAGILIS*, or *DDX4*, highlighted in ESCs or as orthologs in the germ cell lineage of other species, have not been characterized in either hPGCs or hEGCs. The expression of *STELLAR* is relevant as it forms part of the cluster at 12p13 that includes *NANOG* and *GDF3*, which is frequently overrepresented in teratocarcinogenesis and contained within 12p isochromosomes in several aneuploid EC lines [71, 72].

Evidence for Pluripotency of hEGCs: In Vitro and In Vivo

Self-renewal of hEGCs has been documented by continuous cultures that retain markers such as OCT4 and SSEAs (Table 2). The significance of hTERT, present within hPGCs and hEGCs, is less clear. This catalytic component of the telomerase ribonucleoprotein is a marker of nonsenescing cells, which maintain telomere length. This includes stem cells, but also other cell types, such as cancer cell lines or those from human fetal development (i.e., postgastrulation) [73]. Nevertheless, loss of

Table 2. Characterization of pluripotency markers in published reports of human embryonic germ cell derivation

	Marker										
Report	**AP**	**SSEA-1[a]**	**EMA-1[a]**	**SSEA-3**	**SSEA-4**	**TRA-1–60[b]**	**TRA-1–81**	**OCT4**	**NANOG**	**hTERT**	**Karyotype**
Shamblott et al. [3, 77]	√	√	—	√[c]	√	√	√	√	—	√	√ Passages 8 and 10
Turnpenny et al. [4, 70]	√	√	√	ND	√	—	—	√	√	√	√ Passages 10 and 12
Park et al. [6]	√	√	—	ND	√	√	√	√	—	—	√ Passage 20
Liu et al. [5]	√	√	—	√[c]	√	—	—	√	—	—	√ Passage 15
Pan et al. [34]	√	√	—	ND	√	√	√	√	—	√	√ Passages 7 and 13
hESC/hECC	√	ND	—	√	√	√	√	√	√	√	

√, positive test; —, not recorded.
[a]SSEA-1 and EMA-1 are very similar or identical antigens [81].
[b]Antibody recognizes the same epitope as GCTM2.
[c]Staining was weak and inconsistent.
Abbreviations: AP, alkaline phosphatase; hECC, human embryonal carcinoma cell; hESC, human embryonic stem cell; ND, not detected; SSEA, stage-specific embryonic antigen.

this gene expression profile coincides with the onset of differentiation via haphazard EB formation to ectodermal, endodermal, and mesodermal end points. This satisfies in vitro criteria for pluripotency and has been achieved by all four groups who have derived hEGCs [3–6]. Unlike ESC-derived structures, however, no spontaneous contractions consistent with mesodermal differentiation toward cardiomyocytes have been reported. Furthermore, since the original report, which maintained the presence of LIF, Fgf2, and forskolin [3], the subsequent groups have all withdrawn LIF and, in some instances, the other factors from the culture medium [4–6]. These apparently conflicting observations, possibly due to factors intrinsic or extrinsic to hEGCs, or a combination of both, require clarification.

The issue of hEGC pluripotency becomes particularly interesting with in vivo analyses, for which the only true test is the ability to form chimeric embryos, not permitted with human cells for ethical reasons. By these criteria, mouse ICM cells, mESCs, mEGCs of either sex, and the recently derived mouse multipotent germline cells (mMGCs) can give rise to all somatic cell lineages, as well as functional gametes [32, 74–76]. In contrast, mPGCs are nullipotent [41]. Without recourse to these experiments for human cell types, teratoma formation is taken as standard evidence of pluripotency in vivo and has been achieved for hESC and hECC [41]. Further, this property (unlike efficiency of derivation) is not dependent on genetic background [74, 75]. Intriguingly, no group has reported teratomas from hEGCs. Our experience has been to engraft early-passage hEGCs into the thigh muscle of immunocompromised mice. The cells expressed AP at the time of engraftment. Whereas the positive control of human N-TERA2 ECCs, cultured in parallel, yielded characteristic teratomas, no such tumors have been observed from equivalent numbers of hEGCs, consistent with findings elsewhere [77]. The multiple cell lineages within a teratoma confirm pluripotency. However, failure to form a tumor does not mean hEGCs lack pluripotency. Teratomas arise from unrestricted local proliferation of the undifferentiated cells that then differentiate to derivatives of all three germ layers. Our preliminary evidence suggests that at least some human cells persist within the immunocompromised mice, but their phenotype is, as yet, unclear. This insinuates that hEGC proliferation was restrained sufficiently to avoid tumorigenesis, but potentially in its place cells have differentiated (if so, presumably in response to cues from their respective individual murine environments).

So, at present, in the absence of the definitive chimeric experiment, the in vivo pluripotency of hEGC remains unresolved. Two therapeutic considerations may lessen the importance of this: first, in vivo transplantation of EGC-derived cells has generated functional responses; second, lack of teratoma formation is highly desirable for therapy in human recipients.

Therapeutic Potential of Human EGCs

Important studies toward therapeutic exploitation of hEGCs have progressed. The in vitro differentiation capacity of hEGC, via ongoing culture of EB-differentiated (EBD) cells, has been well described by the original deriving group [78], including in vitro characterization in response to BMP2 and transforming growth factor $\beta 3$ of a musculoskeletal phenotype [79]. EBD cells, capable of significant expansion in culture, also provided the material to demonstrate that imprinting appears appropriate with monoallelic expression of several relevant genes, a critical consideration for potential therapeutic applications [35]. Further accumulating evidence reflects a propensity for neuronal differentiation [6, 34] (our unpublished data). Pan et al. [34] found that, as for hESCs, retinoic acid or increases in intracellular cAMP signaling enhanced neural differentiation of hEGC derivatives, including tyrosine hydroxylase-positive cell types relevant to therapy for Parkinson's disease. Whereas this study was restricted to in vitro investigation, cellular derivatives of hEGCs have also improved motor function in rats in vivo. Interestingly, after diffuse motor neurone injury, the grafts of transplanted human cells did not directly reinnervate tissues; instead, they appeared to support the survival and regrowth of endogenous rat neurones [80]. Taken together, these studies argue for the inclusion of hEGCs in stem cell research programs.

Future Directions

Now that several groups have successfully established hEGC cultures, an increase in protocols for directed differentiation is likely to follow. The goals of these experiments are no different from other aspects of human stem cell research: namely, to generate physiologically normal cells with the desired function, ideally by normal regenerative or developmental pathways. It remains to be seen whether the undifferentiated cells ever produce teratomas. Potentially, this will be an unforeseen advantage of hEGC research, in which case-comparative study of hEGCs and hECCs also offers an informative model on the origin of gonadal tumors.

Better understanding is needed of the derivation process and long-term culture so that true robust hEGC lines can be banked equivalently to hESCs. This needs to include transition to human feeder cells, or, ideally, their complete avoidance, and gearing up of cell culture efforts toward good manufacturing practice. To achieve these goals, our current focus is on trying to understand the determinants of derivation. For this, better knowledge of the starting hPGC population will allow definition of the differences that arise between species and after derivation. Unique to PGC-EGC research, this requires consideration of the complex somatic-germ cell interactions that differ between male and female and the propensity for meiotic differentiation. All of this research will be hindered without refined methods for isolating pure PGC populations away from their somatic neighbors. Conversely, achieving this will allow better gene expression analyses and standardized culture experiments that enable more thorough analysis of additive factors and comparison of EGCs with ESCs and ECCs. It will be revealing to determine whether the differences apparent at the cell surface, currently demonstrated by the variable requirement for exogenous factors, translate to intracellular differences or whether the same underlying molecular pathways are truly common to all human pluripotent stem cells.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

NOTE ADDED IN PROOF

Since electronic publication of this article, a further group has reported on aspects of the human PGC-EGC lineage [82] and their use in cell therapy.

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Origin of pluripotent germ cell tumours: The role of microenvironment during embryonic development

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ABSTRACT

Carcinoma *in situ* (CIS) testis, known also as intratubular germ cell neoplasia, is the cancer stem cell from which the great majority of testicular germ cell derived tumours (TGCTs) of the testis arise. TGCTs can proliferate into morphologically homogeneous seminomas or can differentiate into virtually any type of tissue and form teratomas (non-seminomas). CIS cells display a close phenotypic similarity to fetal germ cells (primordial germ cells or gonocytes) suggesting an origin due to a developmental delay or arrest of differentiation of early germ cells. The pluripotency of these neoplasms has recently been explained by a close resemblance of their expression profile to that of embryonic inner cell mass cells studied in culture as embryonic stem cells, with high expression of transcription factors associated with pluripotency, such as NANOG and OCT3/4, as well as proteins found in several tissue specific stem cells, such as TFAP2C (AP-2γ) or KIT. CIS and seminomas highly express a number of pre-meiotic germ cell specific genes, which are down-regulated during development to non-seminomas, while the expression of other embryonic markers, such as SOX2, is up-regulated. The mechanistic pathways and causative factors remain to be elucidated of both the initial transformation of fetal germ cells into CIS cells and the progression of CIS cells into an invasive tumour in the young adult. However, evidence supported by epidemiological studies indicate that disturbances in the hormonal microenvironment of the differentiating gonads may results in both the neoplasia and a host of other problems later in life, such as genital malformations, decreased spermatogenesis, and signs of hypogonadism.

1. Introduction

More than 35 years ago the first paper describing atypical spermatogonia in testicular biopsies from two patients was published (Skakkebaek, 1972a). Both patients later developed overt testicular tumours indicating that the abnormal cells were precursor cells of the malignant tumours (Skakkebaek, 1972b). This original study is to our knowledge one of the first descriptions of a true tumour/cancer stem cell (Nielsen et al., 1974). Today, carcinoma *in situ* (CIS) (also known as intratubular germ cell neoplasia or testicular intraepithelial neoplasia) is generally accepted as the common pre-invasive precursor cell that gives rise to testicular germ cell tumours (TGCTs) of the adolescents and young adults (Ulbright et al., 1999; Oosterhuis and Looijenga, 2005). There are two other types of TGCTs, which develop without a CIS stage: spermatocytic seminoma of elderly men and infantile germ cell tumours. Both of these tumour types are rare and have remained at a steady low incidence level (Visfeldt et al., 1994). The opposite is true for TGCTs, originating from the CIS precursor stage, which have increased in incidence over the last several decades and now constitute the most prevalent malignant cancer among young male adults in Northern and Western Europe, with a life time frequency in Denmark of approximately 1% (Richiardi et al., 2004).

The TGCTs originating from CIS are traditionally subdivided into two histologically distinct groups, seminomas (also called classical seminoma to distinguish from spermatocytic seminoma) and non-seminomatous germ cell tumours, each constituting approximately 50%. Whereas the seminoma is a generally uniform tumour and resembles early fetal germ cells and CIS cells, the more malignant non-seminomas are very heterogeneous and encompass

Abbreviations: CIS, carcinoma *in situ*; TGCT, testicular germ cell tumour; EC, embryonal carcinoma; PG, primordial germ (cell); TDS, testicular dysgenesis syndrome; ES, embryonic stem (cell).

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embryonal carcinoma (EC), choriocarcinoma, teratomas, yolk sac tumour, and can also contain elements of seminoma (Ulbright et al., 1999). EC cells of the non-seminomas have the ability to differentiate into a wide range of cell types, and show a phenotypic resemblance to the inner cell mass cells and epiblast from the early embryo. Hence, EC cells represent a malignant caricature of the human embryonic stem (ES) cells that are derived in culture from the inner cell mass (Andrews, 1998; Solter, 2006). However, many EC cells show limited developmental potency, or even nullipotency, which might be regarded as the ultimate consequence of adaptation occurring in tumours that have developed over a period of 20 or more years (Baker et al., 2007). Intriguingly, a recent report suggested that non-seminomas, most likely the yolk sac tumour component, also may have the potential to differentiate to germ cells, although this needs independent confirmation (Honecker et al., 2006). Taken together, the observations from pathology suggest that the common pre-invasive CIS cells have the capability to develop into many different germinal and somatic tissues and may be regarded as pluripotent or truly totipotent. In that sense, we believe that CIS is a cancer stem cell for both TGCT types, seminoma and non-seminomatous tumour. However, it is debated that CIS has to be first re-programmed to EC, which then is a stem cell for somatic components of non-seminomas.

The remarkable potential of CIS cells raises the immediate question of their origin. A few years after their initial discovery, a morphological similarity to gonocytes was noted and it was proposed that CIS cells could be remnants of undifferentiated embryonic/fetal cells (Nielsen et al., 1974; Skakkebaek et al., 1987). Their fetal origin was supported by immunohistochemical studies of proteins present in CIS also shown to be present in primordial germ (PG) cells and gonocytes, and by identifying CIS cells in prepubertal patients, who later developed TGCTs, indicating that the cells had originated prior to puberty (Muller et al., 1984). Additional support was provided by epidemiological studies showing that the increase in TGCTs was related to birth cohort rather than year of diagnosis (Møller, 1989). Remarkably, further epidemiological studies demonstrated that first-generation immigrants to Sweden had the same risk for developing TGCTs as in their country of origin, whereas second-generation immigrants had the same risk as native Swedes (Hemminki et al., 2002; Hemminki and Li, 2002). Besides strongly indicating the fetal origin of TGCTs in young adults, these epidemiological studies also showed the aetiological importance of environmental factors in development of the CIS cells, although some genetic predisposition also plays a role. Hence, TGCTs in young adults have been proposed to be part of a larger testicular dysgenesis syndrome (TDS) that includes mild genital malformations and certain forms of infertility, all thought to be highly influenced by environmental exposures (Skakkebaek et al., 2001). So, 35 years from the initial description of CIS cells, it has become increasingly clear that the attention of investigators dealing with the TGCTs and their origin has to focus on both the early *in utero* gonadal development and the impact of the multitude of different environmental factors that can negatively affect this process, including the so-called endocrine disruptors. In this review, we will summarise findings concerning the phenotype and genotypic signature of CIS cells and discuss the hypotheses concerning their origin and how these embryonic cells in the adult develop into a malignant tumour.

2. Expression of embryonic marker genes in CIS cells and during invasive progression: the development of a pluripotent tumour?

CIS cells have many morphological similarities with PG cells and early gonocytes (Nielsen et al., 1974; Albrechtsen et al., 1982; Skakkebaek et al., 1987). Relatively early on it was identified that the three cell types share the expression of immunohistochemical markers such as placental-like alkaline phosphatase (PLAP) or the stem cell factor receptor KIT (Giwercman et al., 1991; Rajpert-De Meyts and Skakkebaek, 1994; Jorgensen et al., 1995). These markers are now commonly used in the clinical setting to help identify CIS in surgical biopsies. More recently, the expression profiles of CIS cells studied by microarrays (Almstrup et al., 2004; Skotheim et al., 2005) provided a series of candidate marker genes, such as *TFAP2C* (AP2γ) (Hoei-Hansen et al., 2004), which has shown promising results as a possible marker of CIS cells in semen samples (Hoei-Hansen et al., 2007).

A landmark in the understanding of CIS and the derived invasive cancers was the identification of *POU5F1* (OCT3/4) and *NANOG* expression, which are known for their association with pluripotency in ES cells (Fig. 1) (Looijenga et al., 2003; Rajpert-De Meyts et al., 2004; Hoei-Hansen et al., 2005). These two transcription factors are, together with SOX2, key regulators of the formation and maintenance of the inner cell mass during pre-implantation development and for sustaining self-renewal in ESCs (Scholer et al., 1990; Nichols et al., 1998; Niwa et al., 2000; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). Among others, knock-out studies of both *Pou5f1* and *Nanog* in mice have implicated these genes in the first and second initial fate decisions in mammalian development, respectively (Cavaleri and Scholer, 2003; Chambers et al., 2003).

Although very useful, experimental studies in animals have demonstrated differences in developmental biology between mice and humans. Stem cells have been isolated from adult mouse testes that exhibit properties similar to ES cells (Guan et al., 2006; Seandel et al., 2007), and these studies showed that a subset of spermatogonial stem cells in mouse testes retain the embryonic phenotype of PG cells and gonocytes. In humans a different scenario is seen, where NANOG and OCT3/4 (together with KIT and AP2γ) are expressed in PG cells and gonocytes, but are down-regulated during the second half of pregnancy and until 2–4½ months of post-natal age (Jorgensen et al., 1995; Rajpert-De Meyts et al., 2004; Hoei-Hansen et al., 2004, 2005; Honecker et al., 2004). During this early infantile period, a transient increase in the production of testicular hormones occurs, known as the 'mini-puberty' (Forest et al., 1973), which coincides with the final stage of differentiation of gonocytes into infantile spermatogonia (Hadziselimovic et al., 1986). Although NANOG and OCT3/4 are down-regulated, downstream factors identified in ES cells, UTF1 and REX1 (Niwa, 2001; Kristensen et al., 2005; Nishimoto et al., 2005), continue to be expressed throughout the male development in spermatogonia and are still present in the adult testes (Kristensen et al., 2008). The continued expression of UTF1 and REX1, apparently uncoupled functionally from OCT3/4 and NANOG, shows that the partial embryonic phenotype of the germ cells is still evident in the mature human testes. The link between CIS cells and embryonic cells has been further supported by a study showing a substantial overlap between human ES cells and CIS cells' gene expression profiles (Almstrup et al., 2004).

There are, however, marked differences in the expression of embryonic genes among the histological types of overt tumours derived from CIS. The phenotype of seminoma is very close to that of CIS, whereas the phenotype of EC, considered the undifferentiated stem cell compartment of non-seminomas, resembles much closer that of ES cells, suggesting a re-programming or a de-differentiation event (Sperger et al., 2003; Almstrup et al., 2005; Skotheim et al., 2005; Korkola et al., 2006). This is supported by the high expression of SOX2 in EC, for example, while in seminoma this marker is virtually negative (Sperger et al., 2003; Santagata et al., 2007; Perrett et al., in press). A further somatic differentiation of EC is observed in nearly all non-seminomas and this event is associated with down-regulation/inactivation of the embryonic

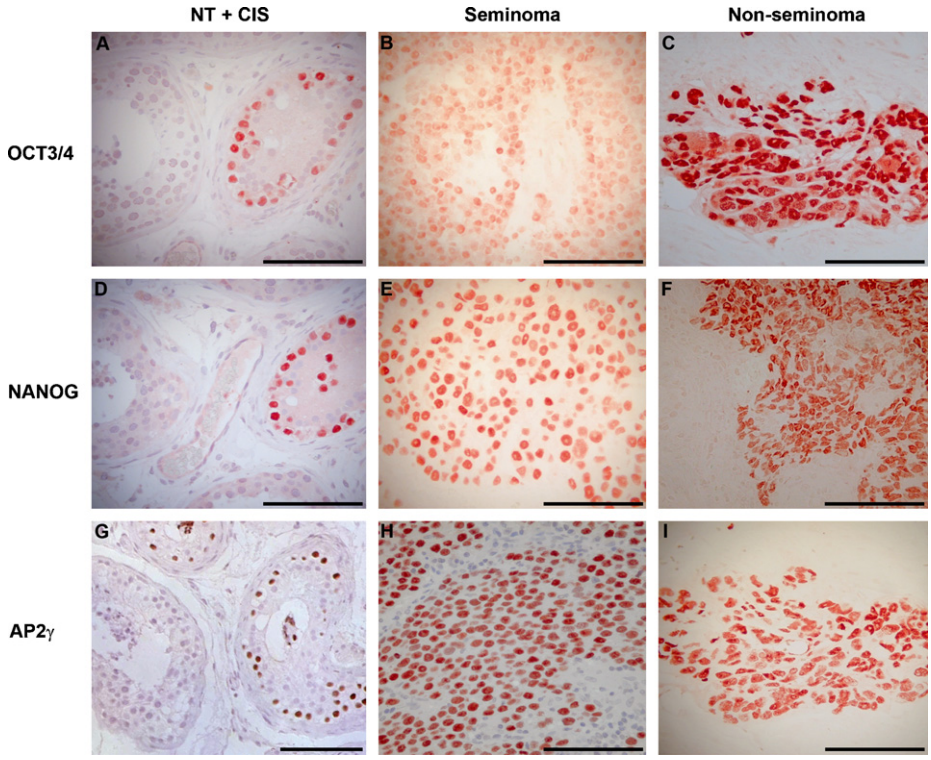

Fig. 1. Expression of pluripotency associated proteins OCT4, NANOG, and AP2γ in carcinoma *in situ* (CIS) cells and testicular germ cell tumours. Images (A), (D), and (G) show area of normal testis (NT, unstained) adjacent to tubules with CIS cells (stained positively for OCT4, NANOG, and AP2γ). Images (B), (E), and (H) shows example of classical seminoma strongly positive for the same transcription factors. Images (C), (F) and (I) show the expression of the three proteins in non-seminomas; note the intensely positive clusters of embryonal carcinoma (EC), whereas the surrounding teratomatous tissues or atrophic tubules without malignancy (in F) show no staining (scale bar 100 μm).

pluripotency genes (Figs. 1–3) (Skotheim et al., 2005; Korkola et al., 2006).

The similarities between the ES cell lines and CIS cells do not stop at gene expression patterns, there are also remarkably similar patterns of genomic aberrations in the two cell types. CIS cells are known to undergo polyploidization and DNA content in the triploid to hypotetraploid range has been identified in CIS adjacent to both seminoma and non-seminoma (Ottesen et al., 2004b). Although polyploidization seems to appear prior to the establishment of extra 12p material (Geurts van Kessel et al., 1989; Oosterhuis et al., 1989; Ottesen et al., 2004b), the specific stage at which tumour development is initiated and genomic instability introduced remains unresolved. Due to technical problems only a small number of cases of CIS have been examined for chromosomal aberrations,

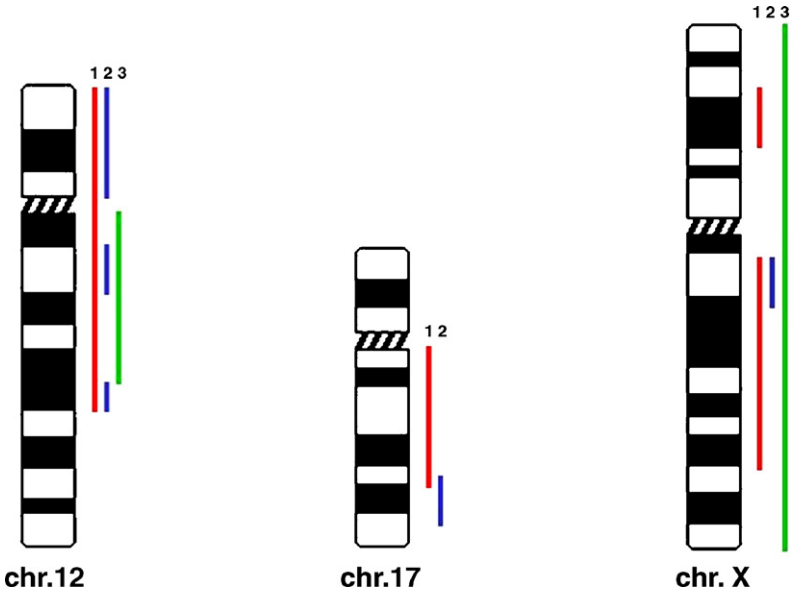

Fig. 2. Schematic figure showing relative gains in regions of chromosomes (chr) 12, 17, and X detected in three studies analysing CIS adjacent to overt tumours. On the right side of the chromosomes red, blue, and green bars show the extension of the gains observed in study 1, 2, and 3, respectively. Study 1: Looijenga et al. (2000); study 2: Ottesen et al. (2003); study 3: Summersgill et al. (2001).

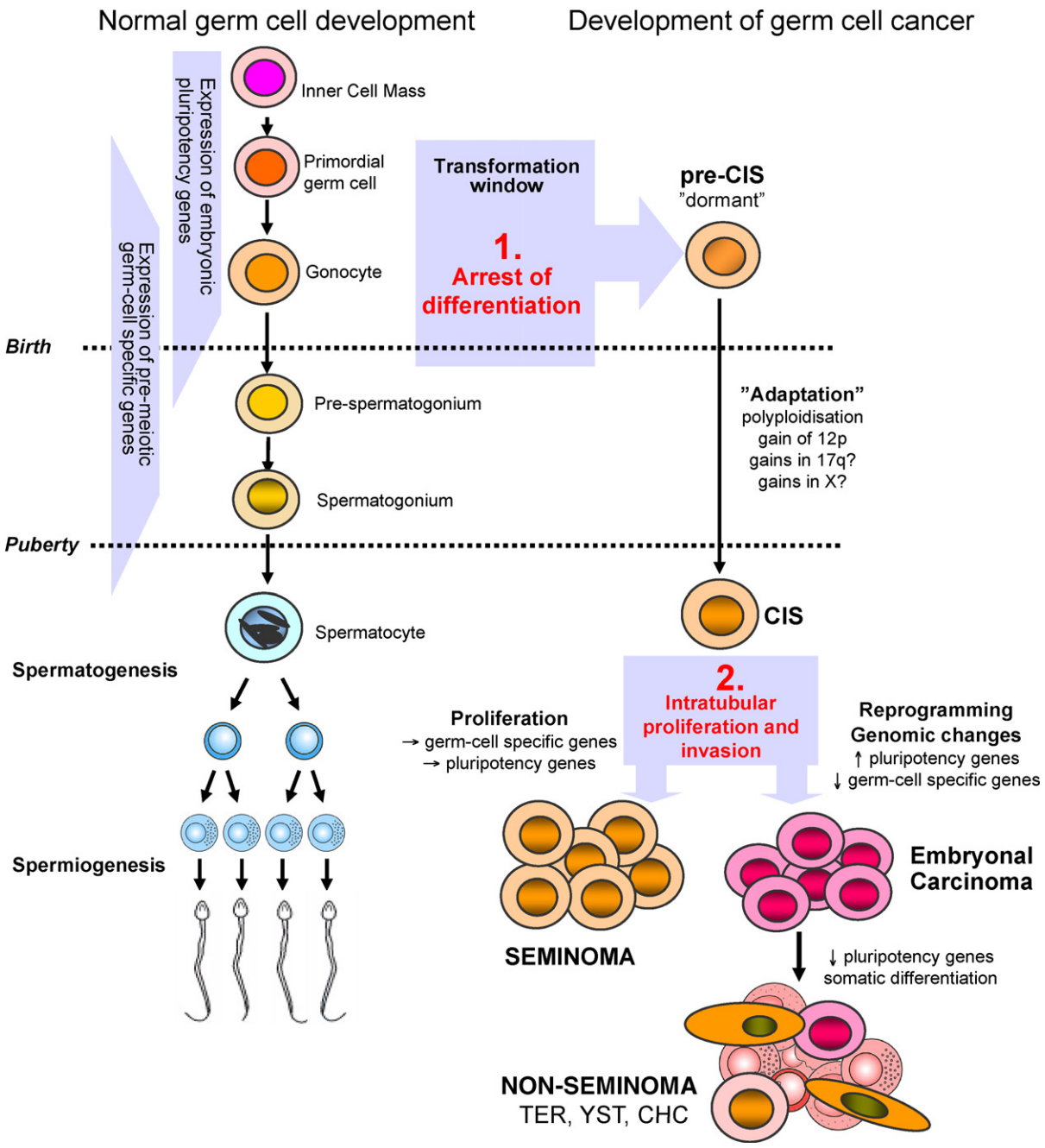

Fig. 3. A model of the origin of carcinoma *in situ* (CIS) of the testis in early development and hypothetical pathways of progression to overt testicular germ cell tumours (TGCTs). The key events in the origin and progression of TGCT are represented in red: (1) the arrest of differentiating primordial germ cells or gonocytes and (2) the increased invasiveness with (in embryonal carcinoma, EC) or without (in seminoma) loss of germ cell phenotype. In the case of non-seminomas, EC is the presumed stem cell compartment that may undergo down-regulation of the pluripotency genes and differentiate to somatic non-seminomatous components such as teratoma (TER), yolk sac tumour (YSC) or choriocarcinoma (CHC) (modified from Rajpert-De Meyts, 2006).

however a range of imbalances were detected, including gains of regions of the chromosomes 7, 8, 12, 14, 15, 17 and X (Looijenga et al., 2000; Summersgill et al., 2001; Ottesen et al., 2003, 2004a,b) (Fig. 2). A recurrent gain of 12p material was demonstrated in the vast majority of overt TGCTs, together with chromosomal aberrations established as typical for TGCTs (Atkin and Baker, 1982; Summersgill et al., 2001; and reviewed in von Eyben, 2004). Fascinatingly, the extra 12p material was also observed in several cases of CIS adjacent to overt tumours but was absent in CIS cells with no evidence of invasive growth (Looijenga et al., 2000; Ottesen et al., 2003, 2004a,b; Skotheim et al., 2006). Whereas gains of 17q were only detected in a few CIS cases (Ottesen et al., 2003, 2004b), a recurrent gain of 17q has been associated with non-seminomas in particular, perhaps illustrating the high proliferation potential of this more aggressive tumour (Kraggerud et al., 2002; Skotheim et al., 2002).

Gains in largely the same chromosomal regions are seen in cultured ES cells after prolonged culture indicating that the microenvironment may play a role in increasing genomic instability (Draper et al., 2004; Andrews et al., 2005; Baker et al., 2007). Evidence suggests that the ES cells 'adapt' through these genomic gains, proliferating faster and becoming easier to main-

tain with time in culture. In addition, when cells from such an 'adapted' culture were inoculated into a SCID (severe combined immunodeficient) mouse, a teratocarcinoma containing histologically recognisable stem cells was formed, which grew out when the tumour was explanted into culture and exhibited properties of the starting ES cells (Andrews et al., 2005). Therefore, the 'adapted' ES cells resembled the malignant non-seminoma EC cells. As such, ES cells show adaptive changes during culture which mimic changes occurring in CIS and later the derived EC cells during tumour progression. Thus, the non-random genomic aberrations seen in CIS cells may be caused by selection of cells with a proliferative advantage during tumour development and progression (Fig. 3), for example those with an over-expression of the pluripotency-related genes located on the short arm of chromosome 12 (Clark et al., 2004; Korkola et al., 2006). The simplistic hypothesis would be that only a few genes are involved, perhaps only one from each region amplified. However, the situation may be more complex, which is supported by the historical difficulty in identifying single genes that underlie common chromosome amplifications in various cancers, not only TGCTs (Baker et al., 2007). The fact that the microenvironment plays such a defining role in genomic instability in ES cells could indicate that the same is true during the formation of CIS cells. It is quite possible that a disturbance or disruption of the original niche of gonocytes or PG cells may have led to imbalance between proliferation and differentiation resulting in the genetic instability and neoplastic transformation. As time passes, the CIS cells also develop through selection, eventually resulting in the progression to a malignant seminoma or non-seminoma.

3. The CIS cancer stem cells

The pathogenesis of TGCTs in young men has not yet been elucidated in detail. However, it is obvious that unravelling the biology of CIS cells is fundamental for the understanding of the aetiology of TGCTs. The CIS cell represents an interesting variant of cancer stem cell since it originates before the tissue that it propagates in is fully differentiated and functional. The concept that cancers arise from 'stem cells' or 'germ cells' was already proposed about 150 years ago and recently, due to advances in stem cell biology, has been given a revival with the proposal of the 'cancer stem cell hypothesis' (reviewed in Wicha et al., 2006). The two related concepts of this hypothesis are that (i) tumours originate in either tissue stem cells or their immediate progeny through malfunctions in the process of self-renewal. As a result of this (ii) tumours contain so-called 'cancer stem cells' as a cellular subcomponent that retains key stem cell properties. These properties include self-renewal, which drives tumourigenesis, and differentiation that contributes to the cellular heterogeneity seen in most cancers. However, in contrast to TGCTs, the cellular heterogeneity is limited to the same tissue type in the vast majority of somatic cancers. Cancer stem cells are thought to function in a similar way as normal epithelial tissue stem cells that constantly replenish a tissue with committed differentiated cells not capable of self-renewal. Within the last couple of years experimental evidence in a variety of tumours has lent support to the cancer stem cell hypothesis that represents a paradigm shift in the understanding of carcinogenesis and tumour cell biology (Wicha et al., 2006).

The initiation of the neoplastic transformation generating CIS cells, which is discussed in detail below, is most likely triggered by a disturbance in the embryonic, fetal, or – perhaps in some cases – also early postnatal microenvironment of differentiating germ cells. Thus, CIS cells are different from the somatic cancer stem cells due to their origin in early development long before the tissue is fully differentiated and also in their ability to give rise to tumours with pluripotent potential. Regardless of these differences, CIS cells should be regarded as genuine cancer stem cells. The cells self-renew and are often found in the periphery of these solid tumours. The CIS cells are malignant cells that have the ability to invade all tubules in the testis and form solid seminomas tumours. In another cases, which tend to be relatively younger men than those who develop seminomas, CIS cells undergo a further development and give rise to non-seminomas, which include a variety of histologically different invasive tumours. This was already shown in the first description of the cells in 1972, where two patients with CIS were followed to the point where they developed invasive tumours (Skakkebaek, 1972b, 1978). Furthermore, later studies showed that patients with CIS, who seemed cured after chemotherapy, developed cancer after the treatment (von der Maase et al., 1988; Dieckmann, 1988; Dieckmann and Loy, 1991). This indicates that the cells have the capacity to repopulate the testis from a single or only few cells, which is a classic characteristic of cancer stem cells. Still, it is unresolved to what degree CIS cells make the transition to the invasive tumours and whether all CIS cells are destined to become invasive. It seems likely that only one or a few cells at a time make the transition to develop into an invasive cancer. However, testes with both seminoma and non-seminoma tumours separated by a non-malignant tissue are not uncommon, indicating a possible polyclonal origin of these tumours from at least two CIS cells.

Much remains unknown regarding the formation of solid tumours from CIS cells, especially the development of non-seminomas. The development of CIS cells to EC is particularly intriguing since it seems to encompass more than just a classic differentiation, and resembles a de-differentiation to a more original embryonic cell, mimicking cells from the inner cell mass of the early embryo.

4. Does disturbance of the stem cell niche result in development of CIS cells? The possible role of endocrine disrupters

The differentiating cells in the child are very susceptible to environmental damage, because development is a highly integrated process in which massive proliferation and extensive differentiation are synchronized with each other and with apoptosis (Birnbaum and Fenton, 2003). The extensive growth rates allow for mutagenic and epigenetic alterations. Likewise, differentiation represents a controlled process in which patterns of gene expression undergo large changes. Consequently, both cell division and differentiation offer multiple opportunities for the initiation of lesions as well as the promotion of the growth of altered cells initiating the complex process of developing a cancer. Moreover, physiological barriers protective of external factors such as the blood–brain barrier and blood–testes barrier are not complete *in utero*. Furthermore, the metabolising and elimination capabilities of the developing organism are not fully developed until after birth, leaving the fetus and newborn particularly susceptible to the adverse effects of environmental compounds (Birnbaum and Fenton, 2003).

Several recent studies have elucidated the mechanism of early differentiation of sexually bipotential primordial germ cells into the male cell lineage, however, much deeper insight into the early human embryogenesis is needed to understand the development of CIS cells. Rare genetic disorders of sex differentiation have yielded insight into the possible mechanisms of CIS cell formation. For example, in some individuals with mutations in *SRY* and 46,XY gonadal dysgenesis, CIS cells and/or gonadoblastoma may be seen in the streak or in severely dysgenetic streak gonads (Muller et al., 1992; Cools et al., 2006). In addition to rare instances of a mutation in *SRY*, a more commonly observed lack of the Y chromosome

in a subset of cells in the developing testis (a mosaic sex chromosome aneuploidy) would cause a focal lack of masculinisation and a varying degree of gonadal dysgenesis, often with the occurrence of gonadoblastoma or CIS. However, in the vast majority of cases of sporadic CIS/TGCT in adult men the signs of dysgenesis are very discreet but nearly always consistent with focal lack of proper virilization and gonadal maturation, manifested by clusters of distorted tubules and undifferentiated Sertoli cells (Hoei-Hansen et al., 2003; Nistal et al., 2006). These studies combined with lessons from disorders of sex differentiation and epidemiological observations support the notion that interference with early male development, either from genetic mutation or environmental factors, can lead to the development of CIS cells and hence neoplasia.

The differentiation of human gonocytes into infantile spermatogonia spans a relatively long period with a gradual down-regulation of the embryonic pluripotency genes and up-regulation of germ cell-specific genes with a role in spermatogenesis. Analysis of the CIS phenotype and the presence of CIS cells in certain conditions associated with testicular dysgenesis have provided strong evidence that developmental arrest in the differentiation of the early germ cell lineage is a core pathogenetic event leading to neoplastic transformation into CIS (Fig. 3) (Rajpert-De Meyts et al., 1996, 1998; Rajpert-De Meyts, 2006). Thus, it seems plausible that the long duration of differentiation leaves a window for disturbances in the hormonal balance that may topple the delicate balance and delay or arrest the differentiation of PG cells or gonocytes into infantile pre-spermatogonia. Moreover, the differentiation of germ cells occurs in an intimate contact with somatic cells in the microcellular "niche", which consists of Sertoli cells, Leydig cells, and peritubular myoid cells, and it is in these few cell types that hormonal and paracrine factors responsible for the differentiation process are produced. These cells are therefore the likely targets of environmental factors such as endocrine disrupters, which is supported by a clear association of germ cell neoplasia with insufficient function of the androgen-producing Leydig cells, and with decreased proliferation of Sertoli cells in association with small testes with reduced spermatogenesis (Holm et al., 2003). Problems with poorly developed or partially hyalinised tubule walls are also a common feature of CIS tubules. All these features can be reproduced in experimental animal models by exposure to selected endocrine disrupters, in particular various anti-androgenic compounds, including phthalates (Mylchreest et al., 1998; Fisher et al., 2003; Sharpe, 2006). There is still only indirect evidence from human studies, but the association of testicular cancer with exposures to some of these compounds has already been suggested by several studies, indicating that the development of CIS cells could be directly coupled to endocrine disruptors disturbing early male development (Hardell et al., 2003; Swan et al., 2005; Main et al., 2007).

5. Concluding remarks

The study of CIS cells has its focus on two major events: (i) the initial development *in utero* of the CIS cell and (ii) the progression of CIS cells in adulthood to an invasive malignant tumour. There is growing evidence that disturbances of the cellular microenvironment of developing germ cells, also known as their "niche", are of a primary importance in the development of the CIS cells and also in the origin of other disorders of testicular function, even those manifested in adulthood. As these disorders are becoming increasingly common, we must consider the negative impact of the modern lifestyle and strive to find the responsible factors together with the pathways they target, especially during early human embryogenesis. Numerous studies have demonstrated that germ cell tumours derived from CIS cells mimic embryonic development and constitute a model to study pathways of pluripotent cell differentiation. In this way germ cell tumours and ES cells are highly similar and it has been shown that during extended culture human ES cells recapitulate features of the malignant transformation seen in germ cell tumours. Hence, the knowledge obtained from studies of human ES cells *in vitro* can help to understand the mechanisms of neoplastic transformation of germ cells and invasive progression of tumours.

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