ABSTRACT

Title of Document: TOWARDS THE DERIVATION OF BOVINE EMBRYONIC STEM CELLS.

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The ability of embryonic stem cells (ESCs) to self-renew and differentiate into a wide range of cell types has encouraged researchers to attempt to isolate ESCs from embryos of domestic species for the past two decades. Success has been limited. The aim of the current study was to investigate whether colonies derived from inner cell masses (ICMs) of bovine blastocysts expressed the same markers of pluripotency and candidate genes representing the various signaling pathways as those found in human or mouse ESCs. The ability of selected cytokines to maintain the major transcription factors associated with pluripotency (NANOG, POU5F1 and SOX2) in the ICM explants was also tested. The results of the study showed that the three major transcription factors (NANOG, POU5F1 and SOX2) were expressed initially in culture but were lost with continued culture and passaging. Markers of differentiation (BMP4, HNF4, NCAM, and CDX2) were also expressed in the initial days of culture. The candidate genes representing the various signaling pathways were expressed in the initial days of culture as well as in subsequent passages. Noggin, a cytokine inhibiting the BMP4 pathway successfully up-

regulated the relative expression of NANOG in the ICM explants with respect to controls. The results indicate that signaling pathways associated with regulating pluripotency are expressed in ICM explants and that with cytokine supplementation pluripotency may be maintained. An alternate approach in which differentiating cells in the primary colonies were selectively ablated to eradicate cells secreting pro-differentiation signals was tested. Bovine embryos that carried the hygromycin resistance gene driven by the NANOG promoter were generated by SCNT. Any pluripotent colonies generated from these embryos should survive in the presence of hygromycin. When cultured in the presence of Noggin and hygromycin, colonies were generated; however they failed to proliferate on passaging. This suggests that the culture conditions were not optimal for the NANOG promoter to remain active over extended culture.

TOWARDS THE DERIVATION OF BOVINE EMBRYONIC STEM CELLS

By

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Dedication

To my loving husband.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BMP4	Bone Morphogenic Protein 4
cDNA	Complementary Deoxyribonucleic Acid
CDX2	Caudal Homeobox Gene 2
CK	Casein Kinase
CM	Conditioned Medium
Ct	Cycle Threshold
DMAP	Dimethyl Amino Purine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
Dsh/Dvl	Dishevelled
EC	Embryonic Carcinoma
EDTA	Ethylene Diamine Tetra Acetate
EG	Embryonic Germ
ESC	Embryonic Stem Cell
ESCM	Embryonic Stem Cell Culture Medium
FBS	Fetal Bovine Serum
FGF2	Fibroblast Growth Factor 2
FGFR	Fibroblast Growth Factor Receptor
FM	Feeder Medium
FOXD3	Forkhead Box Gene
Fz	Frizzled
GFP	Green Fluorescent Protein
GP130	Glycoprotein 130
GSK	Glycogen Synthase Kinase
hESC	Human Embryonic Stem Cell
HP	Hygromycin Phosphotransferase
ICC	Immunocytochemistry
ICM	Inner Cell Mass
Jak	Janus Kinase
LIF	Leukemia Inhibiting Factor
LIFR	LIF Receptor
LRP	Lipoprotein Related Receptor
LSM	Least Square Means

MEF	Mouse Embryonic Fibroblast
mESC	Mouse Embryonic Stem Cell
mRNA	Messenger Ribonucleic Acid
NH	Nanog Promoter Hygromycin Phosphotransferase
NHG	Nanog Promoter Hygromycin Phosphotransferase GFP
NT	Nuclear Transfer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGC	Promordial Germ Cells
PHA	Phytohemagglutinin
PI3K	Phosphoinositol 3 Kinase
PN	Pronuclear
POU5f1	Pit-Oct-Unc Class V factor 1
RE	Restriction Endonuclease
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SALL4	Sal-like 4 protein
SAS	Statistical Analysis System
SCNT	Somatic Cell Nuclear Transfer
SEM	Standard Error of the Mean
SOCS	Suppressor of Cytokine Signaling
SOE	Site Overlap Extension
SOF	Synthetic Oviductal Fluid
SOX2	SRY-related Homeobox Gene 2
SSEA	Stage Specific Embryonic Antigen
STAT	Signaling Transducer and Activator of Transcription
STO	Sandos inbred mice 6-Thioguanine and Ouabain resistant
TCM	Tissue Culture Medium
TGF	Transforming Growth Factor
TRA	Tissue Rejection Antigen
UTF-1	Undifferentiated embryonic cell Transcription Factor-1

Introduction

Embryonic stem cells (ESCs) are characterized by their ability to self-renew and capacity to give rise to a broad spectrum of differentiated cell types. Pluripotency is maintained during ESC self-renewal through the promotion of proliferation and the prevention of differentiation. ESCs can proliferate for extended periods of time, be manipulated genetically using recombinant DNA technology, be directed for targeted differentiation and have a capacity for germline transmission. These qualities have made ESCs an excellent tool for genetic engineering (Capecchi 1989) by virtue of which they been used extensively in investigations of functional genomics. As a result, these successes have stimulated research interest for the derivation of ES and ES-like cell lines from livestock and other laboratory species. Despite many efforts to derive ESCs from other mammalian species, ESCs that retain their capacity for germline transmission have only been verified in the mouse.

Promising results with hESCs and adult stem cells have nurtured hope for their potential use in regenerative medicine. However, such an application is still far from reality since substantial research is required to elucidate the yet unknown aspects of the basic biology of pluripotent cells, as well as safety issues associated with their use in therapy. In this context, the derivation, propagation and differentiation of ESC-like cultures from domestic animals as biologically relevant models has gained interest. ES-like cells derived from livestock can also potentially be used for creating transgenic livestock. The practical aspects of these animals include improvement in milk production and composition, increase in growth rate,

improved feed usage, improved carcass composition, increased disease resistance, enhanced reproductive performance and increased prolificacy. In addition, ESCs and ES-like cells are also being viewed as a tool for the production of tissues and organs for xenotransplantation. Partcular interest has been focused on pigs genetically modified with the aim to overcome immune rejection by the human host (Wobus and Boheler, 2005).

However the principle interest for creating transgenic animals is the production of genetically modified animals to serve as bioreactors for commercially important proteins such as Anti-thrombin III, Factor IX, α-antitrypsin (Ebert et al., 1991; Schnieke et al., 1997; Wright et al., 1991) to name a few. Despite the lower costs of producing biomolecules in microorganisms, like bacteria and yeast, these organisms do not properly execute several post-translational modifications, and correct folding in order to produce fully active human proteins (Melo et al., 2007). At the same time, the price of human biomolecules produced in vitro by mammalian cell culture is extremely high. This makes the creation of transgenic animals with the capability of secreting these products in their fluids potentially lucrative (Melo et al., 2007).

Traditionally, transgenic livestock have been generated employing the procedures of pronuclear (PN) microinjection and somatic cell nuclear transfer (SCNT). PN microinjection allows addition of DNA fragments to the genome however they integrate randomly (Wolf et al., 2000). Schnieke et al. (1997) showed that SCNT was more efficient for the production of founder animals (sheep) as compared to DNA microinjection. However, primary somatic cells used for SCNT

procedures have a limited lifespan in vitro and clonal selection and transfection of these cells further compromise cell vigor and usability for this purpose (Denning and Priddle, 2003). Bovine fetal fibroblast cells, which are commonly used to make transgenic cattle, have 30–50 population doublings before senescence (Polejaeva and Campbell, 2000). Clarke and coworkers (2000) have estimated that gene targeting requires around 45 population doublings in sheep. ESCs and ES-like cells with their capability to proliferate for extended periods of time would alleviate this problem.

Successful production of chimeric cattle was achieved when ES-like cells were isolated from early embryos, transfected with exogenous DNA, reintroduced into pre-implantation embryos. The transgenic cells were shown to contribute to tissues of the resulting calves; however, these ES-like cells did not contribute to the germline of these chimeric animals (Cibelli et al., 1998). Furthermore, ES-like cells thus far obtained have been difficult to passage or grow clonally which would hinder use of sophisticated genetic manipulations.

Efficient procedures for production of in vitro embryos in cattle make bovine embryos an abundant source for the derivation of ESC-like procedures. While numerous studies have attempted to derive ES cells from bovine embryos (Milatipova et al., 2001; Strelchenko 1996; Stice et al., 1996; Cibelli et al., 1998; Iwasaki et al., 2000; Saito et al., 2003; Wang et al., 2005), success has been limited. Whether the difficulties result from inadequate knowledge or inherent recalcitrance within the system is not known. The overall goal of this research was to investigate some of the factors which may be contributing to these hurdles.

The main objectives of this research were to determine whether;

1. Bovine embryos and explants express the same core of pluripotency determining factors as hESCs and mESCs,
2. Bovine explants respond to the same cytokines as either hESCs and mESCs, and
3. By selectively ablating differentiating cells, the proliferation and maintenance of pluripotent cells would be encouraged.

It is hoped that the findings of this research will provide insights into the reason that makes derivation and maintenance of ES-like cells from bovine embryos difficult. At the same time it is anticipated to provide leads that will enable the derivation of ESCs form bovine embryos possible.

Characteristics of Embryonic Stem Cells

There are three basic types of stem cells that exist in mammals: somatic, germinal and embryonal stem cells. Embryonic carcinomas (EC) cells were the first pluripotent cells isolated from teratocarcinomas; teratocarcinomas are complex tumors comprising of a mixture of germ cells and derivatives of all the three lineages: endoderm, mesoderm and ectoderm (Martin and Evans, 1975). As pluripotent cells, EC cells are capable of multilineage differentiation, but they have had limited applications due to frequent aneuploidy and restricted ability to colonize germ lines (Martin, 1980). Primordial germ cells (PGCs) are another pluripotent cell population. PGCs are isolated from the genital ridge of the post-implantation embryo (Shamblott et al., 2001; Resnick, 1992; Liu et al., 2004). Embryonic stem cells (ESCs) are pluripotent cells derived from pre-implantation embryos that are also capable of differentiating into all the three cell lineages as well as into germ cells. Murine ESCs were first derived by Evans and Kaufmann (1981) and Martin (1981) from the inner cell mass (ICM) of blastocysts and since then have also been derived from blastomeres of morulae (Tesar, 2005; Eistetter, 1989) and 8-cell embryos (Tesar, 2005; Delhaise et al., 1996) and primitive ectoderm of implantation-delayed blastocysts (Prelle et al., 2002).

The derivation of ESC lines entails diversion of the pluripotent epiblast or blastomeres from their fated differentiation. Most murine ESC lines have been isolated from embryos of the inbred strain 129 and its various sub-strains. Most other

mouse strains have been refractory to isolation of ESCs indicating a strong genetic component to ESC derivation (Kawase et al., 1994). At the same time, though these cells have demonstrated competence to form all cell types within the fetus, a strong ES cell contribution to the entire fetus (including embryonic derived placental tissues) following chimera formation has not yet been demonstrated in either mice or non-human primates. However, under certain limited conditions mouse ESCs can form trophectodermal cells in vitro (Ralston and Rossant, 2005) and in vivo (Beddington and Robertson, 1989), while human and other non-human primate ESCs can differentiate readily into trophectodermal cells (Thomson et al., 1995; Xu et al., 2002).

ESC lines are capable of sustained self-renewal and wide-ranging differentiation plasticity. They can be propagated clonally as a homogenous, uncommitted cell line without losing their pluripotency or stable karyotype for prolonged periods of time. Murine ESCs are capable of integrating into the early developing embryo even after extensive genetic manipulation, with the ESC descendants being represented among all cell types, including functional gametes (Kehler et al., 2005). These properties make murine ESCs an important tool for genetic engineering especially via homologous recombination to make precise modifications to the germline (Boiani and Scholer, 2005). Human ESCs share all the same properties except for the fact that the germline capability cannot be tested due to ethical considerations (Pera et al., 2000). However, unless germline transmission is proven in the pluripotent cells derived from embryos of species other than primates, they are generally referred to as ES-like cells. These wide-ranging properties of ESCs

have made them an important instrument in the study of developmental biology. The broad ranges of applications of mouse and human ESCs have generated strong interest among scientists working with other species such as cattle and pigs.

Creation of transgenic livestock

In farm animals, transgenic research is focused on production characteristics such as growth and body composition, lactation performance as well as disease resistance and immune resistance (Wheeler 2007). Transgenic farm animals have the potential to become important tools for biomedical research, either as disease models for production of therapeutic proteins, tissues or organs. Once available, ESCs from domestic species can potentially be used for the production of transgenic animals that will not only increase our understanding of basic developmental biology but also be important for biopharming. Production of transgenic livestock as 'biopharms' expressing proteins with potential therapeutic and commercial applications in their milk of livestock species was first demonstrated by Clark et al. in 1989. The most popular methods employed to generate transgenic animals is by the use of PN microinjection and somatic cell nuclear transfer (SCNT) technologies.

One of the techniques employed to generate a transgenic animals is pronuclear microinjection in which the DNA is inserted into the pronucleus of a fertilized oocyte. The success of pronuclear injection with respect to transgene integration ranges from around 1% for farm animal (cattle, pigs and sheep) to 3% for laboratory animals (mice, rats and rabbits) (Wall, 1996). This method usually results in mosaics in which not all cells of the animal contain the transgene. The time and cost of screening for

germline transmission in mosaic animals such as cattle can be substantial. Also there is a high variability in transgene expression between animals not only due to mosaicism but also to chromosomal position effects as a result of the random integration of the transgene. Microinjection is also limiting as it only allows for the random addition of exogenous DNA rather than targeting to specific sites (Hodges and Stice, 2003).

Somatic cell nuclear transfer (SCNT) is a technique that can be used to create a transgenic animal. It involves the transfer of a donor nucleus into the cytoplasm of an enucleated oocyte. Prior to SCNT, donor cells are transfected, propagated and tested for the incorporation of the transgene by molecular techniques. SCNT allows for not only the addition of DNA at random sites but also targeted insertion of DNA by homologous recombination which enables modulation of specific gene expression and creation of gene knockouts. The success rate for SCNT averages between 1-3% in most animals including cattle (Solter, 2000). A large number of transplanted embryos are lost during pregnancy and perinatal development. These neonatal losses are not due to any one anomaly but rather complications that can range from increased birth weight, pulmonary abnormalities, respiratory problems, to metabolic deficiencies and placental abnormalities (Hodges and Stice, 2003).

Other popular methods of generating transgenic mice include the use of viral vectors such as replication-defective retroviruses (Xia et al. 2007) and adenoviruses (Tsukui, 1996), which have a high efficiency of stable single copy integration. In addition, mammalian spermatozoa which have the ability to bind exogenous DNA molecules (Brackett et al. 1971) as well as internalize them have been used as carriers

of DNA, although the results of sperm-mediated gene transfer are controversial due to the low efficiency and repeatability (Gandolfi, 2000).

Ever since it was demonstrated that a targeted mutation could be introduced into ESCs by homologous recombination (Thomas and Capecchi, 1987) and germline transmission of a targeted mutation could be obtained allowing chimera production (Thompson et al., 1989), ESCs have become the most popular method for creating transgenic mice. Due to their rapid proliferation, ESCs provide an inexhaustible supply of cells for genetic manipulation. Individual transfected clones can be screened in vitro for integration and expression of exogenous DNA construct before creating germline chimeric animals (Wheeler et al., 1995), which increases the efficiency of producing transgenics. Numerous endogenous genes have been targeted by homologous recombination in pluripotent ESCs in culture (Ma et al., 2003; Pfiefer et al., 2002; Stanford et al., 2001). Unfortunately, despite intensive efforts, this technology is limited to the mouse as no germline competent ESCs have been described for any other mammalian species.

Current status of embryonic stem cell research in domestic species

Efforts to derive ESCs from pig, goat, sheep and horse have most often been attempted with in vivo blastocysts. Peer-reviewed reports of porcine ES, ES-like or ICM cell lines have been published, using in vivo-derived (Notarianni et al., 1990, 1991; Piedrahita et al., 1990; Hochereau-de Reviers and Perreau, 1993; Wheeler, 1994; Chen et al., 1999; Li et al., 2003, 2004), as well as, in vitro produced (IVP) embryos (Li et al., 2004). Most attempts at deriving ESCs from bovine pre-

implantation embryos has involved in vitro-produced embryos that have lesser developmental competence than in vivo derived embryos (Bavister, 2004). Production of ovine (Notarianni et al., 1991; Zhua et al., 2007), equine (Saito et al., 2002) and caprine ES-like cell lines has also been reported (Keefer et al., 1996). However, the few attempts with in vivo derived embryos from bovine and other species have not had any more success. Similarly, a few ES-like cell lines from rodents other than the mouse (hamster, Doetschman et al., 1988; rat, Iannaccone et al., 1994; Vassilieva et al., 2000) and from rabbit (Schoonjans et al., 1996) have been reported. At the same time, there are several reports of embryonic germ (EG) cell lines derived from pig, goat and bovine PGCs derived from primordial germ cells found in the early genital ridge tissue, (Piedrahita et al., 1997; Shim et al., 1997; Mueller et al., 1999; Tsung et al., 2003; Rui et al., 2004).

Establishment of ESCs from ungulate embryos including bovine has been problematic. There are several published reports of bovine ES or ES-like cell lines from IVP early blastocyst-staged embryos (Saito et al., 2002, 2003; Sims and First, 1994; Cibelli et al., 1998; Iwasaki et al., 2000; Mitalipova et al., 2001; Wang et al., 2005). Some of these bovine ES-like cell lines have been reported to exhibit pluripotency both in vitro and in vivo. However, teratoma formation in immunocompromised mice has rarely been reported for putative ungulate ESC lines, with a few exceptions (Hochereau-de Reviers and Perreau 1993; Anderson et al. 1996). While ES-like cells can be used in SCNT to create cloned animals (Keefer et al., 1994; Stice et al, 1996; Chen et al., 1999; Saito et al., 2003), few of the putative ES-like cells morphologically resemble mESCs or express markers that are normally

associated with ESC lines (Mitalipova et al., 2001; Stice et al., 1996; Cibelli et al., 1998; Saito et al., 2003). For instance, POU5F1 is found to be associated with the pluripotency of ES-like cells in many species, however, few of the previously reported bovine ES-like cell lines were POU5F1 positive (Mitalipova et al., 2001; Stice et al., 1996; Cibelli et al., 1998; Saito et al., 2003) except for one report by Wang et al. (2005) where they found NT derived ES-like cell lines positive for POU5F1. However, no such results were reported for IVP bovine embryos in that study. Furthermore, POU5F1 protein is expressed in both the ICM and trophectoderm of ungulates, i.e., pigs, cattle and goats (van Eijk et al., 1999; Kirchhof et al., 2000; He et al., 2004). Cell surface markers, SSEA1 and SSEA4 have been shown in ICM and trophectoderm of caprine embryos (He et al., 2006) and in ICM cultures of NT embryos (Wang et al., 2005). NANOG, a key marker of pluripotency is found in the ICM caprine blastocysts (He et al., 2006) but there are no such published reports for expression of NANOG protein in bovine embryos.

ESCs are commonly derived from preimplantation embryos by placing a hatched blastocyst or zona-free pre-blastocyst stage embryos on a feeder layer and thereafter sub-culturing the ES-like cells. Another method employed for isolation of the ICM from the blastocyst is immunodissection, where the trophectoderm of the blastocyst is targeted and destroyed using antibodies. Immunodissection is the preferred method for derivation of hESC (Trounson 2006; Pera et al., 2000) where persistence of trophectoderm is a problem whereas whole embryo culture is usually employed for derivation of mESC (Bryja et al., 2006). Murine ESCs are propagated by enzymatically dissociating colonies and plating individual cells for new colony

formation (Thomson et al., 1998; Evans and Kaufmann, 1981). Human ESCs are also routinely passaged by enzymatically dispersing the cells with dispase or collagenase (Yao et al., 2006). However, bovine ES-like cells fail to form colonies after enzymatic disassociation with trypsin, collagenase, protease etc. (Mitalipova et al., 2001; Cibelli et al., 1998; Wang et al., 2005). Trypsin is the only reported enzyme that dissociates bovine ES-like cells, but it also causes a failure of these cells to self-renew and to induce spontaneous differentiation (Wang et al., 2005). This refractoriness of ES-like cells to enzymatic dissociation has made it difficult to pursue a clonal propagation of most ES-like cells derived from domestic species including bovine ES-like cells.

Signaling pathways and transcription factors in stem cell biology

Transcription factors: major players

POU5F1 (Oct3/4 or Oct4)

POU5F1 is encoded by the POUF51 gene and is a member of the POU (Pit-Oct-Unc) transcription factors. In mESCs DNA binding is mediated by the 75-amino acid POU-specific domain (POU_S) and the 60-amino acid carboxy-terminal POU homeodomain (POU_{HD}). POU5F1 binds to the octamer motif ATGCAAAT. A proximal enhancer (located about 1.2 kb upstream) is responsible for POU5F1 expression in the epiblast, and a distal enhancer region (located about 2 kb upstream) drives expression in the morula, ICM, and primordial germ cells. This distal enhancer is also required for ESC-specific expression (Yeom et al., 1996). Orthologs of POU5F1 share a high

degree of genomic structural organization and sequence conservation across other mammalian species including bovine, human and mouse (van Eijk et al., 1999).

Prior to zygotic gene activation, maternally derived POU5F1 mRNA can be detected in the ovum through the four cell stage in mouse and 8 cell stage in bovine embryos (Kurosaka et al., 2004). During mouse pre-implantation development, POU5F1 zygotic expression is initiated at the four-cell stage and is later restricted to the pluripotent ICM cells of blastocysts and, thereafter, to the epiblast and finally to the germ cell lineage (Boiani et al., 2002; Yeom et al., 1996).

POU5F1 is expressed also in human and mouse ESCs, and its expression diminishes when these cells differentiate and lose pluripotency (Ginis et al., 2004). A knockdown of POU5F1 expression in mESCs correlates with induction of trophectoderm genes CDX2, HAND1, and PL-1, with formation of cells with trophoblast giant cell phenotype. Contrarily reduction of its expression leads to expression of endodermal markers such as Gata6 in both mES and hESCs (Hay et al., 2004). Maintaining POU5F1 expression within a certain range appears to be critical for mESC renewal, with an increase or decrease beyond the threshold of 50% triggering differentiation to endoderm and mesoderm or to trophectoderm, respectively (Niwa et al., 2000). However, POU5F1 expression alone is not sufficient to maintain the undifferentiated phenotype and requires a co-operative signal provided by LIF stimulation in mESCs.

Numerous target genes of POU5F1 in ESCs have been identified, and these include FGF4, UTF1, OPN, REX1/ZFP42, FBX15, and SOX2 (Chew et al., 2005; Table 1A). The POU octamer elements within the enhancers of the above mentioned

genes are found in proximity to SOX2-binding elements. Both POU5F1 and SOX2 are expressed in ESCs and are capable of forming heterodimers both on and off the DNA. A composite SOX2-POU5F1 cis-regulatory site on the NANOG promoter and has been determined to be necessary for pluripotent expression and both SOX2 and POU5F1 have been shown to bind this module both in vitro and in mouse and human ESCs (Kuroda et al. 2005; Rodda et al., 2005; Okumura-Nakanishi et al., 2004). POU5F1 and SOX2 regulate the expression of NANOG, and POU5F1 is required for the efficient binding of SOX2 to the NANOG promoter (Rodda et al., 2005; Kuroda et al., 2005; Figure 1). These three transcription factors, NANOG, POU5F1 and SOX2 work in concert to regulate expression of genes in pluripotent cells and a substantial proportion of the POU5F1-bound genes (44.5%) have been demonstrated to be occupied by both NANOG and POU5F1 (Loh et al., 2006).

SOX2 (SRY-related HMG box 2)

SOX2 is a transcription factor belonging to the SRY-related HMG (high mobility group) box containing gene family and is a transcription factor essential for pluripotent cell development (Avilion et al., 2003). It has an expression pattern similar to that of POU5F1 during mouse pre-implantation development, as it is expressed in all blastomeres of the four-cell embryo and becomes restricted to the ICM and epiblast of the blastocyst (Avilion et al., 2003). SOX2 is required to maintain cells of the epiblast in an undifferentiated state, and in its absence they change their identity, becoming trophectoderm or extra-embryonic endoderm (Avilion et al., 2003). SOX2-null cells differentiated into trophoectoderm-like cells (Masui et

al., 2007; Figure 2). Two regulatory regions (SRR1 and SRR2) in SOX2 are known to confer ESC-specific expression (Tomioki et al., 2002). SRR2, located 1.2 kb downstream of the transcription start site, contains the composite POU5F1-SOX2 element. It acts synergistically with POU5F1 and silencing of POU5F1 or SOX2 leads to the down-regulation of POU5F1 and SOX2 enhancer activities and reduction in the endogenous transcripts and proteins (Chew et al., 2005). However, involvement of multiple Sox factors such as Sox4, Sox11 or Sox15 in activation of SOX2-POU5F1 enhancers in ESCs has shown that SOX2 function can be redundant (Masui et al., 2007) and they can functionally replace Sox2. However, SOX2 is necessary for regulating multiple transcription factors that affect POU5F1 expression and forced expression of POU5F1 rescues the pluripotency of SOX2-null ESCs. These results indicate that the essential function of SOX2 is to stabilize ESCs in a pluripotent state by maintaining the requisite level of POU5F1 expression. On the other hand, Boer et al. (2007) demonstrated that elevating SOX2 levels inhibits the endogenous expression of five SOX2:POU5F1 target genes (SOX2, FGF-4, NANOG, UTF1 and POU5F1) that are regulated by closely spaced HMG and POU motifs (referred to as an HMG/POU cassette), which bind SOX2 and POU5F1, respectively (Table 1B). In addition, SOX2 repression is dependent on the binding sites for SOX2 and POU5F1. Although over-expression of POU5F1 and NANOG also inhibits their own promoter, their over-expression does not appear to broadly inhibit the promoters of other SOX2:POU5F1 target genes.

NANOG

NANOG is a divergent NK2 homedomain (HD) transcriptional factor that functions to maintain self-renewal of embryonic stem (ES) cells (Mitsui et al., 2003; Chambers et al., 2003). In mouse embryos, NANOG mRNA is detectable as early as the morula stage. Its expression is prominent in the inner cell mass of the blastocyst (Palmieri et al., 1994; Avilion et al., 2003). After implantation, it is detectable at embryonic day 6 in the proximal epiblast in the region of the presumptive streak and, thereafter, in the pluripotent cells of the nascent gonad at E11.5-E12.5 (Hart et al., 2004). NANOG expression is restricted to pluripotent tissues, ESC lines and human germ cell tumors (Hart et al., 2005) and is dramatically reduced by retinoic acid-induced differentiation. NANOG over-expression in hESCs enables their propagation for multiple passages during which the cells remain pluripotent (Darr et al., 2006; Chambers et al., 2003). Its over-expression in mESCs renders them independent of LIF supplementation (Chambers et al., 2003; Mitsui et al., 2003) as well as resistant to differentiation by retinoic acid (Loh et al., 2006). Reduction in NANOG expression correlates with induction of extraembryonic endoderm genes GATA4, GATA6, and laminin B1, with subsequent generation of groups of cells with parietal endoderm phenotype (Hough et al., 2006; Figure 2). A similar cell type is formed upon ectopic GATA6 expression in mESCs (Fujikura et al., 2002) raising the possibility that NANOG may prevent primitive endoderm differentiation via GATA6 repression. Lin et al. (2004) reported that tumor suppressor p53 promoted differentiation of ESCs by suppressing NANOG expression. The p53 protein can also bind to the NANOG promoter after DNA damage to ESCs resulting in suppression of NANOG expression

and triggering differentiation to maintain genomic stability. NANOG over-expression has also been shown to cause proliferation of NIH3T3 cell by promoting them to enter into S phase (Zang et al., 2006).

The NANOG promoter region has two transcription start sites and has binding sites for POU5F1 (Wu et al., 2005). Analysis of mouse and human NANOG revealed that the C-terminal domain is responsible for trans-activation (Pan et al., 2005; Oh et al., 2005). Although NANOG and POU5F1 have discrete functions in self-renewing ESCs, NANOG cannot function in the absence of POU5F1, suggesting interdependent modes of action (Chambers et al., 2003).

Although NANOG has been shown to be positively regulated by POU5F1 and SOX2, there is evidence that they are not the only players involved in its regulation (Chambers et al., 2003). It was found that greater than 90% of promoter regions of various genes that are bound by both POU5F1 and SOX2 are also occupied by NANOG and their binding sites are in close proximity to each other. Together they co-occupy the promoter regions of transcription factors (eg. POU5F1, SOX2, NANOG, STAT3, etc.), members of the TGF-β and WNT signaling pathways, genes involved in differentiation into various lineages, and genes encoding components of chromatin remodeling and histone-modifying complexes (Boyer et al., 2005). Loh et al. (2005) proposed that NANOG sustains self-renewal and the undifferentiated state through the modulation of POU5F1 and SOX2 levels (Figure 1). These two transcription factors in turn control the downstream genes important for maintaining pluripotency or inhibiting differentiation. In addition, NANOG also controls important molecular effectors of ESC fate by regulating genes transcribing histone

methyltransferases, telomeric proteins and those responsible for transcriptional repression in the epiblast.

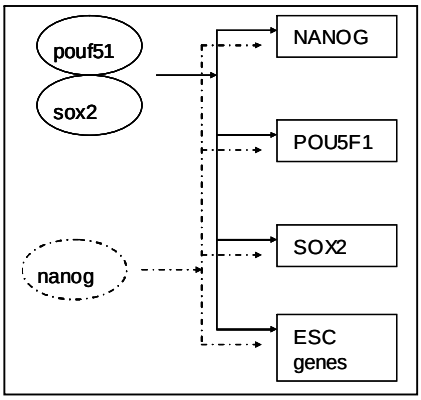

Figure 1: Transcriptionally regulated circuitry in ESCs. The transcription factors NANOG and the dimerized form of POU5F1 and SOX2 regulate the transcription of their own genes as well of other genes expressed in ESCs. Adapted from Boyer et al., 2005. The solid arrows indicate the genes regulated by the Pou5f1:Sox2 dimer and the dotted lines indicate the genes regulated by Nanog.

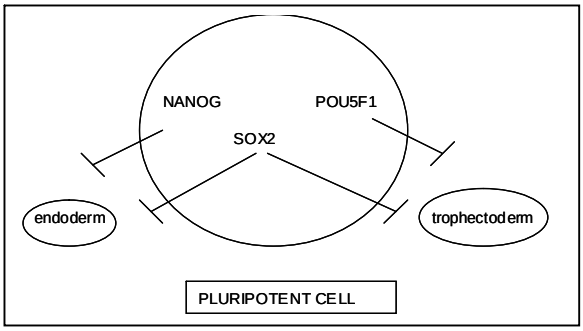

Figure 2: Role of transcription factors in maintenance of pluripotency. NANOG, POU5F1 and SOX2 function by preventing the pluripotent cell from differentiating into specific lineages and, thereby, maintaining self-renewal of ESCs.

Table 1 A: Genes regulated by POU5F1

Genes	Function	Reference
FGF4	Early embryonic development	Dailey et al., 1994
UTF1	Transcription co-activator/repressor, chromatin associated	Nishimoto et al., 1999; van den Boom et al., 2007
OPN	Negatively regulates the pool size of hemapoetic stem cells in bone marrow	Botquin et al., 1998
REX1	Zinc-finger protein	Rosjford et al., 1994
FBX15	Phosphorylation-dependent ubiquitination	Tokuzawa et al., 2003
SOX2	Transcription activator, maintains pluripotency	Catena et al., 2004
FGFR4	Early embryonic development	McDonald and Heath, 1994
FOXD3	Trophoblast progenitor cell differentiation	Hanna et al., 2002

Table 1B: Genes regulated by POU5F1:SOX2 dimer

Genes	Function	Reference
NANOG	Transcription activator, maintains pluripotency	Rodda et al., 2004
UTF1	Transcription co-activator/repressor, chromatin associated	Nishimoto et al., 1999;
OPN	Negatively regulates the pool size of hemapoetic stem cells in bone marrow	Botquin et al., 1998
SOX2	Transcription activator, maintains pluripotency	Tomioka et al., 2002
FGF4	Early embryonic development	Yuan et al., 1995
POU5F1	Transcription activator, maintains pluripotency	Chew et al., 2005

Transcription factors: minor players

REX1: REX1 is a developmentally regulated acidic zinc finger protein gene (ZFP-42). REX1 mRNA is detected in a limited range of cells and tissues: undifferentiated ESCs and EC cells, mouse blastocysts including trophectoderm, and meiotic germ cells of the adult mouse testis (Rogers et al., 1991). . Knockdown of NANOG in embryonic stem cells results in a reduction of REX1 expression. NANOG, POU5F1 and SOX2 can transactivate REX1 promoter (Ben-Shushan et al., 1998; Shi et al., 2006). Though REX1 has been shown to be regulated by pluripotency related transcription factors, it has not yet been demonstrated to influence transcriptional factor networks or signaling pathways in ESCs.

SALL4: SALL4 is a member of spalt-like protein family. It is a zinc finger protein thought to act as a transcription factor. It is downstream of the WNT pathway and is regulated by TCF/LEF1 (Bohm et al., 2007). It is also known to be expressed predominantly in the ICM of early mouse embryos (Yoshikawa et al., 2006), in embryonic carcinoma cells and in the adult testis and ovary. Disruption of both alleles of SALL4 leads to embryonic lethality during peri-implantation stage (Kohlhase et al., 2002). Wu et al. (2006) showed that SALL4 null-ESCs also have reduced proliferation in vitro. Furthermore, SALL4 bound to NANOG, POU5F1 and SOX2 upstream regulatory sequences. These early results show that SALL4 is involved in transcription factor network in ESC; however, its role needs to be investigated further.

FOXD3: FOXD3, a member of the forkhead family of transcriptional regulators, is required for maintenance of embryonic cells of the early mouse embryo. FOXD3 expression is detected during early embryogenesis in the epiblast and later in neural crest cells (Dottori et al. 2001). It has been implicated in the control of differentiation in multiple systems (Hanna et al., 2002). FOXD3 null embryos die after implantation at approximately 6.5 days postcoitum with a loss of epiblast cells. Moreover, it has not been possible to establish FOXD3 null ESC lines or to generate FOXD3 null teratocarcinomas (Hanna et al., 2002).

Factors and inducers of pluripotency

Recent studies have further investigated the induction and maintenance of pluripotency by attempting to induce pluripotency in differentiated mouse fetal and adult cells by introducing four selected transcription factors, POU5F1, SOX2, c-MYC and KLF-4 (Takahashi et al., 2006; Okita). The authors started with a panel of 24 transcription factors and narrowed it down to the above mentioned four factors based on the ability of the transcription factors to maintain pluripotency. The retroviral introduction of these factors transformed the cells into an ES-like state in terms of morphology, proliferation and teratoma formation (Takahashi et al., 2006). Using improved selection strategies it was possible to obtain germline-competent iPS cells (induced pluripotent cells) which exhibited increased ESC-like gene expression and DNA methylation patterns (Okita et al., 2007; Meissner et al., 2007). Another study by Yu et al. (2007) with a similar set of transcription factors (OCT4, SOX2, NANOG, and LIN28) showed that introduction of these factors is sufficient to reprogram

human somatic cells to pluripotent stem cells to exhibit the essential characteristics of hESCs.These were landmark studies in terms of attempting to unravel what a pluripotent state entails.

Signaling pathways

LIF-Jak STAT Pathway

Mouse ESCs have historically been maintained in a co-culture with mitotically inactivated mice fibroblast (Evans and Kaufmann, 1981; Martin, 1981). Supplementation with LIF (leukemia inhibitory factor) eliminated the need for the co-culture system (Smith et al., 1988). LIF signaling is largely, though not wholly responsible for maintenance of pluripotency in mESCs. LIF is a member of the LIF-oncostatinM-Il-6 superfamily of cytokines. It acts by engaging a heterodimeric cell surface receptor complex comprising the LIF receptor subunit (LIFR; Gearing et al., 1992) and glycoprotein 130 (GP130; Davis et al., 1993). A family of related cytokines, including cardiotrophin 1, oncostatin M and ciliary neurotrophic factor, that interact with the LIFR/GP130 complex can substitute for LIF and support ESC self-renewal (Boiani and Scholer, 2005).

Dani et al., (1998) demonstrated that embryos lacking LIFR or GP130 can develop beyond gastrulation, which suggests the existence of an alternative pathway(s) governing the maintenance of pluripotency in vivo. They generated mESCs in which both copies of the LIF gene were deleted. Though these cells showed a significantly reduced capacity for regeneration of stem cell colonies, self-renewal was not abolished and undifferentiated ESC colonies were still obtained in the complete absence of LIF. $LIF^{-/-}$ embryos can survive beyond implantation in a normal uterus; however $LIF^{-/-}$ females fail to support embryo implantation (Dani et

al., 1998). In the absence of maternal LIF, blastocysts fail to implant and enter a stage similar to that seen during delayed implantation (Stewart et al., 1994).

LIF binding to a LIFR induces LIFR-GP130 heterodimerization which results in the activation of receptor-associated kinases of the Janus family (Jak). Activated Jaks phosphorylate specific tyrosines on GP130 signaling complex creating docking sites for proteins on the activated receptor complex (Matsuda et al., 1994). When GP130 is phosphorylated, several signaling pathways are activated involving STAT 1 and 3 including the extracellular signal receptor kinases (ERK1 and 2) and the phosphatidylinositol-3 kinases (PI-3K) (Figure 3; Cavaleri and Scholer, 2003). In addition, LIF induces SOCS (suppressor of cytokine signaling) proteins which are negative feed-back inhibitors. The transcription of SOCS inhibits the tyrosine phosphorylation of GP130 and STAT3 (Heinrich et al., 1998).

The stimulation of Ras/Raf/MEK/ERK signaling pathway by LIF leads to differentiation of mESCs (Figure 3; Burdon et al., 1999). Interference with this pathway by mutation of Grb2 or Shp2, inhibition of the activation of MEKs with the inhibitors PD98059 and UO126, or by dephosphorylating ERKs by mitogen activated protein kinase phosphatase 3 (MKP-3), promotes self-renewal by limiting differentiation (Burdon et al., 1999).

In absence of LIF signaling, induced either by LIF withdrawal or by the expression of a dominant interfering form of STAT3, mESCs differentiate into a morphologically mixed population of endoderm and mesoderm (Niwa et al., 1998). Constitutive activation of STAT3 in mESCs eliminates the requirement of LIF in mESC for maintenance of pluripotency (Matsuda et al., 1999). The inhibition of the

MEK/ERK pathway enhances the propagation of mESCs (Burdon et al., 1999) and facilitates the isolation of ESCs from normally refractory murine CBA blastocysts (Lodge et al., 2005).

Human ESCs express LIF, IL-6, and GP130 receptors, as well as the downstream signaling molecules. Although stimulation of hESCs with GP130 cytokines results in a robust phosphorylation of downstream ERK1, ERK2, and Akt kinases, as well as the STAT3 transcription factor, the activation of STAT3 is insufficient to maintain hESCs an undifferentiated state. Continuous receptor or STAT3 activation is not sufficient to block hESC differentiation (Humphrey et al., 2004; Daheron et al., 2004) demonstrating that this pathway is not sufficient for maintenance of pluripotency in hESCs.

Bovine LIF (bLIF) has been cloned and used in culture (Yamanaka et al., 1999, 2001), but there is no commercially available bLIF. Therefore, most researchers have used human LIF (hLIF) to supplement the culture medium for bovine embryos and colonies derived from them because of its greater sequence homology compared to murine LIF (mLIF). While supplementation of embryo culture media with bLIF has been described to increase TE cell counts without affecting the ICM (Yamanaka et al., 1999, 2001), hLIF has been noted to increase (Sirisathien et al., 2003; Funston et al., 1997), decrease (Vejlsted et al., 2005) or have no effect on the ICM (Rodrigues et al., 2006) of the bovine blastocyst. At the same time, bovine ES-like cells have been derived in the presence hLIF (Saito et al., 2003) and the absence of exogenous LIF (Mitalipova et al., 2001). Furthermore, the generation of cell colonies from blastomeres has been demonstrated to not be

influenced by exogenous hLIF (Vejlsted et al., 2005; Rexroad et al., 1997). Based on the puslished results, it appears that bovine pluripotent cells resemble hESCs in terms of their response to LIF and that LIF does not seem to play a role in the maintenance of pluripotency in bovine ES-like cells.

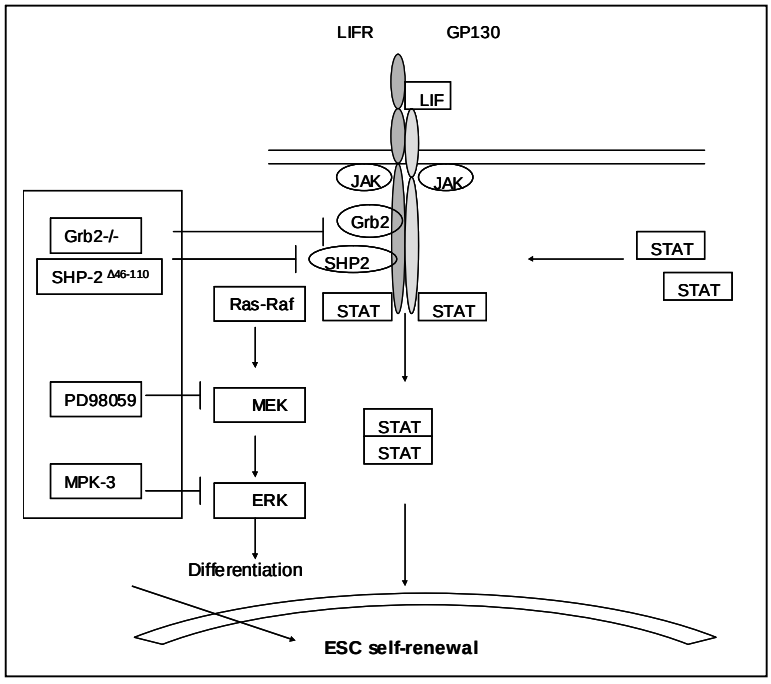

Figure 3: LIF regulated pathway in mESC. LIF binds to LIFR and GP130 bringing the Janus kinases in proximity allowing them to phosphorylate each other, thereby further facilitating the recruitment and phosphorylation of STATs. The activated STATs dimerize and translocate to the nucleus where they cause transcription of other genes. Binding of LIF to these receptors also stimulates the MEK/ERK pathway that leads to differentiation. Inhibition of this pathway enables self-renewal of mESCs. Adapted from Burdon et al., 2002.

TGF-β (Transforming Growth Factor) Superfamily

The TGF-β superfamily has been shown to play an important role in the maintenance of pluripotency in mESCs and hESCs. The TGF-β superfamily exhibit two distinct modes of the ligand-receptor interaction: one exemplified by members of the BMP subfamily and the other represented by TGF-βs and Activins. BMP ligands such as BMP2 and BMP4 exhibit a high affinity for the extracellular ligand binding domains of the type I BMP receptors. The preassembled type I receptor-ligand complex binds the type II receptor (Shi and Massague, 2003). In contrast to the BMPs, TGF-β and Activin bind tightly to the type II receptor allowing the subsequent incorporation of the type I receptor, forming a large ligand-receptor complex involving a ligand dimer and four receptor molecules. Binding of the dimeric ligand to both receptors facilitates the phosphorylation and subsequent activation (phosphorylation of multiple serine and threonine residues in the GS region) of the type I receptor by the type II receptor kinases (Shi and Massague, 2003).

The intracellular messengers downstream from the activated receptors are the Smad proteins which can be divided into three classes: (1) receptor-mediated Smads (R-Smads; Smad 1, 5 and 8) that are phosphorylated in a ligand-specific manner by activated receptor complexes, (2) the common mediator Smad (co-Smad; Smad 4), and (3) the inhibitory Smads (I-Smads; Smad 6 and 7) that negatively regulate the Smad signal transduction pathway. The R-Smads on phosphorlyation form a complex with Smad 4 and the complex translocates to the nucleus where it can bind directly, or through transcriptional partners, to specific sequences in the promoters of target genes to regulate transcription (Varga and Wrana, 2005; Figure 4).

Among the three classes of Smads, only R-Smads are directly phosphorylated and activated by the type I receptor kinases. Smad2 and Smad3 respond to signaling by the TGF-β subfamily (which includes TGF-β, Activin, nodal etc.) and Smads 1, 5, and 8 primarily to signaling by the BMP subfamily (which includes BMP 2/4). In the basal state, R-Smads are predominantly localized in the cytoplasm, whereas the I-Smads tend to be nuclear. Smad4 is distributed in both the cytoplasm and the nucleus. After receptor activation, the phosphorylated R-Smads are translocated into the nucleus (Figure 4). Dephosphorylation by phosphatases as well as ubiquitination by ubiquitin ligases, leads to the termination of Smad signaling (Shi and Massague, 2003).

The access of TGF-β ligands to their receptors is restricted by a diverse group of soluble proteins that act as ligand binding traps, sequestering the ligand and barring its access to membrane receptors. Noggin is employed to inhibit the BMP4 induced signaling cascade. It mediates its effect by competively binding to BMP receptors thereby obstructing BMPs to bind to them.

BMP4 has been shown to act synergistically with LIF and prolonged the self-renewal of mESCs in serum-free medium (Ying et al., 2003).The requirement of serum during clonal expansion and *de novo* derivation of mESCs and has been shown to be replaceable by BMP4 (Ying et al., 2003). However, the effect of BMP4 on self-renewal is dependant on the presence of LIF. In its absence, BMP4 is a strong inducer of mesodermal differentiation. In contrast, without BMP4, neural differentiation ensues; hence it appears that BMP4 blocks neural differentiation. BMP2/4 stimulates the transcription of Id (inhibitor of differentiation) genes (Hollanagel et al., 1999;

Ying et al., 2003) and constitutive expression of Id1 circumvents the requirement for BMP4 (Ying et al., 2003). Id family members encode negative regulators of the basic helix-loop-helix (bHLH) transcription factors. They are negative regulators of differentiation and positive regulators of proliferation (Hollanagel et al., 1999). Transient inhibition of BMP4 signaling by Noggin has been shown to induce cardiomyocyte differentiation of mouse embryonic stem cells (Yuasa et al., 2005). BMPs further support self-renewal of mESCs by inhibiting MAPK pathways (Qi et al., 2004) in mESCs. Specific inhibition of ERK or p38 kinases using pharmacological agents in mESCs dramatically improves self-renewal (Qi et al., 2004). Pharmacological inhibition of Smad 2/3 encourages maintenance of pluripotency in ICM of mouse blastocysts outgrowths but not the maintenance of the undifferentiated state in mESCs (James et al., 2003).

Studies with hESCs suggest that BMPs promote differentiation which contrasts with their role in mESCs (Pera et al., 2004). Treatment with exogeneous BMP4 antagonist, Noggin prevents spontaneous differentiation into primitive endoderm. Noggin has been used to block the effects of BMP4 in order to derive neural cells (Pera et al., 2004; Lim et al., 2000). hESCs cultured in serum-free unconditioned medium (UM) are subjected to high levels of intrinsic BMP4 signaling activity, which is reduced in conditioned media (CM; media containing MEF secreted factors). hESCs cultured in the absence of feeders in CM supplemented with basic fibroblast growth factor (bFGF) and BMP4 tend to differentiate to trophoblast lineage (Xu et al., 2002). Replacing BMP4 with Noggin, Nodal or Activin A sustains

undifferentiated proliferation of hESCs in the absence of fibroblasts or CM (Xu et al., 2005; Wang et al., 2005).

During early embryonic development, Nodal/Activin signals establish the embryonic axes, induce mesoderm and endoderm, pattern the nervous system, and determine left-right asymmetry in vertebrates (Schier, 2006). Nodal and Activin A bind activin receptors and activate Smad2 by phosphorylation. Activin A has been implicated in differentiation of mESCs into mesoderm, differentiation of human pancreatic precursor cells into beta cells, inhibition of neural differentiation and induction of hESCs into endoderm (Beattie et al., 2005). In undifferentiated hESCs maintained with CM, the TGF-β/Activin/nodal branch acts through Smad 2/3 mediated signaling. On differentiation of hESCs, Smad 2/3 signaling is decreased while Smad 1/5 is increased (James et al., 2003). hESCs cultured in feeder-free conditions in the absence of CM can be maintained in an undifferentiated state upon supplementation with Activin A (Xiao et al., 2006; Levenstein et al., 2006; Beattie et al., 2005; Vallier et al., 2005). On withdrawal of Activin A or addition of the Activin inhibitor, follistatin, the cells differentiate (Beattie et al., 2005). Nodal also binds to Activin receptors and acts via the Smad 2/3 signaling pathway. Blocking of this pathway using a pharmacological inhibitor induces differentiation of hESCs which can be reversed using Activin/nodal (Vallier et al., 2005).

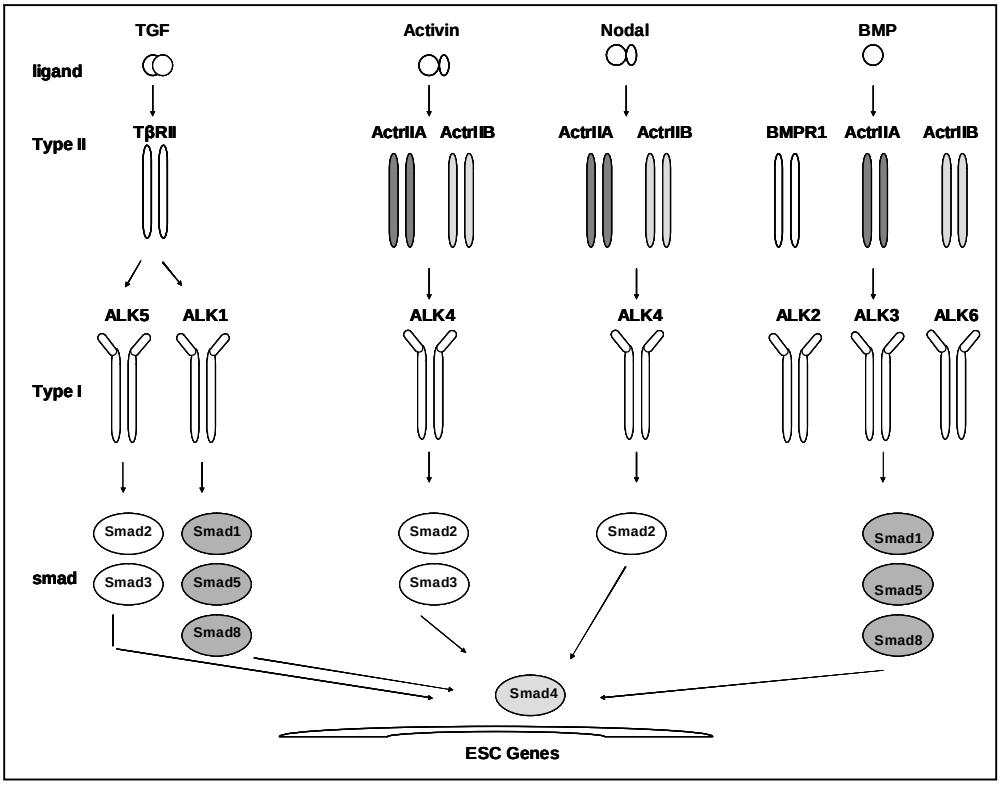

Figure 4: TGF-β signaling network in ESCs. The respective ligands bind to their respective Type I and Type II receptors. This ligand binding activates the receptors and they further phosphorylate and activate the respective Smads. The activated R-Smads bind to Smad4 and are translocated to the nucleus where they facilitate the transcription of various ESC related genes. The ActRIIA receptors are represented in dark grey, ActRIIB in light grey and BMPRI and TGFRII in white. Smad 1/5/8 represented in dark grey respond to activation by BMPs, Smad 2/3 represented in white respond to activation by the TGF branch, and Smad 4 acts as a cofactor to both the branches of the TGF-β superfamily.

FGF2 (Fibroblast Growth Factor 2)

FGFs mediate cellular responses by binding to and activating the receptor tyrosine kinases (RTKs), FGF-receptors. FGF-stimulation leads to tyrosine phosphorylation of the docking protein FRS2a and FRS2b, followed by recruitment of multiple Grb2/Sos complexes resulting in activation of the Ras/MAP kinase, PLC-γ, and PI3K signaling pathways (Eswarakumar et al., 2006; Figure 5). FGFR signaling plays critical roles at different stages of embryonic development (Ornitz et al., 2001). FGF2, also known as basic FGF, has an octamer-containing enhancer downstream of the coding region which is activated synergistically by POU5F1 and SOX2. FGF2 has been proposed to facilitate chromatin remodeling by suppressing methylation of histone 3 (H3) at STAT binding site (Song and Ghosh, 2004). The long-term culture and maintenance of human ESCs in the presence of serum does not require the addition of exogenous FGF2, however, in serum-free medium; FGF2 increases the initial cloning efficiency of human ESCs and FGF2 and is required for continued undifferentiated proliferation (Amit et al., 2000). When culturing hESCs in absence of feeders and without CM or serum, supplementation with FGF2 along with other growth factors like Noggin and Activin A, enhances the proliferation of pluripotent cells (Wang et al., 2005; Vallier et al., 2005).

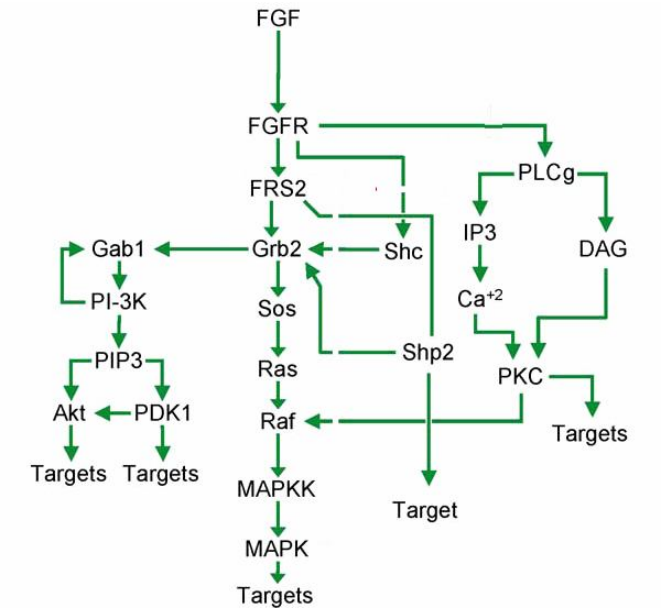

Figure 5: FGF mediated signaling network. Binding of FGF to FGFR results in activation of the Ras/MAP kinase, PLC-γ, and PI3K signaling pathways. Adapted from Eswarakumar et al., 2005.

WNT

The key cytoplasmic events in the canonical WNT pathway include inhibition of GSK-3β (glycogen synthase kinase 3β) mediated β-catenin degradation and selective β-catenin stabilization, nuclear localization and subsequent transactivation. Though the WNT proteins were first discovered as oncogenes in the mouse, they were later determined to perform important roles in axis formation and patterning in the developing embryo (Wang and Wynshaw-Boris, 2004). WNT ligands have been shown to promote proliferation and inhibit differentiation via different mechanisms in different stem cell and progenitor populations.

According to the most widely accepted canonical model of the β -catenin pathway, in the absence of WNT ligand, β -catenin is ubiquitinated, resulting in its degradation by the proteasome thereby reducing its cytoplasmic level. When WNT acts on the cell surface, free β-catenin accumulates and is translocated to the nucleus, where it binds to the promoter of its downstream target genes (Wang and Wynshaw-Boris, 2004). β-catenin displaces transcriptional co-repressors and recruits transcriptional activators (Kikuchi et al, 2006; Figure 6).

The WNT ligands act on mESCs via the canonical pathway. Direct activation of β-catenin fully recapitulates the effect of WNTs on ESCs (Hao et al., 2006; Ogawa et al. 2006). WNTs and LIF have synergistic effects in the regulation of the activity of STAT3. WNT increases STAT3 mRNA, while the LIF promotes the phosphorylation of STAT3 proteins (Hao et al., 2006). Takao et al. (2007) demonstrated a decrease in β-catenin following mESC differentiation caused by LIF withdrawal. Expression of

the activated mutant of β-catenin maintains the expression level of NANOG, as well as the long-term proliferation of ESCs, even in the absence of LIF. Furthermore, β-catenin interacts with POU5F1 to up-regulate NANOG and interacts with NANOG with POU5F1 to assist in the LIF dependent self-renewal of ESCs. Sato et al. (2004) showed that addition of recombinant WNT3a to hESC under feeder-free conditions can stimulate proliferation; however it was later demonstrated that this does not suffice to maintain or expand undifferentiated status for longer periods of time (Dravid et al., 2005). Increasing β-catenin signaling by treatment with WNT3a-conditioned medium or by over-expression of β-catenin promotes neural lineage commitment by hESCs (Otero et al., 2004). In a different study, Lako et al. (2001) showed that the over-expression of WNT3 up-regulates brachyury expression (mesodermal marker) and encourages differentiation towards the haematopoietic lineage in mESCs.

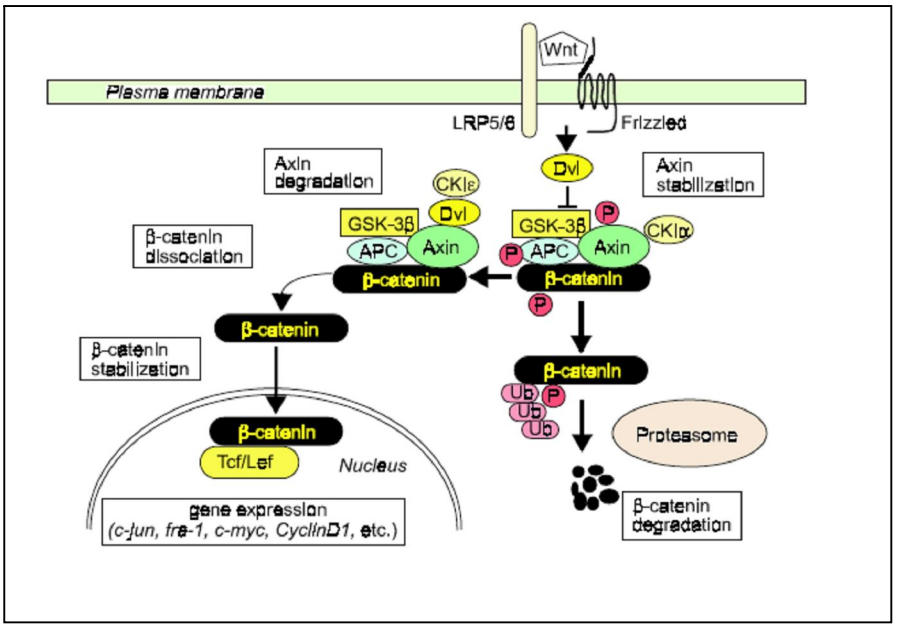

Figure 6: Canonical WNT pathway in ESC (Kikuchi et al., 2006). Binding of Wnt to its receptor, Frizzled and co-receptor LRP5/6 facilitates the liberation of β-catenin from its sequestration complex. The now stabilized β-catenin translocates to the nucleus where it binds to the promoter of its downstream target genes through interaction of Tcf and Lef.

PI3K (Phosphoinositide 3-kinases)

PI3Ks are enzymes that phosphorylate phospholipids at the plasma membrane. On being activated, PI3K phosphorylates PtdIns(3,4)P_2 and generates PtdIns(3,4,5)P_3 which is a target of PH domain (pleckstrin homology domain)-containing proteins and acts as a second messenger. Proteins such as AKT (also known as PKB) interact with PtdIns (3,4,5)P_3 via PH domains and are subsequently translocated to the plasma membrane. Activation of AKT plays important roles in cell proliferation and survival through phosphorylating various substrates. PI3K and AKT proteins can be detected throughout murine pre-implantation development and inhibition of AKT activity results in significant delay in blastocysts hatching (Riley et al., 2005).

The PI3K pathway is activated by several growth factors and cytokines including insulin and LIF via tyrosine kinases. In addition to these exogenous factors, the PI3K pathway is endogenously activated by the constitutively active Ras family protein ERas (ESC-expressed Ras; Takahashi et al., 2005). The PI3K pathway utilizes multiple downstream effectors, including mTOR (mammalian target of rapamycin), which have shown to be essential for proliferation in mouse ESCs and early embryos (Takahashi et al., 2003; Murakami et al., 2004; Figure 7). Forced expression of a dominant-negative mutant (Paling et al., 2004) and treatment with a specific inhibitor of PI3K (LY294002; Paling et al., 2004; Armstrong et al., 2006) demonstrated that PI3K was important for maintenance of the undifferentiated state of mouse and human ESCs (Figure 7). It has also been shown that PI3K may promote self-renewal in both mouse and human ESCs by inhibiting the Ras/MAPK pathway, but precise mechanisms remain elusive (Li et al., 2007; Paling et al. 2006). Watanabe et al.

(2006) show that myristoylated, active form of Akt (myr-Akt) maintained the undifferentiated phenotypes in mouse ESCs without the addition of LIF (Figure 7). Moreover, the inhibition of PI3K activity with either pharmacological or genetic tools results in decreased transcription of NANOG and decreased NANOG protein levels (Storm et al., 2007). There is evidence that PI3K pathway is important for the self-renewal and proliferation of mESCs, however the exact mechanisms are not yet clear.

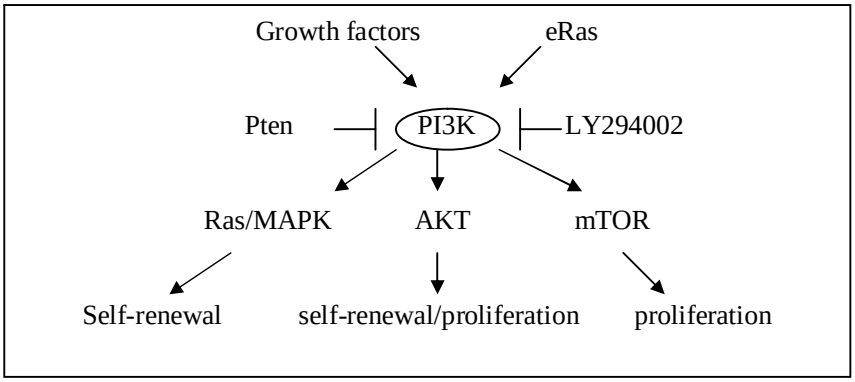

Figure 7: Role of PI3K signaling in ESCs. Activation of PI3K activates the Ras/MAPK, AKT and mTOR signaling pathways which, are involved in the self-renewal and proliferation of ESCs.

SRC Family of Tyrosine Kinases

The SRC family of non-receptor protein tyrosine kinases regulates diverse processes such as cell division, motility, adhesion, differentiation, and survival. Anneran et al. (2004) showed that cYES, a member of the SRC family of non-receptor tyrosine kinases, is highly expressed in mouse and human embryonic stem (ES) cells. The expression of kinase active mutants of Src and Hck can maintain ESCs in an undifferentiated state when LIF concentrations are reduced but not absent (Boulter et al., 1991; Ernst et al., 1996). cYes kinase activity is regulated by LIF and serum and is down-regulated when cells differentiate. Selective inhibition or knock-down of SRC family kinases decreases growth and expression of pluripotency genes such as POU5F1, FGF4 and NANOG.

Comparison of human and mouse embryonic stem cells

The derivation of murine ESCs was reported in 1981 (Martin; Evans and Kaufmann) followed almost 20 years later by derivation of human ESCs (Thomson et al., 1998). Whereas mESCs have been instrumental in answering numerous questions related to mammalian developmental biology, hESCs are considered promising sources for therapeutic cell transplantation.

ESCs have core key characteristics:

- They have the ability to self-renew for extended periods of time.
- They exhibit lack of contact inhibition.
- They have a high nucleo-cytoplasmic ratio and a short G1 cell cycle phase.
- ESCs can spontaneously differentiate and can be stimulated to differentiate in vitro into various cell types including germ cells.
- In suspension they form embryo-like aggregates called 'embryoid bodies' comprising of derivatives of all the three germ layers.
- On in vivo ectopic transplantation ESCs give rise to teratocarcinomas and when combined with normal pre-implantation embryos, these cells contribute to all tissues and organs including the germline.

Despite sharing key core characteristics, mouse and hESCs differ in morphology and growth properties. mESCs have a high alkaline phosphatase activity that can be measured by enzyme-based reactions or antibodies. mESCs grow in tight round colonies whereas hESCs generally grow in flat colonies with distinct borders. mES have a shorter doubling time (Burdon et al., 2002) as compared to hESCs (Amit et al., 2004). mESCs are more amenable to dissociation with enzymes such as trypsin,

a prerequisite for clonal proliferation; whereas hESCs are routinely cultured via mechanical passaging and are prone to differentiation on enzymatic dissociation into single cells. In serum-free culture conditions, mESCs require supplementation with LIF (Smith et al., 1988). In defined culture conditions, the addition of BMP4 complements the actions of LIF (Ying et al., 2003). In contrast, LIF does not support pluripotency in hESCs. Human ESCs are routinely grown in the presence of FGF2 (Amit et al., 2000). Moreover, supplementing the culture medium with Activin A (Vallier et al., 2005) or the BMP4 inhibitor, Noggin (Wang et al, 2005) has shown to be beneficial in maintaining pluripotent hESCs.

Studies in both systems support the existence of a core molecular program of 'stemness' that is conserved evolutionarily. There is a panel of surface markers and transcriptional factors shown to be expressed in ESCs that are used to classify them as pluripotent. However, mESCs and hESCs again show differences in the expression of some of these surface and molecular markers (Table 2), in the culture conditions and in their responsiveness to extrinsic signals. Some of these cell-surface markers are also detected in somatic cells that arise later in development, thus making them useful only as markers of undifferentiated cells in a specific temporal window when used with other ESC defining criteria (Koestenbauer et al., 2006). There are three commonly used forms of stage-specific embryonic antigens (SSEA): SSEA-1, SSEA-3 and SSEA-4, which are expressed in different stages of development and patterns in mESCs and hESCs. SSEA-1 is a glycophingolipid with a lactoseries core; SSEA-3 and 4 are also glycophingolipids but with a globoseries core (Draper and Andrews, 2004). SSEA-1 is expressed on the surface of pre-implantation stage murine embryos

and is reported to play a role in cell-cell adhesion between blastomeres. SSEA-3 and 4 are expressed in the ICM and on undifferentiated hESCs but not mESCs. Tumor rejection antigen (TRA-1-60, TRA-1-81) and germ cell tumor monoclonal-2 (GCTM-2) are antibodies against antigens associated with pericellular matrix keratin sulfate/chondroitin sulfate proteoglycan and mark undifferentiated hESCs but not mESCs (Koestenbauer et al., 2006). In addition, CD9 antigen is expressed on both mES and hESCs whereas osteopontin is expressed only on hES and PECAM-1 (CD31) and the Forssman antigen only on mESCs (Koestenbauer et al., 2006; Stern et al., 1978).

Both mESCs and hESCs are pluripotent cell populations derived from the pre-implantation embryo: they exhibit the hallmarks of pluripotent cells, but differ in the signaling pathways that help maintain them and in the surface markers that characterize them. This leads to an important question as to which of these markers are optimal for characterizing putative ESC lines in other species and which extrinsic ligands ought to be included in the culture medium to provide optimal culture conditions. Despite the vast number of studies with mESC and hESCs, it is not known why the efficiency of ESC derivation is so species and strain dependant. Hence, it is more likely than not that, ES-like cells and ESCs derived from other species will display characteristics that they may share with both these cell types or are a unique entity on their own.

Table 2: The difference in the pattern of expression of cell surface markers in hESCs and mESCs.

Surface Markers	hESC	mESC
SSEA 1	-	+
SSEA3/4	+	-
TRA-1-60	+	-
TRA-1-81	+	-
GCTM-2	+	-
Forssman antigen	-	+
CD9 antigen	+	+
Osteopontin	+	-
PECAM-1	-	+

Overview of Objectives and Experimental Design

Characterization of markers of pluripotency in bovine blastocysts

ESCs are pluripotent cells derived from the pre-implantation embryo that have been liberated from their fate of differentiation and are maintained in conditions that promote their proliferation in a pluripotent state. Pluripotent cells derived from bovine blastocysts should express the same markers of pluripotency as the population of pluripotent cells of the ICM of the blastocyst. Therefore, the markers of pluripotency normally used to characterize mouse and human ESCs were first evaluated for their expression in bovine blastocysts. Bovine blastocysts were analyzed by immunocytochemistry for the presence of NANOG, POU5F1, SSEA1 and SSEA4. It was expected that the information obtained would indicate whether the expression patterns of the markers would resemble that of mESC, hESC or would share characteristics with both or neither.

The transcription factors NANOG, POU5F1 and SOX2 regulate pluripotency in the pre-implantation mouse and human embryos and ESCs derived from the ICM. The expression of these transcription factors is an indicator of their pluripotency. Hence, the derivation of a pluripotent cell population from the embryo is likely to be influenced by the presence of the above mentioned transcription factors in the embryo. The expression of NANOG and POU5F1 was measured among individual bovine blastocysts by quantitative RT-PCR. The expression level was normalized to the level of β-actin in the embryos. The results thus obtained would indicate whether these transcription factors are expressed in bovine blastocysts.

Expression of genes related to pluripotency and differentiation in ICM explants

Most published reports of bovine ES-like cells assess the state of pluripotency in the colonies after a few passages. If the culture conditions are not optimal, the colonies will have already progressed towards a path of differentiation by this time point. There are no published reports of the status of the expression of the transcription factors NANOG, SOX2 and POU5F1 during the initial days of explant culture. This study evaluated whether the colonies derived from the ICM expressed these transcription factors in the initial days of culture and how long they could maintain expression when cultured in ESC medium not supplemented by any growth factors. At the same time markers of differentiation were also evaluated to assess any differentiation and if so which lineages the cells of the ICM explant would follow.

Candidate gene expression in ICM explants

Growth factors can promote the proliferation and maintenance of pluripotent cells. In defined culture conditions, LIF supplementation supports mESC proliferation whereas FGF2 supports hESC proliferation. In order to identify which signaling pathways are expressed in the ICM explants, candidate transcripts representative of the various signaling pathways important for mESCs and hESCs were evaluated by semi-quantitative RT-PCR. The candidate genes belonged to the Jak-STAT (GP130, LIFR), WNT (β-CATENIN, FZLD), BMP4 (BMPR1A, BMPR2, ID1, ID3), FGF (FGFR1), and Activin (ALK4, ACTRIIB) pathways. To assess the effects of

passaging on gene expression, the same candidate genes were evaluated in ICM explant colonies in passages 0-2.

Effect of cytokine supplementation

In defined culture conditions, the presence of cytokines and growth factors improves the survival of pluripotent cells. Murine ESCs are routinely cultured in media supplemented with LIF. BMP4 also supports pluripotency of mESCs in defined culture conditions. In contrast, hESCs are cultured in media supplemented with FGF2, Activin A, or Noggin. In order to identify which cytokines might help maintain bovine pluripotent cells, ICM explants were cultured in media supplemented with cytokines (Noggin, BMP4, FGF2, Activin A or Noggin + FGF2; details on page 48). Since there is sufficient published evidence that LIF does not affect the maintenance of pluripotency in bovine ES-like cells, we chose not to study its effect on ICM explants. The expression of the pluripotency-determining transcription factors (NANOG, POU5F1 and SOX2) was measured across passages 0-2 by quantitative RT-PCR. The expression level was normalized to β-actin for each sample. The concentration and the passage number served as the main effects, whereas the replicate served as the random effect. (For detailed methods, see page 62).

Selective ablation of differentiated cells

ESCs and ES-like cells can undergo spontaneous differentiation as is characteristic of pluripotent cells. The differentiating cells secrete ligands that

stimulate other pluripotent cells to follow specific differentiation lineages. The strategy of selective ablation was adopted in order to eliminate the differentiation-inducing cells. To meet this end, a vector was constructed that had a hygromycin phosphotransferase gene under the control of the NANOG promoter. Transgenic pluripotent cells bearing this gene were expected to survive in the presence of hygromycin as long as the cells were pluripotent and the NANOG promoter was active. Cells that differentiate would succumb to the toxic effects of hygromycin, thus allowing pluripotent cells to proliferate. This experimental approach was predicted to improve the conditions for survival of pluripotent cells in ICM explants.

Material and Methods

ICM explant culture

Preparation of feeder layer

Feeders for the culture of ICM-derived explants were made from STO MEFs (mouse embryonic fibroblasts; CRL-1503, ATCC, Manassas, VA). The MEFs were cultured in feeder medium (FM) composed of Knockout DMEM (Cat # 10829, Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS; Cat # SH 30070.03; Hyclone, Logan, UT) and 1% penicillin-streptomycin (10,000 IU each; Cat # 15140-122, Invitrogen). MEFs were mitotically inactivated (blocked) by incubating an 80% confluent layer of STO MEF in 0.01 mg mitomycin (Cat # M0503, Sigma-Aldrich, St. Louis, MO) per ml of FM for 2.5 hrs followed by extensive washing in Dulbecco's phosphate buffered saline (DPBS; Cat # 14190-136, Invitrogen). Blocked cells were frozen as stocks of 4×10^6 cells per ml of cryoprotectant medium (FM supplemented with 10% FBS and 10% DMSO). For creation of feeder layers, blocked MEFs were plated at a density of 5×10^4 cells/ cm^2.

Isolation of ICM

Day 6 IVP bovine morulae were obtained from Bomed Inc. (Wisconsin). In vivo bovine morulae were obtained from the Wye Research and Education Center (University of Maryland, College Park) by flushing superovulated cows using standard techniques. These embryos were cultured overnight in G-2 version 3 embryo culture medium (Vitrolife, Englewood, CO) supplemented with 5% FBS (Cat # SH

30070.03E; Hyclone). Expanded blastocysts were transferred to a dish containing embryonic stem cell medium (ESCM) comprised of DMEM supplemented with 15% FBS, 1% glutamine (Cat # 35050-61; Invitrogen), 1% non-essential amino acids (Cat # 11140, Invitrogen), 1% penicillin-streptomycin (Cat # 15140-122; Invitrogen), and 1% β-mercaptoethanol (Cat # 19470590, ICN Biomedicals Inc., Aurora, OH). The blastocysts were dissected manually under a stereoscope using a microdissection knife (ESE020, AB Technologies, Pullman, WA) and 27-gauge needle to isolate the ICM from the trophectoderm.

Culture of ICM

Isolated ICMs were cultured in ESCM in groups of 2 to 5 on blocked feeders in 12-well tissue culture dishes. Freshly isolated ICMs attached to the feeders and formed outgrowths. The culture medium was changed every other day and the colonies were passaged every 6-7 days. The colonies were passaged by manually dissecting colonies into 0.1 mm pieces using 27 gauge needles. When required, pieces of colony were cryopreserved in cryoprotectant medium (ESCM supplemented with 10% FBS and 10% DMSO).

For the cytokine experiments, ICMs were isolated, cultured and passaged as indicated above. The culture medium (ESCM) was supplemented with one of the following cytokines: rhBMP4 (0, 5, 10, or 15 ng/ml; Cat # 314-BP, R and D Systems, Minneapolis, MN), Noggin (0, 250, 500, or 750 ng/ml; Cat # 120-10, Peprotech, Rocky Hill, NJ), Activin A (0, 10, 25, 50 ng/ml; Cat # 338 AC, R and D systems) or FGF2 (0, 12, 40, or 100 ng/ml; Cat # 133-FB, R and D systems) or Noggin (500

ng/ml) + FGF2 (40 ng/ml). The selection of cytokines was based on their use in hESCs and mESCs and the results obtained from the candidate gene expression analysis of early ICM explants. The concentrations used were based on respective published recommendations for use in mESCs and hESCs. Each study was repeated twice on independent occasions except for Noggin supplementation which was repeated thrice. On each occasion, 3-7 ICM explants were analyzed at each concentration and at each passage.

Sample Collection

For analysis of gene expression during the initial days of culture (days 0-12), 2-4 colony ICM outgrowths were pooled in lysis buffer (Absolutely RNA Nanoprep Kit, Cat # 400753; Stratagene, La Jolla, CA) for RNA extraction and further analysis (pages 71 and 76). Day 0 samples represent freshly isolated ICM explants that have not been cultured. For analysis of candidate genes (page 80) and for analysis of effect of cytokine supplementation (page 86), individual colonies were sampled at the end of each passage. On each occasion, a small portion of the colony (0.1mm) was removed for further propagation prior to sample collection. Then the remainder of the colony was collected in lysis buffer for RNA extraction and further analysis.

Individual embryos (n=16) were collected in lysis buffer for RNA extraction to analyze the gene expression pattern (page 66).

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Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Semi-quantitative RT-PCR

Sample treatment: Embryos, individual and pooled colonies were collected in lysis buffer. Total RNA was extracted from embryos and cell colonies using the Absolutely RNA Nanoprep Kit. The total RNA yield from pooled ICM explants was 298.14±444.57 ug and it ranged from 10.2 ng-1570.5 ng. The total RNA yield from individual ICM explants in the cytokine study was 261.24±268.69 ug and it ranged from 23.9 ng-1527.3 ng. The RNA yield was lower for samples collected during Passage 0 and between Days 1- 6. Non-reverse-transcribed RNA was used as a control for genomic DNA contamination. Dnase treatment was conducted during the RNA extraction protocol to avoid contamination with genomic DNA. Total RNA was quantified using the Spectrophotometer (ND-1000, NanoDrop Technologies; Wilmington, DE) or was quantified at Center for Advanced Research in Biotechnology (CARB), University of Maryland. For each study, the amount of total RNA used for cDNA synthesis was adjusted such that similar amounts of RNA were being used for each sample in the study to enable semi-quantitative PCR analysis. First strand cDNAs were synthesized using Superscript III First-Strand Synthesis System for RT-PCR (Cat # 18080-051; Invitrogen).

PCR primers were designed based on published nucleotide sequences when available or were based on sequence similarity between the mouse and human nucleotide sequences for the respective genes (Table). Primers were designed using the online software available at Sigma-Genosys (http://www.invitrogen.com/content.cfm?pageid) and Intergrated DNA Technologies

(IDT; http://www.idtdna.com/analyzer/Applications/Oligo Analyzer). cDNAs were amplified with PfuUltra hotstart PCR master mix (Cat # 600250; Stratagene) or recombinant Taq DNA polymerase (Cat # 10342-053; Invitrogen) according to the manufacturer's instructions. For each set of PCR reactions, a cDNA sample positive for the respective gene(s) and a cDNA sample negative for the respective gene(s) were included to confirm the specificity of reaction (Table 7). In addition, a reaction without cDNA was also included to test for contamination by genomic DNA. The PCR thermocycling conditions were the following: an initial denaturation step at 94°C for 3 min followed by 35 cycles (except ACTB where 20 cycles were run) of 94°C for 30 sec, primer specific annealing temperature for 30 sec, 72°C for 45 sec extension, and a final extension at 72°C for 10 min. PCR products were separated by electrophoreses through a 1.5% Tris-acetate-EDTA agarose gel stained with GelStar (Cat # 50535; Cambrex Bio Science, Rockland, ME). Gels were imaged with Chemidoc XRS system (BioRad Inc., Hercules, CA). To retrieve amplified PCR products, DNA bands were excised from the gel and extracted with Qiaquick gel extraction kit (Cat # 28704; Qiagen, Valencia, CA). The extracted DNA was sequenced at the sequencing facility at University of Maryland (College Park, MD).

Quantitative RT-PCR

Total RNAs were extracted, and first strand cDNAs were synthesized as indicated above. PCR primers were designed based on published nucleotide sequences when available or were based on sequence similarity between the mouse and human nucleotide sequences for the respective genes (Table 8). Primers were

designed using the online software available at Sigma-Genosys and Intergrated DNA Technologies.Gene transcripts were quantified by real-time PCR using the iCycler thermocycler (BioRad Inc.) and detected using SYBR Green fluorochrome (Cat # 170-8882; IQ SYBR Green Supermix, BioRad Inc.). All reactions were run in duplicate or triplicate. In most of the studies, the difference in Ct values among replicates for each of the genes varied between 0-0.3. If the difference between these technical replicates exceeded 0.5, the data was not included in the analysis. Relative levels of expression were determined with the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference}}$, and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$. β-actin served as the internal reference gene, and samples with no cDNA served as controls for the specificity of the reaction. A pool of cDNAs for each study served as the calibrator. The specificity and integrity of PCR products was confirmed through the melt-curve analysis.

Single-cell nested PCR

Single transfected fibroblasts were placed in microcentrifuge tubes (containing a 5 µl solution of 400 ng/µl Proteinase K/17 µM SDS) using a fine pulled glass pipet. The tubes were incubated at 50°C for 1 h followed by denaturation at 99°C for 30 min. 45 µl of PCR master mix containing 25 pmol external primers (specific for HP) was added and the product (451 bps) was amplified. The external PCR product was used as a template for the next round of nested PCR using 10 pmol of each internal primer (specific for HP). PCR products (97 bps) were separated by electrophoreses through a 2% Tris-acetate-EDTA agarose gel stained with GelStar. Untransfected fibroblasts served as the negative control and plasmid DNA carrying

the hygromycin phosphophotransferase (HP) served as a positive control. A total of 48 transfected fibroblasts maintained under G418 selection for more than 4 weeks were screened for the presence of the gene. All showed a positive band whereas the untransfected fibroblasts did not show the presence of the gene.

Immunocytochemistry

Blocked STO MEFs served as a negative control whereas hTera-2 cells (human embryonal carcinoma, Cat # CRL-1973, ATCC) and mESC (R1, ATCC) served as positive controls for ICC. Blastocysts and ICM-derived colonies were fixed in 4% formaldehyde for 20 min and washed three times with TBST buffer (Tris buffered saline Tween-20; 20mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20, pH 7.4). Permeabilization was performed in a solution composed of 0.2% Triton X-100 and 0.1% Tween-20 in distilled water for 10 min. Nonspecific reactions were blocked with 10% normal goat (SSEA-1, SSEA-4, POU5F1) or donkey (NANOG) serum (Sigma-Aldrich) for 30 min at room temperature. The samples were incubated overnight at 4^{o}C with primary antibodies [(POU5F1, 1: 50 dilution; Cat # SC-9081, Santa Cruz Biotechnology), (SSEA-1, 1:50 dilution; Cat # SC-21704; Santa Cruz Biotechnology), (SSEA-4, 1:50 dilution; Cat # SC-21704 Santa Cruz Biotechnology), NANOG, 1:150 dilution; Cat # 500-P236, Peprotech)]. The samples were washed extensively with TBST buffer, and exposed to secondary antibody (1:200 dilution, AlexaFluor 448, Invitrogen) for 30 minutes at room temperature. The primary antibody was omitted for use as a negative control. These were then washed and stained with 1µg/ml Hoechst 33343 (Sigma-Aldrich) per ml DPBS for 10 minutes

and whole mounted onto slides using Antifade mounting solution (Cat # P-7481; Molecular Probes, Invitrogen). Images of the embryos and colonies were taken with a Leica DM IRE2 inverted microscope (Vashaw Scientific, Norcross, GA).

Alkaline Phosphatase staining

The tissue culture dish containing the respective colonies was rinsed with PBS and fixed using 80% cold ethanol at 4°C overnight. The fixed cells were incubated with freshly made alkaline phosphatase (AP) solution. The AP solution was made from 0.5% diazonium salt of fast red (Sigma-Aldrich), 0.01% alpha-naphthyl phosphate (Sigma-Aldrich), 0.06% magnesium chloride (Sigma-Aldrich) and 0.45% borax in deionized water. The cells were exposed to the solution for 20 min, rinsed with PBS and scored immediately for the number of stained colonies. The positively stained colonies represented alkaline phosphatase activity.

Vector construction

Bovine genomic DNA was extracted using DNeasy Tissue Kit (Qiagen) and 446 base pairs (bps) of the NANOG promoter were cloned via PCR from the genomic DNA. The Hygromycin phosphotransferase (HP) gene (1021 bps) was cloned from the pEF5/FRT/V5-DEST vector. The primers were designed to add sites specific for digestion by restriction endonucleases (RE) and overlap between the two genes (Figure 8, Table 3). After the PCR amplification, a Bam HI site was located 5' to the NANOG promoter sequence; the HP gene carried a Kozak sequence and *Sca* I site 5' and a *Hind* III site 3' to it. Two products were designed: one with a stop codon after

the HP gene (NANOG promoter- HP; NH) and one without the stop codon after GFP (NANOG promoter- HP -GFP; NHG; Figure 8).

After amplifying the two DNA products individually (NANOG promoter and HP), both the products were pooled into the same tube and subjected to two rounds of PCR in the absence of primers to enable annealing of the products. This was followed by further rounds of PCR to amplify the fusion product of the two genes (site overlap extension, SOE). The fusion product was cloned into a vector using the pcDNA 3.1/V5 His TOPO TA Expression Kit (Invitrogen). One Shot® TOP10 Chemically Competent Cells (E.Coli; Invitrogen) were transformed with the DNA according to the manufacturer's recommendations. The transformed bacteria were plated on LB (Luria Bertani) agarose plates containing carbenecillin (100 µg/ml) and cultured overnight at 37ºC to select for the bacteria carrying the plasmid. Individual colonies were screened by PCR for the presence of the DNA insert and amplified in LB broth supplemented with carbenecillin (100 µg/ml). The plasmid carrying the insert was extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen).

The NANOG promoter-HP gene product was cloned via PCR from the pcDNA 3.1 vector using forward primer specific to NANOG promoter and reverse primer specific to HP. The DNA and the target vector (pAcGFP; Clonetech, Mountain View, CA) were both individually digested overnight with *Bam* HI and *Hind* III REs (NEB, Opswich, MA) at 37ºC and purified using Purelink PCR Purification Kit (Invitrogen).

Both fusion products (NH and NHG) were individually ligated overnight with the target vector at 16ºC using T4 DNA ligase (NEB) and cloned into the multiple

cloning site of the pAcGFP1-1 vector. The ligated vector-DNA inserts were transformed into One Shot® TOP10 Chemically Competent Cells (E. Coli; Invitrogen). The transformed bacteria were plated on LB (Luria Bertani) agarose plates containing kanamycin (100 µg/ml) and cultured overnight at 37ºC to select for the bacteria carrying the plasmid. Individual colonies were screened by PCR for the presence of the DNA insert. Bacterial clones positive for the presence of DNA insert were cryopreserved in LB broth supplemented with 80% glycerol. The bacteria carrying the plasmid were amplified in LB broth supplemented with kanamycin (100 µg/ml). The plasmid carrying the insert was extracted and purified using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen).

The plasmid was further tested by restriction mapping and was then sequenced at CBR, University of Maryland to ascertain the presence of the fusion product in the multiple cloning site of the plasmid.

Table 3: Primer sequences for the Site Overlap Extension PCR

Primers	Sequences
NANOG promoter forward (1)	CGTCCAAGCTTAAGGTGGAAAGAAGGTAAGC
NANOG promoter reverse (2)	CATGG*CCATGGT*AGTACTGTTGCTGAGTTGAAGGAGAAGG
Hygromycin phosphotransferase forward (3)	CCTTCTCCTTCAACTCAGCAAC AGTACT*ACCATGG*ATGAAAAAGCCTG AACTCACC
Hygromycin phosphotransferase reverse (4)	C T A G T GGATCCTTATTCCTTTGCCCTCGGA (stop codon) or ACCGGTGGATCCAAATTCCTTTGCCCTCGGA (no stop codon)

Figure 8: Strategy for the Site Overlap Extension PCR

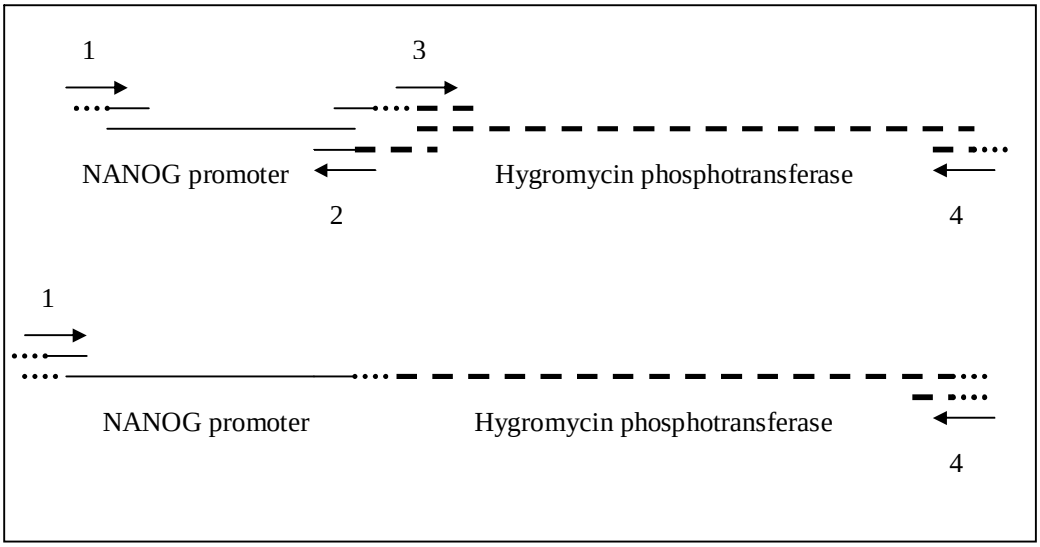
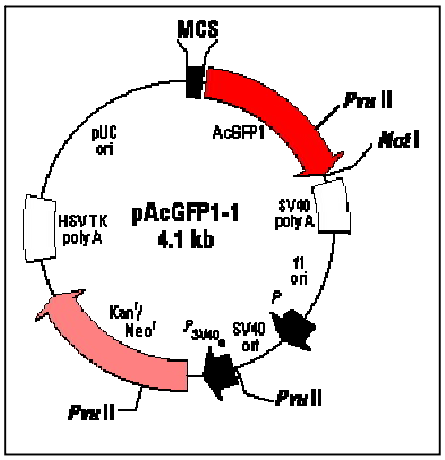
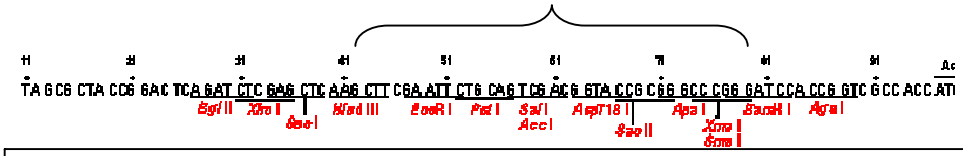


Figure 9: Map of the plasmid and sequence of the gene inserted



*Hind*III-NANOG promoter-*Sca*I-Kozak sequence-hygromycin phosphotransferase- *Bam*HI



TTGCTCACATTTNTTTCCTGCGNTATCCCCTGATTCTGNGGATAACCGTATTACCGCCATGC
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AAGGTGAAGCTAGGAGTATCTCATTGCCGGGTTNCATTGTGTTTCCCGTTTCCGAGGTCCT
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GATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGA
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GGGCAAAGGAATAAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGCCGAGCTGTTC
ACCGGCATCGTGCCCATCCTGATCGAGCTGAATGG

Dark grey: NANOG promoter
Light grey: hygromycin phosphotransferase
Underlined: restriction endonuclease specific sites
Italicized: Kozak sequence

mESC culture

Culture and passage

R1 mESCs (ATCC) were cultured in ESCM supplemented with 1000 units/ ml leukemia inhibiting factor (LIF). These cells were cultured on gelatin-coated tissue culture plates at a density of approx 3.5 x 10^4 cells/ cm^2. The media was changed each day and the cells were passaged every 2-3 days by enzymatically dispersing the colonies with 0.25% trypsin-EDTA (Invitrogen).

Hygromycin sensitivity curve

Mouse ESCs were cultured on a 24 well tissue culture dish as mentioned above at a density of 2 x 10^4 cells/ well. Hygromycin was added to two wells each at concentrations of 0, 0.5, 2.5, 25, 50, 100, 200 µg/ml. The culture media was changed each day and cells were not passaged for 5 days. Colonies were stained for the presence of alkaline phosphatase (AP) and the numbers of colonies positive for AP were counted in each well. This experiment was replicated twice.

Transfection and selection of cells for stable integration of the transgene

Both plasmids (NH and NHG) were digested using restriction endonucleases (BglII and NotI), and the fragment containing the transgene was isolated by running on an agarose gel and purifying the excised gel fragment with the QIAquick Gel Extraction Kit (Qiagen).

mESC were suspended at a density of 5 x 10^5 cells/ well in a gelatin coated (0.1%; Cat # G1393, Sigma-Aldrich) 24-well tissue culture dish, and 2 wells each were transfected with 0.8µg DNA each (cut as well as uncut plasmids) using a DNA: liposome ratio of 1:2 (Cat # 11668-027, Lipofectamine 2000, Invitrogen). Hygromycin (100 µg/ml; Cat # 10687-010, Invitrogen) was added to the wells 18 hours after the transfection to select for cells carrying the transgene. Surviving cells were passaged and cultured in selection medium (ESCM supplemented with hygromycin) for 5 passages. Individual colonies (n= 24 each) were picked, and propagated as individual clones while being maintained under hygromycin selection. The clones were cryopreserved in ESCM cryoprotectant medium.

Transfection and selection of bovine fibroblasts

Culture and passage

Bovine fetal fibroblasts (obtained from USDA, courtesy of Ann Powell and Bob Wall) were cultured in Feeder Medium at a density of 5 x 10^4 cells/ cm^2. The cells were passaged every 3-4 days enzymatically using 0.25% trypsin-EDTA.

Geneticin and hygromycin sensitivity analysis

Bovine fibroblasts were cultured as mentioned above on a 24 well plate at a density of 5 x 10^4 cells/ cm^2. Geneticin (Invitrogen) was added to 2 wells each at concentrations of 0, 50, 100, 200, 400, 600, 800, 1000 µg/ml, and media was changed

each day. Cells were not passaged for 5 days. The number of cells in each well was counted 6 days after initiation of geneticin supplementation.

Bovine ICM explants were cultured as described previously. Hygromycin was added to two wells each (each well containing 3 colonies) at concentrations of 0, 0.5, 1.0, 2.5, 12.5, 25, 50, 100 and 200 µg/ml. The culture media was changed every other day and colonies were observed for surviving cells after 5 days. At the end of 5 days there were no surviving cells in the wells treated with 100 and 200 µg/ml hygromycin (Table 9, Appendix).

Transfection and selection of stable integrated transgenic cells

Bovine fibroblasts were suspended at a density of 2 x 10^6 cells/ cm^2 on a gelatin coated 100 mm tissue culture dish and transfected with a 16 µg DNA using a DNA: liposome ratio of 1:2 (Lipofectamine 2000). The cells were selected for the presence of the transgene by adding Geneticin (Cat # 10131-035, Invitrogen) at a concentration of 400 µg/ml. Surviving cells were passaged and continued to be maintained in selection medium (feeder medium supplemented with 400 µg/ml geneticin) for 3 weeks. These bovine fibroblasts were cultured at a low density (1 x 10^5 cells) in a 100 mm tissue culture dish in selection medium. After 10-15 days of culturing the cells in selection medium, clonally growing colonies were harvested and propagated further.

Hand-made cloning

Cytoplast preparation

Bovine oocytes matured for 20 hrs (during shipping from Minitube Inc., Mt Horeb, WI) were stripped of cumulus cells using 1 mg hyaluronidase (Cat # H-3884,

Sigma-Aldrich) per ml in Emcare (Cat # CECFS, Immuno-Chemical Products, New Zealand). All embryo manipulation was performed in Emcare supplemented with 20% FBS (T20). Zona pellucida was dissolved with 2 mg pronase (Cat # P8811, Sigma) per ml in T20. The oocytes were incubated for 10 min in cytochalasin B (2.5 µg per ml T20; Sigma C-6762) and bisected in a tissue culture dish with a micro-dissection knife (ESE020, AB Technologies) under a stereoscope. The halves were incubated in Hoechst solution (Cat # 14533, Bisbenzimide H33342; Fluka Biochemica, Buchs, Switzerland) to label the DNA at a concentration of 5 µg bisbenzimide per ml in Emcare for 10 min and screened under a fluorescent microscope; any halves containing the metaphase plate were used as controls for the activation procedure.

Fusion

Transfected bovine fibroblast clones were maintained at confluency for 4-5 days under G418 selection in a 24 well dish. Fibroblasts from a single well were dispersed using trypsin into a single cell suspension in Emcare.

The cytoplast halves were washed through a dish of phytohemagglutinin (PHA, Sigma L-8754; 0.5 mg per ml Emcare) and a fibroblast cell was sandwiched between two cytoplast halves. Fusion medium consisted of 0.25 M sorbitol, 100 µM calcium acetate, 0.5 mM magnesium acetate, 0.1% fatty acid-free BSA (Sigma-Aldrich) in distilled water. The cytoplast-fibroblast pair was equilibrated in fusion medium and placed between the wires of the fusion chamber (BTX Electroporator Electro Cell Manipulator ECM 2001, UK). The fusion machine was set according to

the following parameters: alternating current (AC) = 20 V, direct current (DC) = 168 V (3.36 kV/cm), pulse duration = 16 ms, number of pulses = 1, and post-AC pulse = 1s. AC/DC pulses were applied and the triplets were moved to droplets in G1 medium and cultured at 37°C, 5% CO_2. During this time duration, the triplets fused to form a single rounded cell.

Embryo culture

The cells were activated 3 hrs after fusion in a solution containing 5 μM calcium ionophore (Ionomycin, Sigma, Cat # I-0634). The reconstructed embryos were cultured for 3-4 hrs in 2 mM DMAP (dimethyl amino-purine; Sigma, Cat # D2692) at 39°C, 5% CO_2. The embryos were thoroughly rinsed with Emcare and further cultured in embryo culture medium (G-1 version 3 supplemented with 10% FBS) in microwells in 30-μl microdroplet overlayed with oil at 39°C in 5% CO_2, 5% O_2, and 90% N_2. The embryos were scored 4 days after activation and blastomeres were selected for derivation of cell lines. Since these embryos lack a covering of zona pellucida, they cleaved to form an aggregate of blastomeres.

Propagation of transgenic cells

The blastomeres were placed on a blocked feeder resistant to hygromycin (SCRC-1045; ATCC) and maintained in ESCM in the presence or absence of Noggin (500 ng/ml). A day after plating the blastomeres, the medium was supplemented with 0.1 mg/ml hygromycin.

Statistical Analysis

Ct (Cycle threshold) values from quantitative real-time PCR were normalized to β-Actin. In addition to β-Actin, the samples were also analyzed for GAPDH and the values were found consistent for both genes. Levels of mRNA reported are the means and SEs of the relative expression levels described.

Results for the ICM explant gene expression studies were analyzed as a One-Way Analysis of Variance (ANOVA) using the PROC MIXED procedure of the SAS. The expression relative to ACTB was the dependent variable and the day of analysis was the independent variable. A pairwise comparison was conducted to compare the expression between two consecutive days using the PDIFF procedure (SAS). Differences were considered significant at $P < 0.05$.

Results for the cytokine studies were analyzed by ANOVA using the PROC MIXED procedure of the SAS. The expression relative to ACTB was the dependent variable. The concentration, passage number and the interaction of the passage and concentration served as fixed effects. The replicates or trial number served as the random effect. Where an interaction was found significant the main effects were omitted from the model. If no effect of the concentration was observed, the concentration variable was also omitted from the model. The changes in the model statements were made in order to maximize the degrees of freedom. A pairwise comparison was conducted to compare the expression at each passage and concentration to the respective control at that passage and concentration using the PDIFF procedure (SAS). Differences were considered significant at $P < 0.05$.

Results

Markers of pluripotency in embryos

Day 7 bovine IVP blastocysts were individually collected, and mRNA transcript levels were quantified by RT-PCR. A total of 16 embryos were evaluated. However, the CT values were found to be consistent for 8 of the embryos for which the data has been presented. The pluripotency-related genes, NANOG and POU5F1 were expressed in all the embryos tested. On further examination, the expression values as determined by quantitative RT-PCR were found to vary relative to the expression of β-actin in the individual embryos. The mean expression (mean ± SEM) of the two genes among embryos was 6.28 ± 13.62 for NANOG and 2.33 ± 4.23 for POU5F1 (Figure 10).

IVP bovine blastocysts were examined for the expression of markers of pluripotency (Nanog, Pou5f1, SSEA1 and SSEA4) by subjecting the embryos (8-10 total blastocysts per marker) to immunocytochemistry (ICC). The ICC was conducted on two separate pools of embryos on two different days. On each occasion 4-5 embryos were tested per marker. Nanog expression in the ICM was localized to the nucleoplasm as well as the nucleoli, whereas it was restricted to the nucleoli of the trophectoderm cells (Figure 11 A-E). Pou5f1 was expressed in the nuclei of ICM as well as trophectoderm (Figure 11 F-J). SSEA 1 (Figure 11 K-O) was expressed in a punctuate manner the entire surface of the blastocyst (ICM as well as trophectoderm). However SSEA 4 (Figure 11 P-T) was not detected on the surface of any blastocyst tested. Ntera cells (human carcinoma cell line) and mESC served as positive controls for the sensitivity of the primary antibodies (nTera: Nanog, Pou5f1, SSEA4; mESC:

SSEA1). Embryos exposed to the respective secondary antibody without prior exposure to the respective primary antibodies served as negative controls for the specificity of secondary antibody. On each occasion, the nTera-2 cells and mESCs stained positive for the respective antibody whereas none of the negative controls exhibited any staining.

Figure 10. Expression of the pluripotent genes, NANOG and POU5F1 in day 7 IVP bovine blastocysts.

Day 7 bovine blastocysts were individually collected and mRNA transcript levels for NANOG and POU5F1 were determined in each embryo via quantitative RT-PCR. The CT values were normalized to β-actin which served as the internal reference. The data was analyzed by the $2^{-\Delta\Delta Ct}$ method. The NANOG expression is represented by the white bars and the POU5F1 expression by black bars.

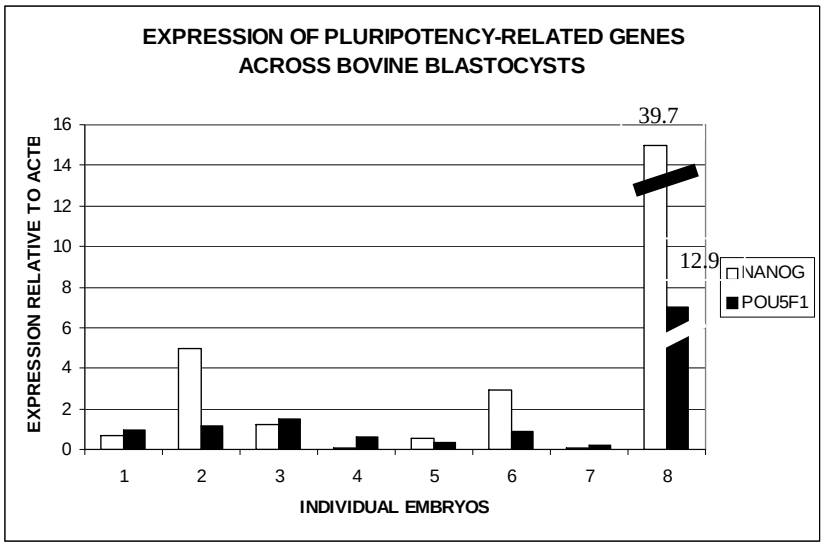
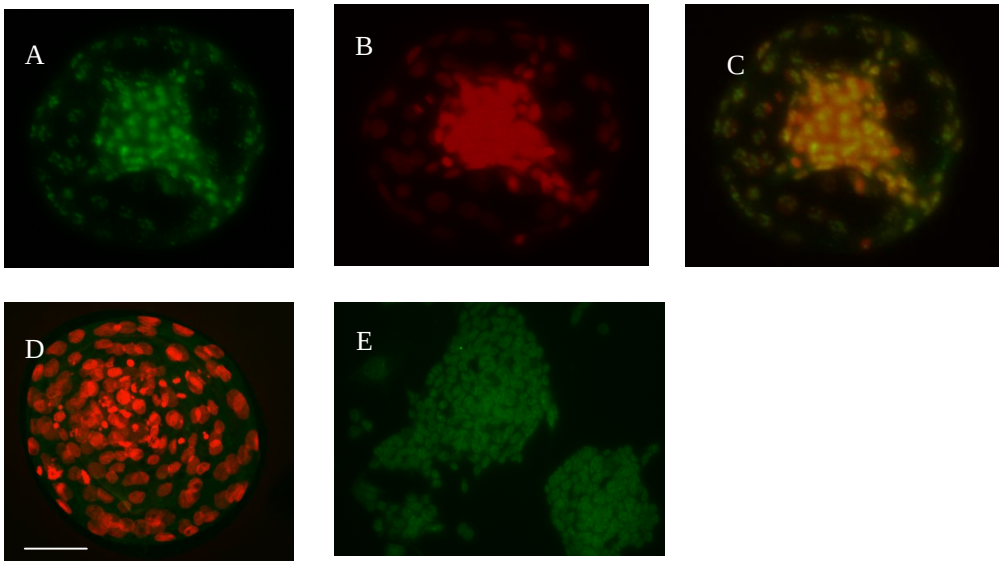
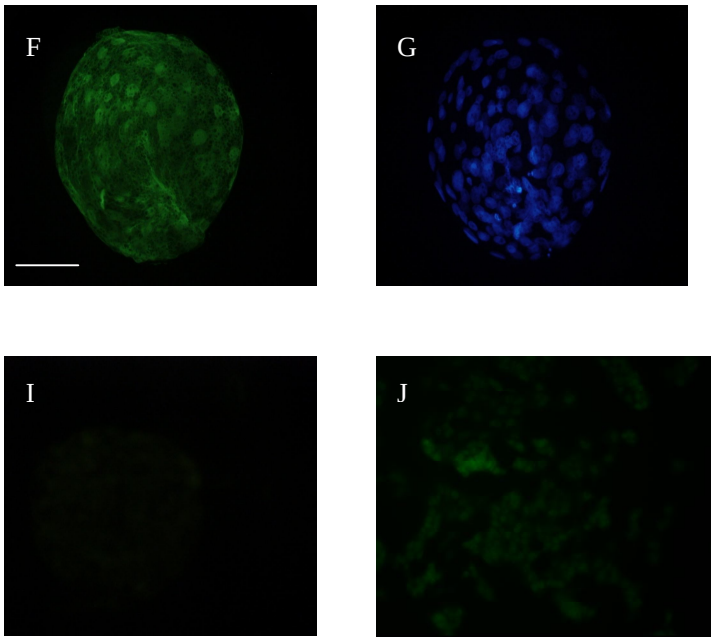


Figure 11. Expression of ESC markers in Day 7 bovine blastocysts.

Bovine blastocysts were fixed and stained with the DNA specific stain H33342 and with antibodies specific to markers of pluripotency. The images were taken with Leica immunofluoresence microscope, bar = 50 µm.

Panel A-T represents the following labeling pattern:
NANOG expression in blastocyst with FITC labeled secondary antibody against primary antibody (green) (A); nuclei labeled with H33342 (red) (B); merge of A and B (C); blastocyst labeled with secondary antibody without exposure to primary antibody and overlaid with H33342 staining (D); nTera-2 with FITC labeled secondary antibody without exposure to primary antibody (E)
POU5F1 expression in blastocyst with FITC labeled secondary antibody against primary antibody (green) (F); nuclei labeled with H33342 (blue) (G); blastocyst labeled with secondary antibody without exposure to primary antibody (I); nTera-2 with FITC labeled secondary antibody without exposure to primary antibody (J)
SSEA 1 expression in blastocyst with FITC labeled secondary antibody against primary antibody (green1) (K); nuclei labeled with H33342 (red) (L) merge of K and L (M); blastocyst labeled with secondary antibody without exposure to primary antibody and overlaid with H33342 staining (N); mESC with FITC labeled secondary antibody without exposure to primary antibody (O)
SSEA 4 expression in blastocyst with FITC labeled secondary antibody against primary antibody (green) (P); nuclei labeled with H33342 (red) (Q); merge of P and Q (R), blastocyst labeled with secondary antibody without exposure to primary antibody and overlaid with H33342 staining (S); nTera-2 with FITC labeled secondary antibody without exposure to primary antibody (T)

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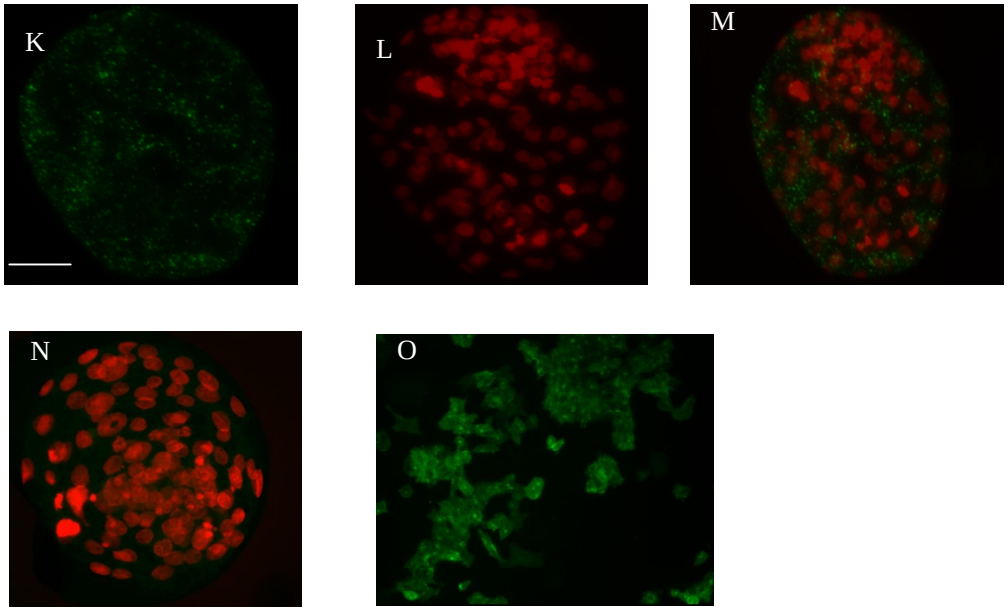
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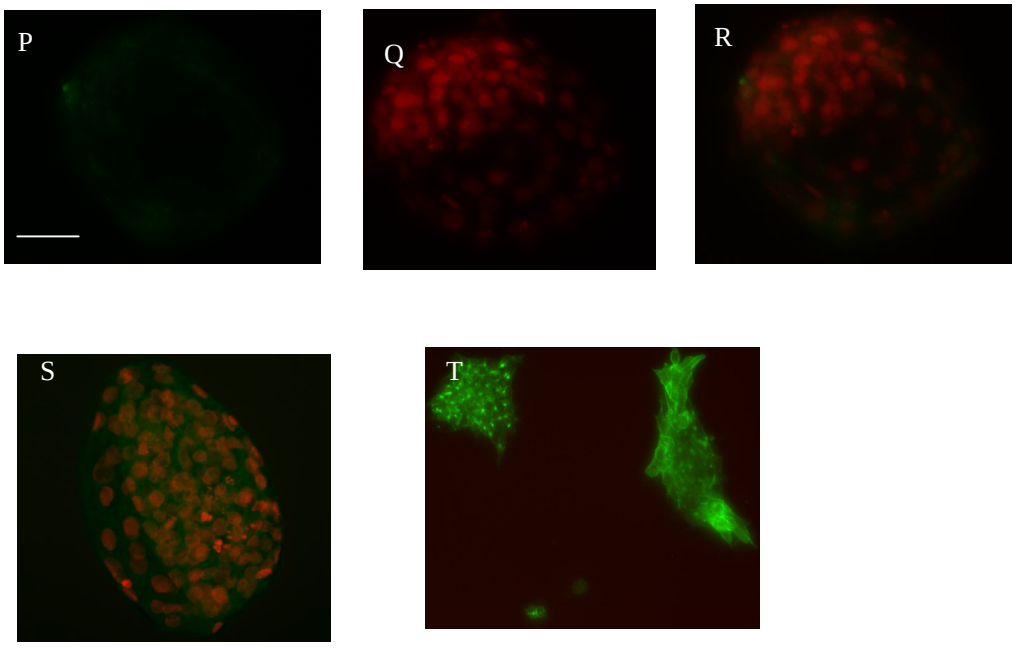
P
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T

Pluripotency and differentiation related gene expression in ICM explants

ICM explants derived from IVP blastocysts were cultured on blocked feeders and the resulting colonies were evaluated on days 0 through 12 for the relative levels of mRNA expression of the pluripotent genes, NANOG, POU5F1 and SOX2. Day 0 samples represent isolated ICM explants that have not been cultured. This experiment was conducted on two independent sets of embryos. On each day of sample collection 2-4 colonies were pooled for analysis. The expression relative to ACTB was the dependent variable and the day of analysis was the independent variable. A pairwise comparison was conducted to compare the expression between two consecutive days using the PDIFF procedure (SAS). Differences were considered significant at $P < 0.05$. Day 0 samples represent freshly isolated ICM explants that have not been cultured. The three pluripotency-determining transcription factors, NANOG, POU5F1 and SOX2 were expressed in the ICM explant in the initial days of culture but their expression reduced significantly with continued culture. The expression of NANOG decreased significantly on day 4 of culture and then on day 7 after which it remained low ($P< 0.01$; Figure 12A). The expression of POU5F1 decreased significantly on day 3 of culture and remained low till day 10 and then increased ($P<0.01$; Figure 12B). The relative expression of POU5F1 seemed to increase after day 10 but there was variability between the two independent replicates. The expression of SOX2 decreased significantly on day 7 of culture after which it remained low ($P<0.01$; Figure 12C).

The above colonies were also evaluated for the relative levels of mRNA transcripts representative of the three lineages, neuroectoderm (NCAM), mesoderm (BMP4) and endoderm (HNF4), as well as the trophectoderm marker (CDX2). CDX2 (Figure 13A) NCAM (Figure 13B), BMP4 (Figure 13C), and HNF4 (Figure 13D) were present on all days of culture. This data suggests that on continued culture the explant cells lose their pluripotent character and differentiate into all three lineages as well as trophectoderm. In addition, the presence of CDX2 in the ICM explants may indicate the presence of trophectoderm cells remaining during the isolation of the ICM. The variability in the expression level of the differentiation related genes from day to day of the culture may be attributed to the fact that the sample collected on each day represents an individual ICM explant and that the particular sample may vary in differentiated character as compared to other explants.

Figure 12. Expression of pluripotency-related genes in cultures of ICM explants derived from in vitro produced blastocysts on Day 0 through Day 12 (D0=isolated ICMs, D1-12=cultured ICM explants).
Relative levels of mRNA expression were determined for the pluripotent genes, NANOG (Panel A), POU5F1 (Panel B) and SOX2 (Panel C). All CT values were normalized to β-actin which served as the internal reference. The panels on the left represent the mean of the expression of the genes relative to ACTB for both the experiments. The standard error bars represent the variation between the two independent experiments. The panels on the right represent the expression of the genes relative to ACTB in the two individual experiments. The standard error bars represent the variation between the technical replicates for each experiment. Each experiment was conducted on a pool of 2-4 ICM explants.

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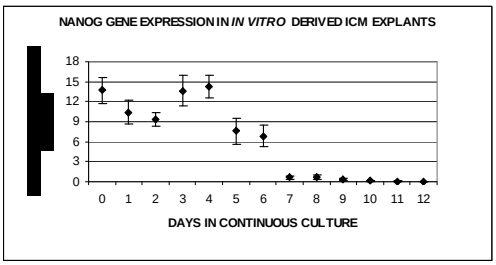

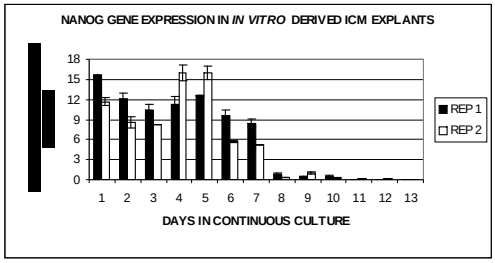

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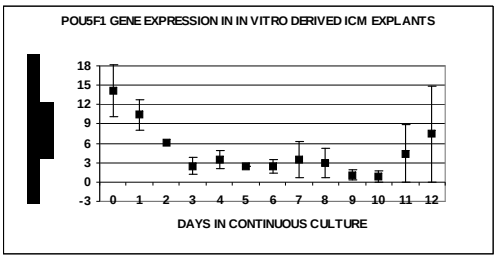

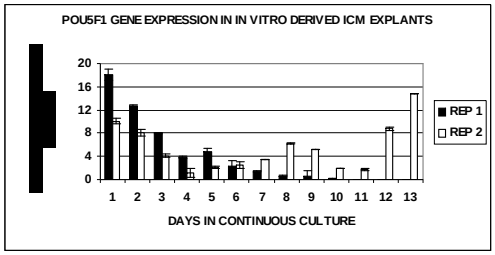

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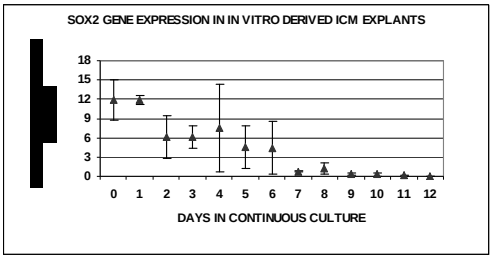


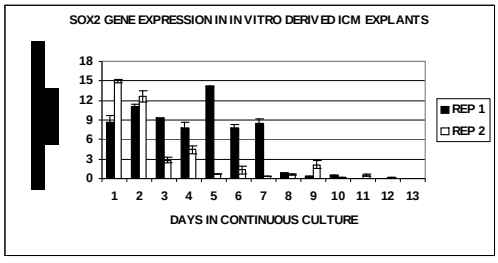
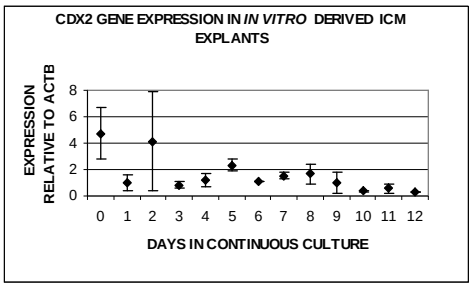
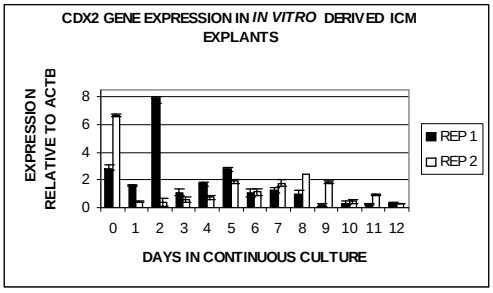


Figure 13. Expression of differentiation-related genes in cultures of ICM explants derived from in vitro produced blastocysts Day 0 through Day 12 (D0=isolated ICMs, D1-12=cultured ICM explants).
Relative levels of mRNA expression were determined for the differentiation related genes, CDX2 (trophectoderm marker; Panel A), NCAM (neuroectoderm marker; Panel B), BMP4 (mesoderm marker; Panel C) and HNF4 (endoderm marker; Panel D). All CT values were normalized to β-actin which served as the internal reference. The panels on the left represent the mean of the expression of the genes relative to ACTB for both the experiments. The standard error bars represent the variation between the two independent experiments. The panels on the right represent the expression of the genes relative to ACTB in the two individual experiments. The standard error bars represent the variation between the technical replicates for each experiment. Each experiment was conducted on a pool of 2-4 ICM explants.

A





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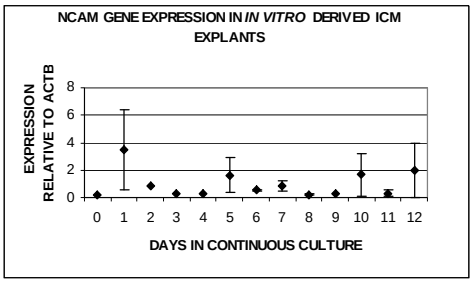

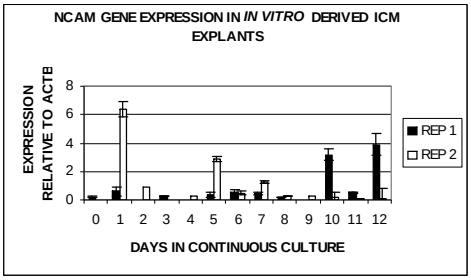

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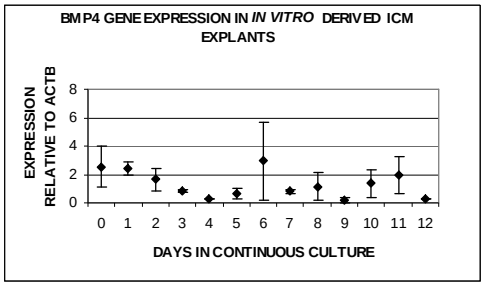
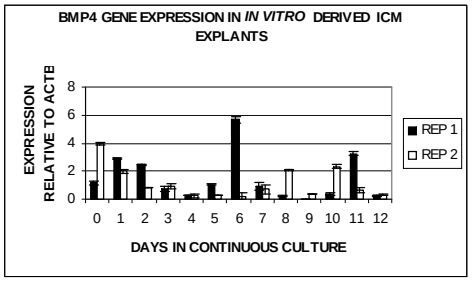

BMP4 GENE EXPRESSION IN *IN VITRO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
0 2 4 6 8
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS IN CONTINUOUS CULTURE


BMP4 GENE EXPRESSION IN *IN VITRO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
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DAYS IN CONTINUOUS CULTURE
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REP 2

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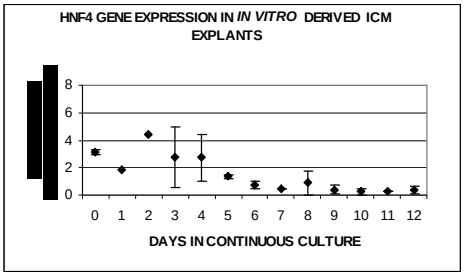
HNF4 GENE EXPRESSION IN *IN VITRO* DERIVED ICM EXPLANTS
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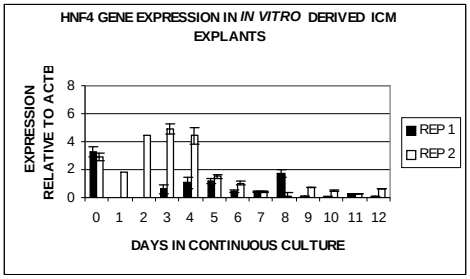
DAYS IN CONTINUOUS CULTURE


HNF4 GENE EXPRESSION IN *IN VITRO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
0 2 4 6 8
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS IN CONTINUOUS CULTURE
REP 1
REP 2

In order to assess whether NANOG, POU5F1 and SOX2 were expressed in ICM explants isolated from in vivo derived bovine blastocysts, ICM isolated from in vivo derived blastocysts were cultured on blocked feeders and the resulting colonies were evaluated on days 2, 4 and 6 for the relative levels of mRNA expression of the pluripotency-determining transcription factors, NANOG, POU5F1 and SOX2. Day 0 samples represent isolated ICM explants that have not been cultured. This study was conducted on 2 individual sets of embryos. On each occasion, 2-4 ICM explant colonies were pooled and collected for analysis on each day of sample collection. The samples were analyzed for relative levels of mRNA of the above mentioned transcription factors and markers of differentiation. All the three transcription factors NANOG (Figure 14A), POU5F1 (Figure 14 B) and SOX2 (Figure 14C) were expressed in the ICM explants for the limited duration the cultures were evaluated.

The same ICM explants were also analyzed for the relative levels of mRNA expression of the markers of the three lineages, neuroectoderm (NCAM), mesoderm (BMP4) and endoderm (HNF4) as well as the trophectoderm marker (CDX2). CDX2 (Figure 15A), NCAM (Figure 15B) and BMP4 (Figure 15C) were present on all days of culture and the level of expression increased with the continued culture. HNF4 (Figure 15D) was not present at the start of culture but began to be expressed with continued culture. This data suggests that the explant cells differentiate into all three lineages as well as trophectoderm. In addition, the presence of CDX2 in the ICM explants may indicate the presence of trophectoderm cells remaining during the isolation of the ICM.

Figure 14. Expression of pluripotency-related genes in cultures of ICM explants derived from in vivo derived blastocysts Day 0 through Day 12 (D0=isolated ICMs, D1-12=cultured ICM explants).
Relative levels of mRNA expression were determined for the pluripotent genes, NANOG (Panel A), POU5F1 (Panel B) and SOX2 (Panel C). All CT values were normalized to β-actin which served as the internal reference. The panels on the left represent the mean of the expression of the genes relative to ACTB for both the experiments. The standard error bars represent the variation between the two independent experiments. The panels on the right represent the expression of the genes relative to ACTB in the two individual experiments. The standard error bars represent the variation between the technical replicates for each experiment. Each experiment was conducted on a pool of 2-4 ICM explants.

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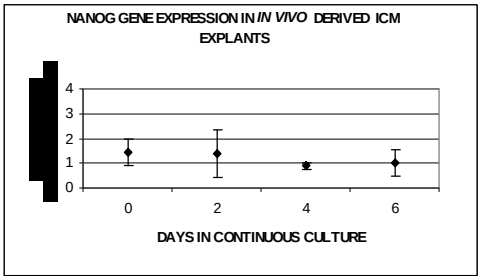

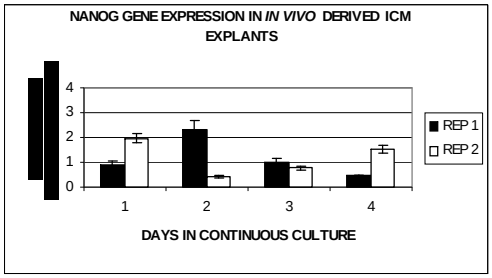

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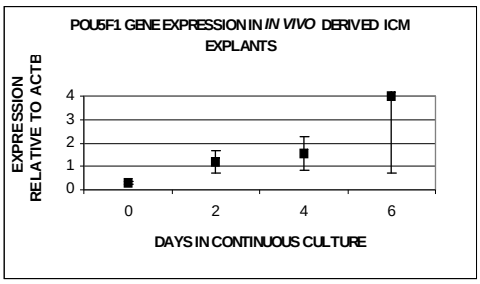

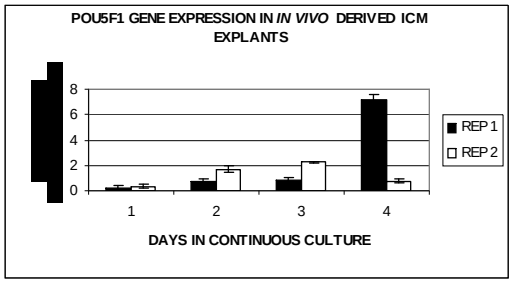

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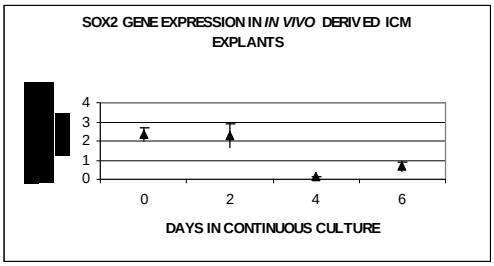


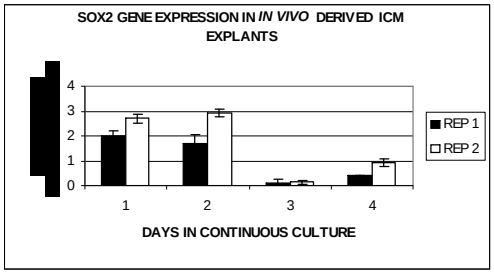
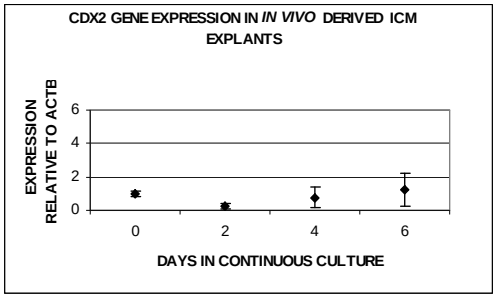
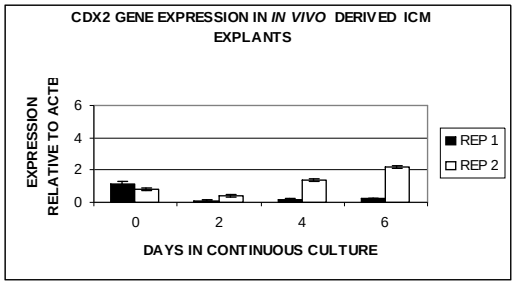


Figure 15. Expression of differentiation-related genes in cultures of ICM explants derived from in vivo derived blastocysts Day 0 through Day 12 (D0=isolated ICMs, D1-12=cultured ICM explants).
Relative levels of mRNA expression were determined via quantitative RT-PCR for the differentiation related genes, CDX2 (trophectoderm marker; Panel A), NCAM (neuroectoderm marker; Panel B), BMP4 (mesoderm marker; Panel C) and HNF4 (endoderm marker; Panel D). All CT values were normalized to β-actin which served as the internal reference. The panels on the left represent the mean of the expression of the genes relative to ACTB for both the experiments. The standard error bars represent the variation between the two independent experiments. The panels on the right represent the expression of the genes relative to ACTB in the two individual experiments. The standard error bars represent the variation between the technical replicates for each experiment. Each experiment was conducted on a pool of 2-4 ICM explants.

A





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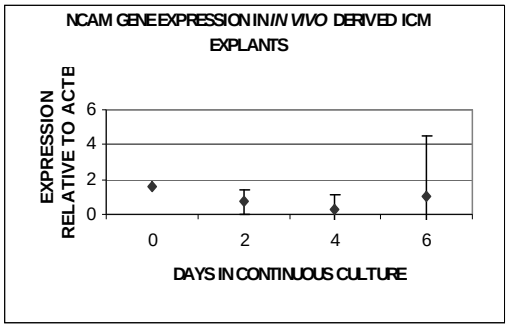

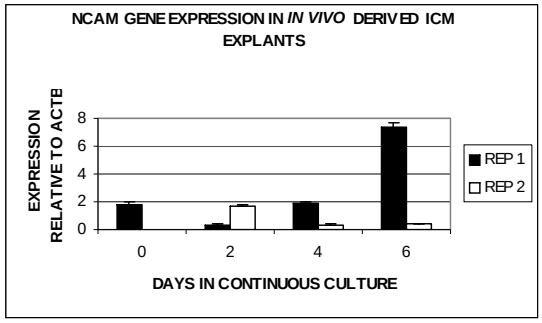

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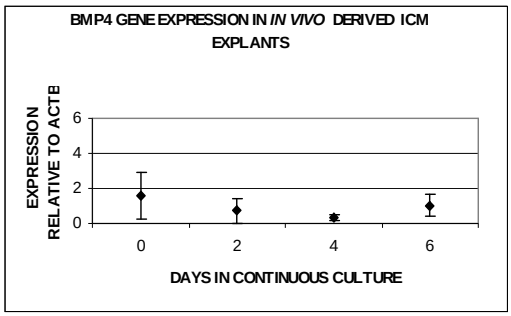
BMP4 GENE EXPRESSION IN *IN VIVO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
6
4
2
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0
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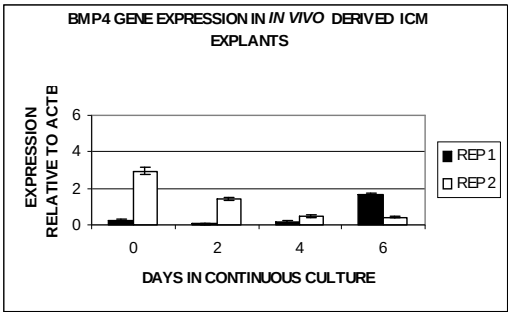
6
DAYS IN CONTINUOUS CULTURE


BMP4 GENE EXPRESSION IN *IN VIVO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
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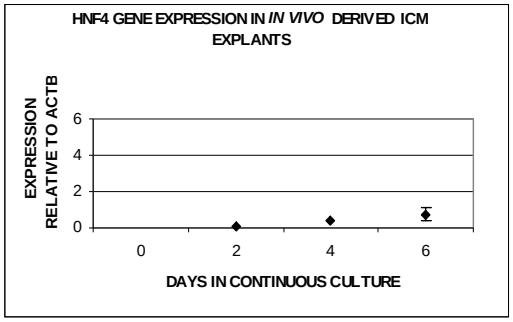
DAYS IN CONTINUOUS CULTURE
REP 1
REP 2

D


HNF4 GENE EXPRESSION IN *IN VIVO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
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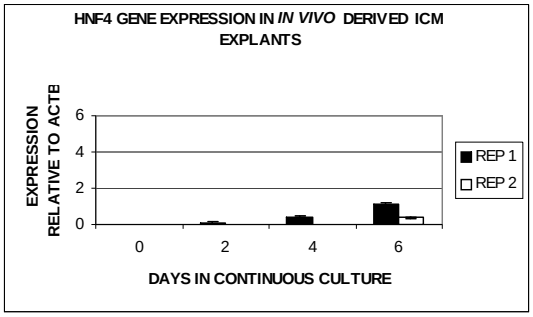
DAYS IN CONTINUOUS CULTURE


HNF4 GENE EXPRESSION IN *IN VIVO* DERIVED ICM EXPLANTS
EXPRESSION RELATIVE TO ACTB
6
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6
DAYS IN CONTINUOUS CULTURE
REP 1
REP 2

The ICM explants were further evaluated by semi-quantitative RT-PCR for the same panel of candidate pathway genes as previously described. The genes belonging to the Jak-STAT (GP130, LIFR), BMP4 (BMPR1A, BMPR2, ID1, ID3), WNT (FZLD, CATENIN), FGF2 (FGFR1) and ACTIVIN (ALK4, ACTR2B) pathways were present on all days of culture, 0-12 (Figure 16). These genes were analyzed for both sets of ICM explant cultures and Figure 16 is a representative of the semi-quantitative RT-PCR data for both the sets.

RNA samples from the ICM explants were further evaluated by semi-quantitative RT-PCR for the expression of candidate genes belonging to the different signaling pathways responsible for pluripotency in mESC and hESC. The genes belonging to the Jak-STAT (GP130, LIFR), BMP4 (BMPR1A, BMPR2, ID1, ID3), WNT (FZLD, CATENIN), FGF2 (FGFR1) and Activin (ALK4, ACTR2B) pathways were present on all days of culture 0-6 (Figure 17). The above experiment was conducted on two independent sets of embryos. On each day of sample collection 2-3 colonies were pooled for analysis.

In a follow up experiment, ICM explants derived from IVP blastocysts were passaged every 6-7 days and individual colonies evaluated at the end of each passage (0-2) for the expression of the same panel of candidate genes via semi-quantitative RT-PCR. The efficiency of colony formation from ICMs (Figure 19 B) isolated from day 7 blastocysts (Figure 19 A) was 60%. The ICM explant colonies grew from 0.1 mm to an average size of 3x3-4x4 mm in a span of 6 days (Figure 19 C). This experiment was conducted on two separate occasions on two independent sets of ICM explants. In both occasions, 4 ICM explant colonies were followed from passage 0

through passage 2.The genes representative of the pathways were present through all the three passages. However, NANOG was not detected in any of the samples, SOX2 was present in samples of passage 0 and POU5F1 was present in all samples passage 0-2 (Figure 18).

Figure 16. Expression of candidate genes in cultures of ICM explants derived from in vitro produced blastocysts grown on feeders on day 0 through day 12 (D0-D12).

Expression levels of candidate genes belonging to the different signaling pathways responsible for pluripotency in mESC and hESC were determined via semi-quantitative RT-PCR. Each band represents a pool of 2-4 ICM explants. cDNA from representative cells and tissues served as positive and negative controls for the respective genes (Table 5).

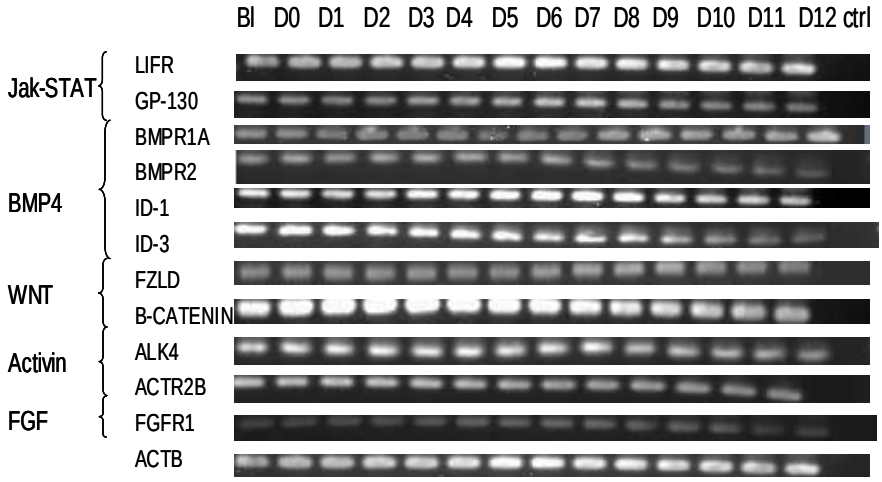


Figure 17. Expression of candidate genes in cultures of ICM explants derived from in vivo derived blastocysts grown on feeders on days 0 through days 6.

Expression levels of candidate genes belonging to the different signaling pathways responsible for pluripotency in mESC and hESC were determined via semi-quantitative RT-PCR. Each band represents a pool of 2-4 ICM explants. cDNA from representative cells and tissues served as positive and negative controls for the respective genes (Table 5).

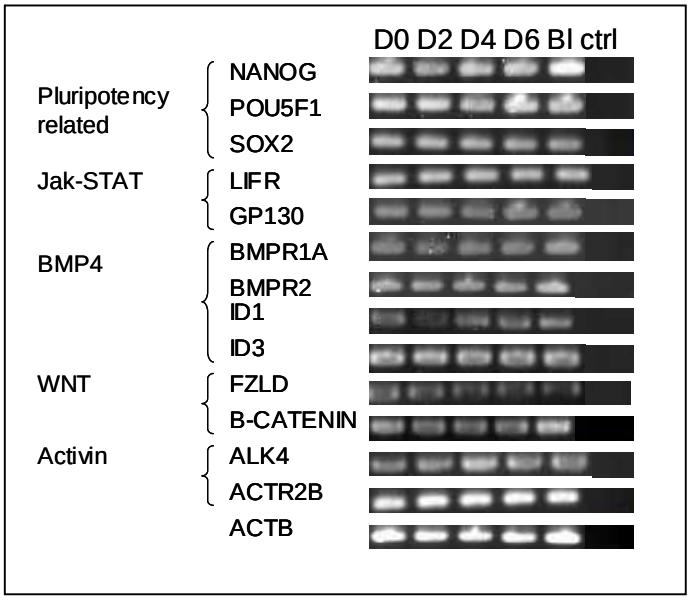


Figure 18. Expression of candidate genes in cultures of ICM explants derived from in vitro produced blastocysts grown on feeders across passages 0-2. In the following panel, 1-4 represent individual colonies/primary cultures that were analyzed across passages 0-2. Expression levels of candidate genes belonging to the different signaling pathways responsible for pluripotency in mESC and hESC were determined via semi-quantitative RT-PCR. This experiment was conducted on two individual sets of ICM explants and each explant was monitored starting passage 0 through 2. The following panel is a representative of one of the sets of cultures. Each band represents an individual ICM explants.

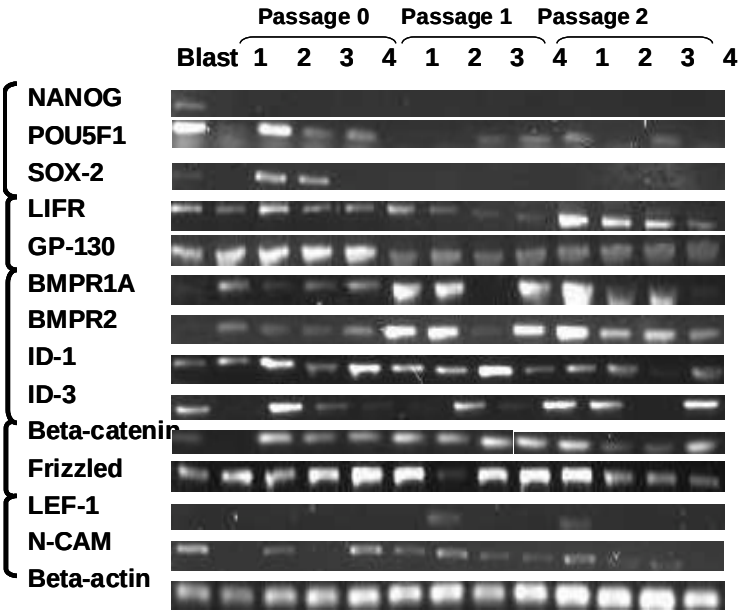
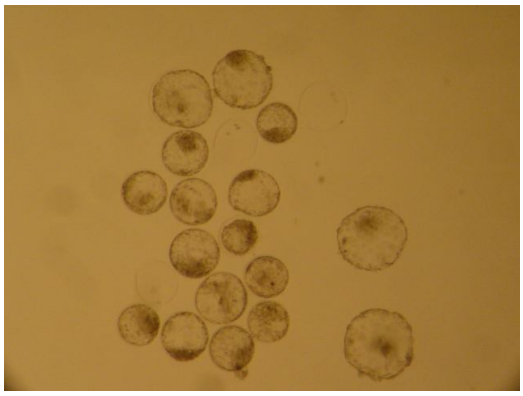


Figure 19. Representative pictures.
Panel A. Day 7 bovine blastocyst
Panel B. Dissected ICMs.
Panel C. Colony growing on MEF feeder

A



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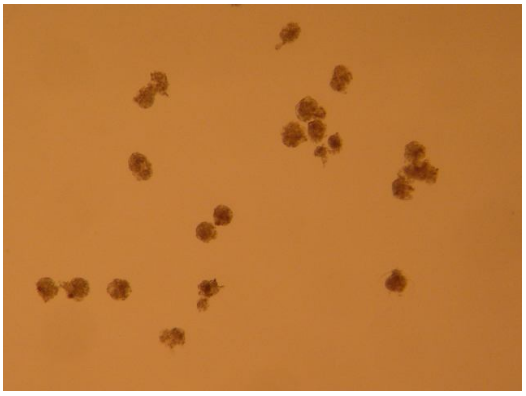
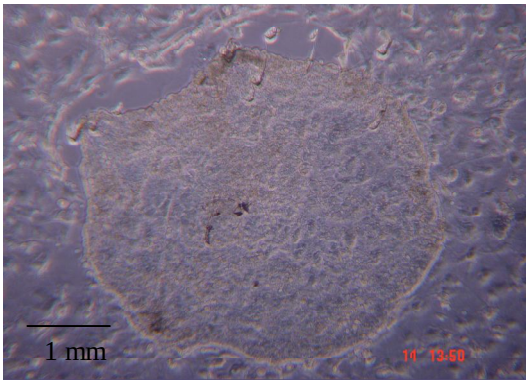


C



Effect of cytokine supplementation on pluripotent gene expression in ICM explants

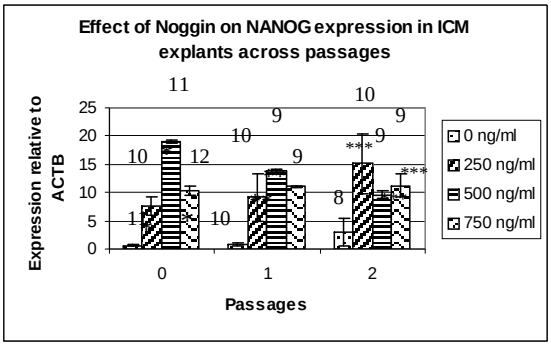
IVP blastocyst derived ICM explants were cultured in the presence of selective cytokines. Samples were collected from the cultures at the end of each passage (0-2). A portion of the ICM explant was used for futher propagation at the end of each passage. Each ICM explant was processed and analyzed individually. The effect of supplementation of each cytokine (Noggin, BMP4, FGF2, Activin A and Noggin + FGF2) on the expression of pluripotency related transcription factors (NANOG, POU5F1, SOX2) was determined by quantitatively measuring the transcripts by qRT-PCR. The data was analyzed as a One- way ANOVA with concentration of the respective cytokine and the passage number as fixed effects and the trial or replicate number as a random effect. The expression relative to ACTB was the dependent variable. An interaction of the concentration and passage number was also assessed as a part of the model statement. Significance was determined at α=0.05.

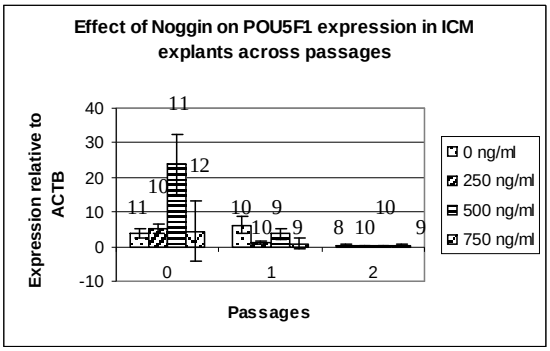
ICM derived explants cultured in ESCM supplemented with 250, 500 or 750 ng/ml Noggin exhibited a significant increase in the expression of NANOG when compared to control (0ng/ml). The experiment was conducted on three different sets of ICM explants on three independent occasions, twice using in IVP blastocysts and once using in vivo produced embryos. This effect of Noggin was independent of the concentration of Noggin used, however, it was affected by the passage number with the relative change in expression being significant at Passage 0 ($P<0.01$) and Passage 1 ($P<0.05$) but not at Passage 2 ($P<0.1$). At the same time there was an interaction

between the passage number and Noggin supplementation. However there was no effect on the expression of POU5F1 or SOX2 (Figure 20).

There was no significant effect of supplementation of either BMP4 (5, 10 or 15 ng/ml; Figure 21), FGF2 (12, 40 or 100 ng/ml; Figure 22), Activin A (10, 25, 50 ng/ml; Figure 23) or a combination of Noggin (500 ng/ml) and FGF2 (40 ng/ml) (Figure 24). Each of these experiments were repeated on two independent sets of embryos on two separate occasions. Although there was no significant effect of the supplementation of the above mentioned cytokines, POU5F1 and SOX2 expression was more susceptible to the passaging and their expression decreased drastically in all studies. Athough the NANOG expression did not increase significantly with respective to the control in the Noggin+FGF2 study, there was variability between the expression levels of the treated samples but not in the controls. This suggests that individual colonies were reacting differently to the cytokines. However, an ICM explant that expressed high NANOG in any one passage did not necessarily show a high NANOG in any other passage. At the same time it did not correlate with high expression of POU5F1 or SOX2.

Figure 20. Effect of supplementation of different concentration of Noggin (0, 250, 500 or 750 ng/ml) on expression of genes related to pluripotency in ICM explants across passages 0-2.
Individual ICM explants were evaluated for NANOG, POU5F1 and SOX2 at the end of each passage by quantitative RT-PCR. The standard error bars represent the variation among individual samples. The numbers above each bar represents the total number of ICMs evaluated at each concentration and each passage. β-actin served as the internal reference gene. Athough the effect of Noggin on NANOG was independent of the concentration of Noggin used, it was affected by the passage number with the relative change in expression being significantly different from the respective controls at Passage 0 (*; P<0.01) and Passage 1 (**; P<0.05) but not at Passage 2 (***; P<0.1).





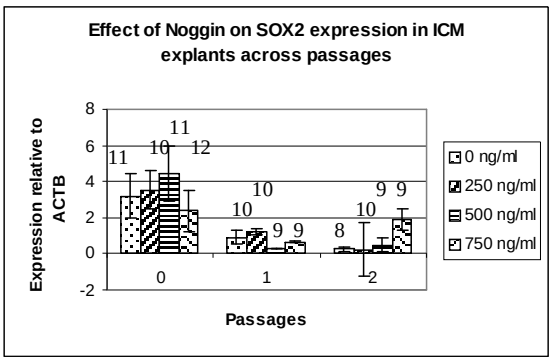
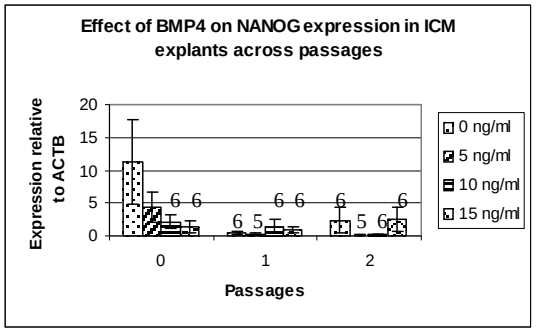
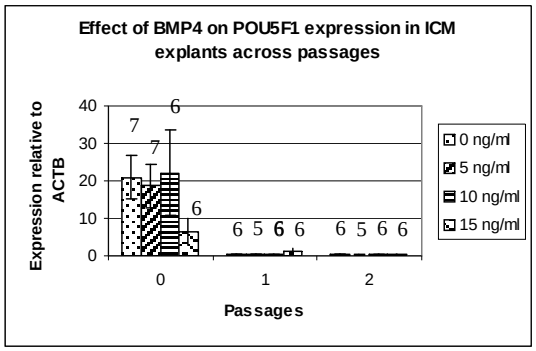


Figure 21. Effect of supplementation of different concentration of BMP4 (with 0, 5, 10 or 15 ng/ml) on expression of genes related to pluripotency in ICM explants across passages 0-2.
Individual ICM explants cultured in ESCM supplemented BMP4 were evaluated for the expression of NANOG, POU5F1 and SOX2 relative to ACTB by quantitative RT-PCR. The numbers above each bar represents the total number of ICMs evaluated at each concentration and each passage.The standard error bars represent the variation among individual samples.





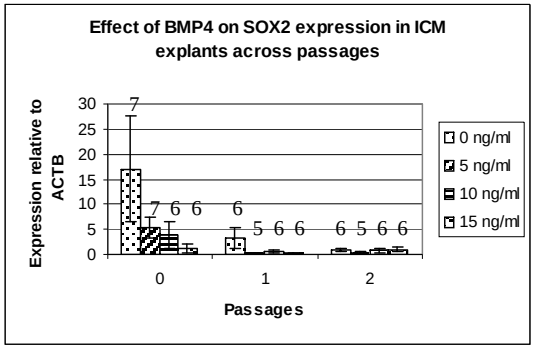
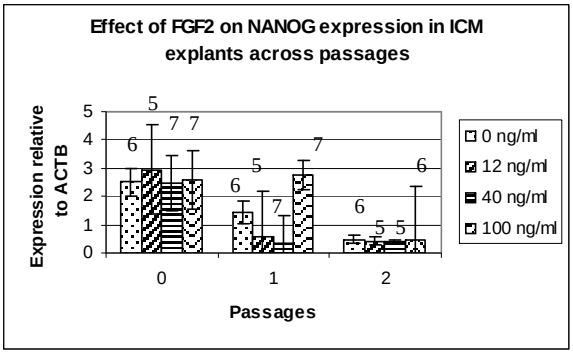
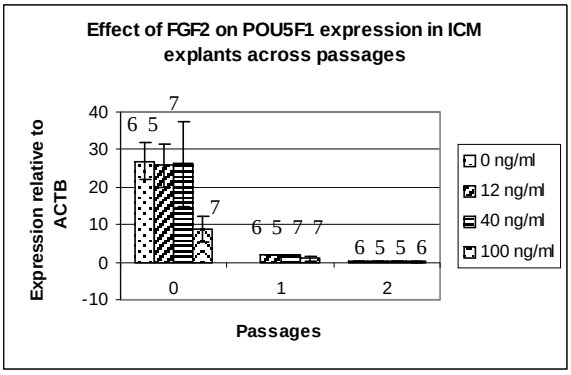


Figure 22. Effect of supplementation of different concentration of FGF2 (0, 12, 40 or 100 ng/ml) on expression of genes related to pluripotency in ICM explants across passages 0-2.

Individual ICM explants cultured in ESCM supplemented FGF2 were evaluated for the expression of NANOG, POU5F1 and SOX2 relative to ACTB by quantitative RT-PCR. The numbers above each bar represents the total number of ICMs evaluated at each concentration and each passage.The standard error bars represent the variation among individual samples.





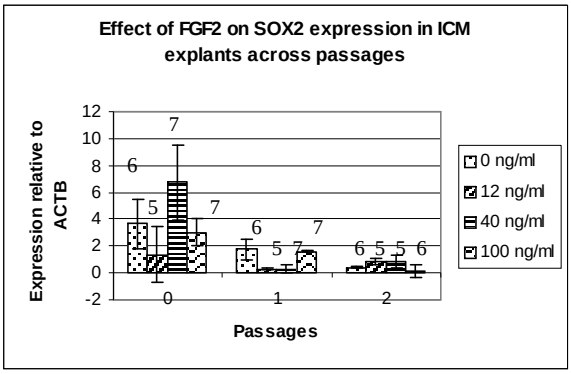
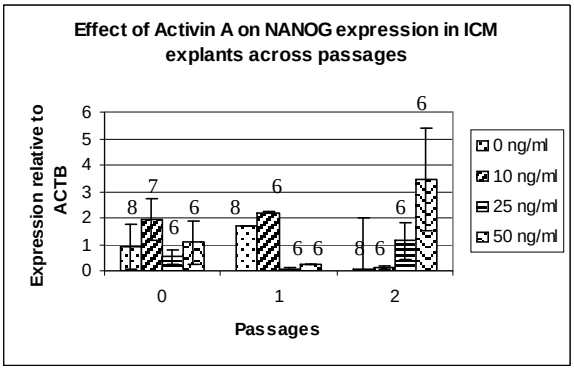
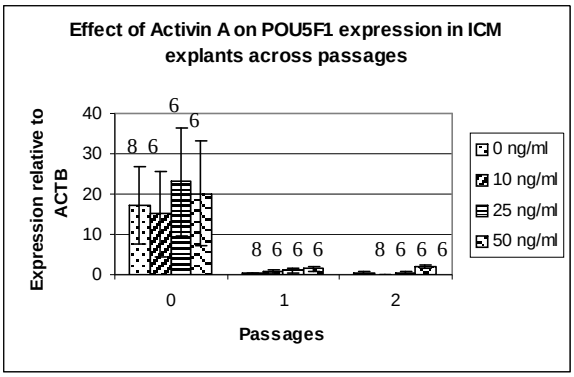


Figure 23. Effect of supplementation of different concentration of Activin A (0, 10, 25 or 50 ng/ml) on expression of genes related to pluripotency in ICM explants across passages 0-2.
Individual ICM explants cultured in ESCM supplemented Activin A were evaluated for the expression of NANOG, POU5F1 and SOX2 relative to ACTB by quantitative RT-PCR. The numbers above each bar represents the total number of ICMs evaluated at each concentration and each passage.The standard error bars represent the variation among individual samples.





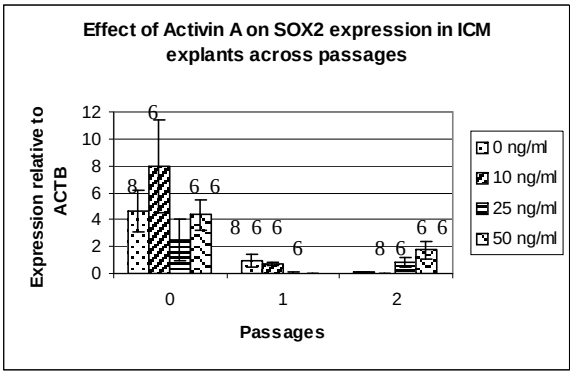
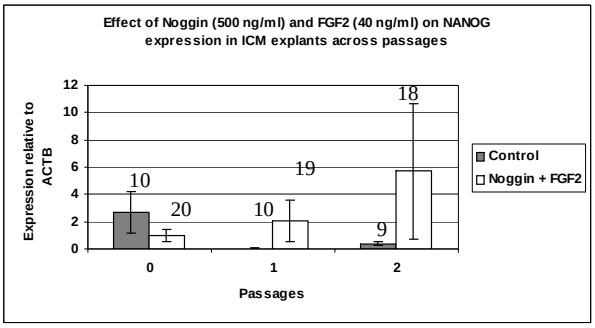
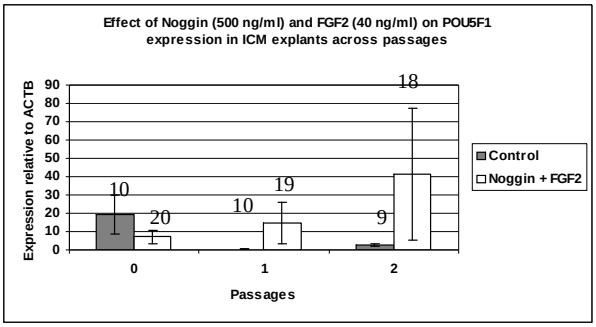
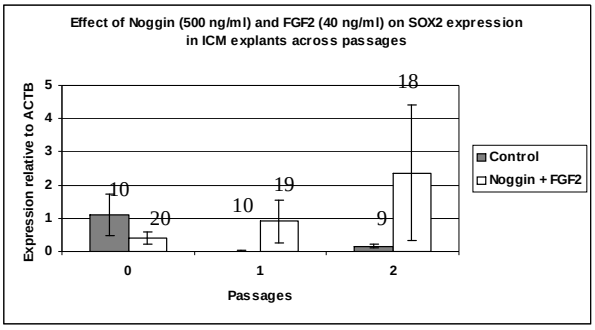


Figure 24. Effect of supplementation of Noggin (500 ng/ml) and FGF2 (40 ng/ml) on expression of genes related to pluripotency in ICM explants across passages 0-2.
Individual ICM explants cultured in ESCM supplemented Noggin + FGF2 were evaluated for the expression of NANOG, POU5F1 and SOX2 relative to ACTB by quantitative RT-PCR. The numbers above each bar represents the total number of ICMs evaluated at each concentration and each passage.The standard error bars represent the variation among individual samples.







Generation of embryos following Hand-made Cloning

The presence of diffentiatiated cells in a population of pluripotent cells can induce the pluripotent cells to differentiate. It was hypothesized that by selectively ablating the diffentiating cells would assist to preserve the existing population of pluripotent cells and allow them to proliferate. To serve this end, a DNA construct, pNANhygro, was designed in which the hygromycin phosphotransferase gene was driven by the NANOG promoter. On transfection into cells, the construct would selectively confer hygromycin resistance to pluripotent cells and ESC progenitors. It has previously been shown that these 446 bases of the NANOG promoter include the regions important for the transcription of NANOG gene in mESCs (Wu and Zhen, 2005). Previous experiments using the GFP reporter have shown the promoter to be active in mESCs.

The effectiveness of the promoter to direct the transcription of the hygromycin phosphotransferase was assessed by creating stably transfecting a R1 mESC line and culturing individual clones for extended periods of time in the presence of hygromycin (0.1 mg/ml). These clones were resistant to the presence of hygromycin and upon differentiation following the removal of LIF from the culture media the cells exhibited cell death (Table 12, appendix).

Once the effectiveness of the vector construct was established, it was transfected into bovine fetal fibroblasts. The fibroblasts were maintained in the presence of G418 over 3 weeks and clones were isolated. However, the clones seneseced when expanded futher in culture. The incorporation of the transgene in the fibroblasts was confirmed by performing a nested PCR on the single cells. The

fibroblasts were then used a donor cells for the somatic cell nuclear transfer procedure.

The procedure of Hand-made Cloning (HMC) is a variant of the Somatic Cell Nuclear Cloning (SCNT) procedure. This procedure was attempted on six independent occasions using different sets of oocytes (n=300). Bovine fibroblasts stably transfected with the DNA construct carrying the NANOG promoter-hygromycin phosphotransferase were used as donor cells for cloning. Prior to HMC, the fibroblasts were maintained at confluency for 5 days to induce them to a quiescent state (G0 stage). After screening for absence of DNA, membrane integrity and cytoplast quality, cytoplast halves were selected for fusion with the donor cells (Table 4, 5). Following fusion of the donor cell with the oocyte cytoplasts, 44 $\pm$ 7.8 of the reconstructed oocytes exhibited healthy, rounded configurations (Table 5) and were activated with calcium ionophore. Control oocytes with intact zona pellucida (31.6 $\pm$ 14.4) and without zona pellucida (21.6 $\pm$ 2.5) as well as cytoplasts containing DNA were activated as well (Table 5). Following activation, for each experiment, an average of 22.3 $\pm$ 8.2 oocytes with intact zona pellucida, 15.5 $\pm$ 2.42 oocytes without zona pellucida and 18.5 $\pm$ 13.2 reconstructed oocytes cleaved to generate blastomeres (Table 5).

The blastomeres were transferred to hygromycin resistant blocked feeders four days after the activation of the reconstructed oocytes and cultured in ESCM. Hygromycin (0.1 mg/ml) was added to the medium the day after plating the blastomeres. Since pluripotent blastomeres of the pre-implantation embryo and progenitors of ES-like cells potentially have the NANOG promoter active, all cells in

which the gene is silenced following differentiation would be susceptible to the toxic effects of the antibiotic. In experiments 1-3, the ESCM was not supplemented with any cytokines (Table 6). The colony formation efficiency of this set of experiments (Expt 1-3) was 8.7%. The blastomeres initiated colonies by Day 2 but the colonies failed to survive any further. In expts 4-6, the ESCM was supplemented with Noggin (500 ng/ml). The blastomeres formed colonies which survived and proliferated upto Day 6 when they were passaged (Table 6). The colony formation efficiency of this set of experiments (Expt 4-6) was 11.36%. The average size of the colony by Day 2 was 1 x 1 mm and the average size by Day 6 was 4 x 4 mm. Blastomeres derived from pronase treated oocytes, oocytes with intact zona pellucida and cytoplast halves containing the DNA served as controls. The control blastomeres from all three groups did not intiate colonies in the presence of hygromycin.

Table 4: Flowchart indicating the procedure of Handmade Cloning (HMC)

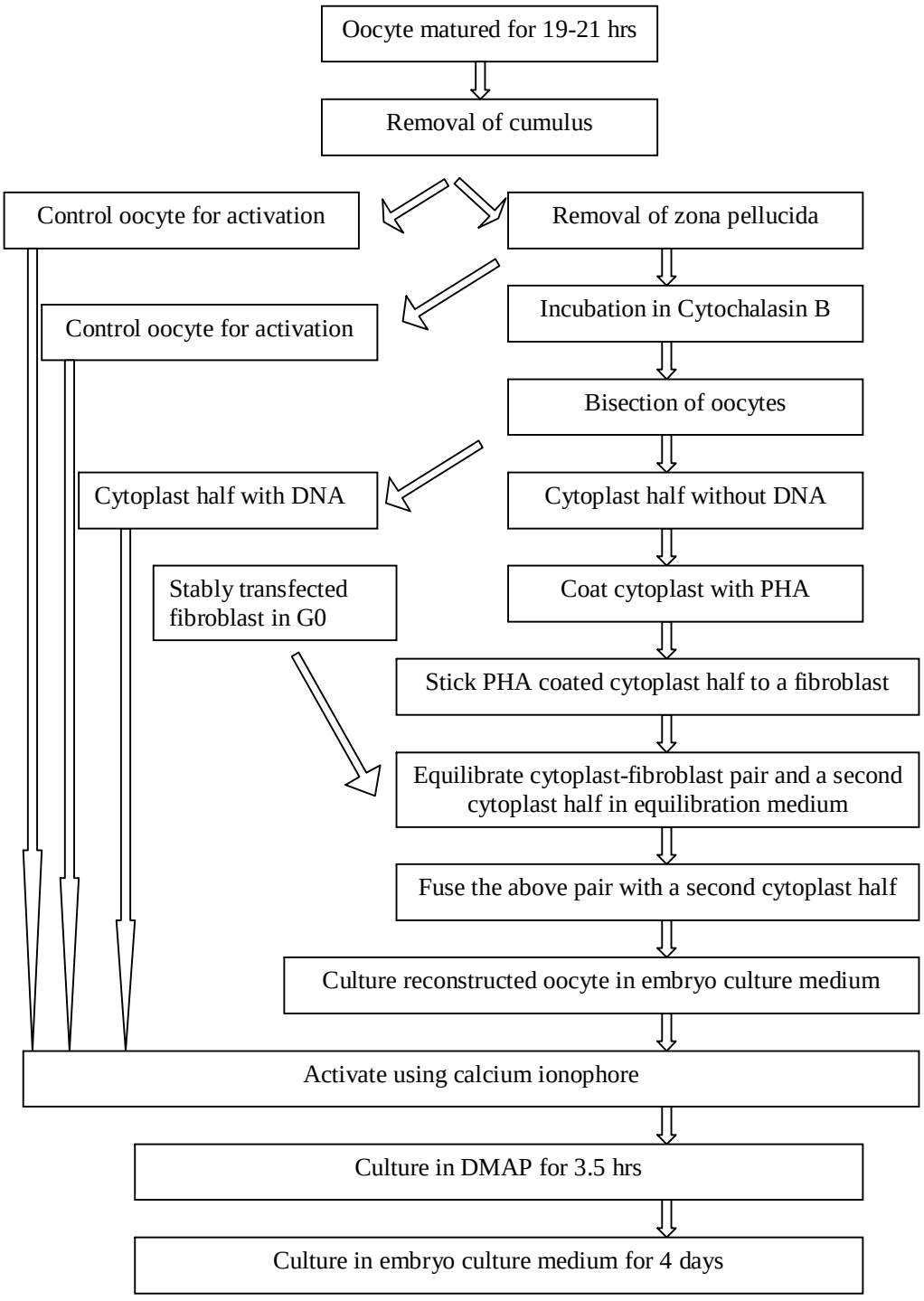


Table 5. Generation of embryos following Hand-made Cloning.

HMC was performed on six independent occasions using separate sets of oocytes (n=300). Oocytes with or without the zona pellucida and cytoplast halves containing the DNA served as controls for the activation process. The reconstructed embryos cleaved to form blastomeres which were then cultured on feeders in the absence (Expt 1-3) or presence (Expt 4-6) of Noggin (500ng/ml).

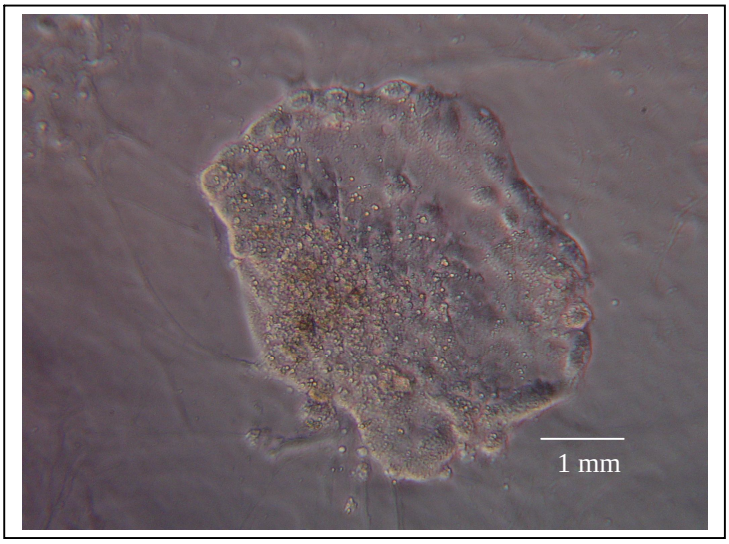
Expt number	Number of reconstructed oocytes	Number of reconstructed oocytes activated (% of reconstructed oocyte activated)	Number of control oocytes without zona pellucida activated	Number of control oocytes with zona pellucida activated	Number of embryos cleaved		
					Control oocytes without zona pellucida	Control oocytes with zona pellucida	Reconstructed embyos
1	36	20 (55.55%)	25	25	20 (80%)	20 (80%)	5 (25%)
2	43	20 (45.5%)	25	25	15 (60%)	18 (72%)	0 (0%)
3	36	25 (69.4%)	20	20	15 (75%)	16 (80%)	18 (72%)
4	47	42 (89%)	20	50	13 (65%)	30 (60%)	29 (69%)
5	57	50 (87.7%)	20	50	16 (80%)	35 (70%)	30 (81%)
6	46	39 (84.7%)	20	20	14 (70%)	15 (75%)	29 (74.3%)
Total	**265**	**196 (73.96 %)**	**130**	**190**	**93 (71.5%)**	**134 (70.52%)**	**111 (56.63%)**

Table 6. Colony formation by blastomeres generated via HMC.

Blastomeres formed by HMC were plated on feeders 4 days after activation. The blastomeres were cultured in ESCM in the presence of hygromycin (0.1mg/ml). The colonies were cultured in the absence or presence of Noggin (500 ng/ml).

Experiment number	Culture condition	Total number of embryos generated	Number of colonies generated by day 2	Number of colonies surviving on day 6	Number of colonies generated after passaging
1-3	ES medium	23	2	0	NA
4-6	ES medium + Noggin	88	10	9	None

Figure 25: Representative picture of a day 4 colony derived from embryos generated by Hand-made cloning. The medium was supplemented with Noggin.



Discussion

Since the isolation of ESCs from mouse embryos more than two decades ago, researchers have been attempting to isolate ESC from various model organisms and domestic species such as the cattle. However, with the exception of the mouse ESCs no other species have exhibited clonal self-renewal and germline transmission. There have been reports of production of germline chimeras from ES-like cells in chicken (Pain et al., 1994; Mc Lavoir et al., 2006) and zebrafish (Ma et al., 2001) however these chimeras were generated using primary cultures and not from true cell lines. Even in mESC, most cell lines existing today have been derived from strain 129 and its sub-strains, and most other strains have proven to be refractory to the derivation of ESC (McWhir et al., 1999). Despite the many peer-reviewed journal articles describing ungulate ES or ES-like cell lines over the past 15 years, no proven ungulate ES cell lines currently exist. Most attempts at isolating ESCs and ES-like cells from cattle have been from the epiblast of the IVP embryos at the blastocyst stage (Cibelli et al., 1998; Mitalipova et al., 2001; Wang et al., 2005). It is unclear whether this lack of success is due to inferior starting material (poor quality embryos), the isolation procedure, the culture conditions or the refractoriness of the species (Keefer et al., 2007). At the same time, oocytes obtained from the slaughterhouse are isolated from ovaries at various stages of the estrus cycle. This has been shown to cause a variation in the developmental competence and gene expression profile of the resulting IVP embryos (Longeran et al., 2003, Niemann et al., 2000; Machatkova et al., 1996). However, studies using in vivo bovine embryos have not had better success at establishing ES-like cells. Real-time RT-PCR analysis

on individual embryos suggested a high variability in the levels of NANOG and POU5F1 of individual IVP blastocysts. Various published studies have indicated that IVP embryos exhibit a variation in the expression of genes regulating various aspects of embryonic growth and development (Lopes et al., 2007; Camargo et al., 2005). The variability in expression of the pluripotent transcription factors may compound the efficiency of derivation of ES-like cells using IVP embryos.

POU5F1 and NANOG, along with a series of cell surface markers (SSEA1, SSEA4, TRA-1-60, TRA1-81) have been used to characterize mouse and human ESCs. Although the expression patterns of ESC surface markers vary between humans and mice, nonetheless, these markers have proven useful in the characterization of ES cell lines (Henderson et al. 2002). For instance, SSEA1 is expressed in mESCs, whereas SSEA3 and SSEA4 are expressed in hESCs (Draper et al. 2002). One of the major problems encountered in monitoring the ES-like cells derived from bovine embryos has been the lack of data on the appropriate markers that can be used to characterize these cells. In this study we found that SSEA1 but not SSEA4 was expressed on the surface of the bovine blastocyst in a punctate pattern. There have been mixed reports regarding the expression of SSEAs in bovine embryos and embryo derived cultures. Mitalipova et al. (2001) reported bovine ES-like cells being positive for the expression of both antigens; Saito et al. (2003) reported these cells positive for SSEA1, whereas Wang et al. (2005) reported IVP blastocysts and ES-like cells derived from them to be SSEA4 positive. The reason behind these contradictory reports is not clear; however the difference in culture systems in different laboratories might be a contributing factor. Furthermore, unlike

the distinct localization of these surface markers to the ICM in mouse and human ESCs, SSEA1 in bovine was localized over both the ICM and trophectoderm in our study and therefore is not a definitive marker for bovine ES-like cells.

Immunolocalized Nanog protein was observed in the nucleoplasm and nucleoli of the bovine ICM cells but it was restricted to the nucleoli in the trophectoderm cells. This pattern is similar to that reported for caprine blastocysts (He et al., 2006), which is in contrast to that observed in the mice where Nanog expression is restricted to the ICM only (Hatano et al., 2005). He et al. (2006) confirmed the nucleolar localization of Nanog in the trophectoderm by double staining with anti-nucleolin and suggested this phenomenon to be a means of sequestration of Nanog in the trophectoderm. Pou5f1 was nuclear localized in the cells of the ICM as well as the trophectoderm. This pattern is in concordance with previously published reports in caprine, bovine and porcine blastocysts (He et al., 2006; Kirchoff et al., 2000; van Eijk et al., 1999). In mouse (Kirchhof et al. 2000; Palmieri et al. 1994), monkey (Mitalipov et al. 2003), and human (Hansis et al. 2004) blastocysts, Pou5f1 expression is restricted to the ICM and is considered a major repressor of the trophoblast lineage. It has been suggested that POU5F1 expression in the trophectoderm of goat, bovine and porcine may be related to the longer period of trophectoderm proliferation before implantation in these domestic animals (Degrelle et al. 2005; Kirchhof et al. 2000). Hence, while Pou5f1 alone cannot serve as a definitive marker of ES-like cells in bovine by itself, it should be a part of the panel of pluripotency markers for characterizing bovine ES-like cells.

In pre-implantation mouse embryos, the pluripotency determining transcription factors, POU5F1, NANOG and SOX2 are expressed in the ICM and epiblast of the blastocyst (Avilon et al., 2003; Palmieri et al., 1994). They are also highly expressed in human and mouse ESCs, and their expression diminishes when these cells differentiate and lose pluripotency (Ginis et al., 2004). The transcripts of NANOG and POU5F1 are routinely used as molecular markers in identifying the pluripotent colonies of mouse and human ESCs. However, few of the previously reported bovine ES-like cell lines were demonstrated to be POU5F1 positive (Mitalipova et al., 2001; Stice et al., 1996; Cibelli et al., 1998; Saito et al., 2003) except for one report by Wang et al. (2005) where they found NT derived ES-like cell lines positive for POU5F1. However, no such results were reported for IVP bovine embryos in that study. There is no published report for presence of NANOG in cultured ES-like cells in any domestic species reported thus far.

One of the aims of this research was to evaluate by RT-PCR if the pluripotency markers, NANOG, POU5F1 and SOX2 are expressed in early ICM derived explants. Our hypothesis is that the early loss of pluripotency-determining transcription factors in culture contributes to the difficulty in self-renewal of pluripotent cells derived from the embryos of domestic species such as cattle. Our results indicated that on culturing ICM cells derived from bovine blastocysts, NANOG, POU5F1 and SOX2 are expressed in the initial days of culture; however, with continued culture their expression diminishes. It is well known that for the maintenance of pluripotency, the relative levels of these three transcription factors needs to be optimum and any change in their ratio can initiate differentiation. At the

same time, markers of differentiation of all the three lineages as well as for the trophectoderm were present on all days of the culture in ICM explants. The presence of the trophectoderm marker, CDX2 suggests that remnants of the trophectoderm may have remained following the dissection. On the other hand it may also indicate the differentiation of ICM explant cells to a trophectoderm lineage. The presence of all the three lineages, endoderm (HNF4), mesoderm (BMP4) and neuroectoderm (NCAM) are also indicators of the spontaneous differentiation of the pluripotent cells of the ICM during culture. This is in accordance with the inherent nature of ESCs and ES-like cells to spontaneous differentiate into various lineages in culture, especially when the culture conditions are not optimal for the maintenance of pluripotency.

During routine derivation of mESCs and hESCs from blastocysts, it takes 6-7 days from the attachment of ICM or the blastocyst before the cells are ready to be passaged for the first time. It is at that moment that pluripotent cells are morphologically identified and usually picked manually and sub-cultured. These cells are propagated to generate a population of pluripotent cells. Our results showed that on passaging the bovine ICM explants, NANOG and SOX2 are not expressed beyond the initial passage (P0); however, POU5F1 is expressed through passage 2. This study has for the first time shown that bovine ICM derived explants express the same molecular markers of pluripotency (NANOG, POU5F1 and SOX2) as mouse and human ESCs, albeit for a short duration. This result indicates that there may be a small window of time during which the cells are pluripotent allowing the possibility that appropriate manipulations may prolong the duration of expression of these

transcription factors. The data also suggest that the ICM explants undergo spontaneous differentiation into all three lineages early in culture.

In addition to the pluripotency-determining transcription factors, there are several signaling pathways that have been implicated in the maintenance of pluripotency in mouse, as well as, human ESCs. However ESCs from these two species vary in terms of which pathways are pertinent for the maintenance of pluripotency. Members of the following signaling pathways are highly expressed in pluripotent mESCs: Jak-STAT (Matsuda et al., 1999; Niwa et al., 1998) and TGF-β families (Ogawa et al., 2007); whereas the members of the following signaling pathways are highly expressed in pluripotent hESCs: FGF (Amit et al., 2000) and TGF-β families (Xu et al., 2005; Wang et al., 2005; Vallier et al., 2005; James et al. 2005). On analyzing bovine ICM explants, transcripts for receptors belonging to the Jak-STAT pathway (LIFR and GP130); TGF-β pathway, BMP4 (BMPR1A, BMPR2) and Activin A (ALK4, ActRIIB); WNT (FZLD); and FGF2 (FGFR) were present in cultures on Days 1-12 and they continued to be expressed on passaging as well. These results suggest that receptors belonging to the respective signaling pathways are actively transcribed. If these pathways are relevant for maintenance of pluripotency in bovine ICM explants, then the pathway may be stimulated by the application of appropriate ligands to act on downstream targets.

The loss of expression of pluripotent markers in the cultures of ICM explants in the initial days of culture is indicative of the fact that the culture environment is not optimal for the maintenance of pluripotency. Mouse and human ESCs are known to spontaneously differentiate in culture, especially if the culture medium is not optimal

for the maintenance of pluirpotency. In order to provide the appropriate environment for culturing ESCs and ES-like cells, growth factors or cytokines are added to the hESC and mESCs culture medium. In mESC, the Jak-STAT pathway stimulated by LIF is most vital, and mESCs differentiate quickly when LIF is withdrawn from the culture medium (Smith et al., 1988; Niwa et al. 2001). Under defined conditions, supplementation with ligands of the TGF-β superfamily such as BMP4 (Smad 1/5/8 mediated responses; Ying et al., 2003; Qi et al., 2004) or Activin A and Nodal (Smad 2/3 mediated responses; Ogawa et al., 2007) increases the number of pluripotent mESC colonies. On the contrary, in hESCs application of Noggin inhibits the Smad 1/5/8 mediated signaling by suppressing the BMP4 pathway. This increases the proportion of pluripotent human ESCs in culture (Wang et al., 2005). Additionally, supplementation with Activin A or Nodal alone or in combination with FGF2 supports the maintenance of hESC in defined medium (Vallier et al., 2005; Beattie et al., James et al., 2005). LIF, however, is not effective in maintaining hESCs in culture.

We hypothesized that in order to extend the duration for which the expression of NANOG, POU5F1 and SOX2 can be maintained in culture, supplementation with cytokines and growth factors is necessary. Therefore, the culture media was supplemented with components of the TGF-β (BMP4, Noggin, Activin A) and FGF (FGF2) pathways. The choice of the factors and the concentration at which they were used was based on their pluripotency-supporting role in mESCs and hESCs (BMP4, Noggin, FGF2, Activin A and Noggin+FGF2). BMP4, Activin A or FGF2 did not affect the upregulation of any of the three transcription factors. Supplementation of

the culture medium with Noggin up-regulated the maintenance of NANOG mRNA in ICM derived explants across passages when compared to control cultures. The results from the supplementation of Noggin in the culture medium of ICM explants suggests that the regulation of pluripotency in bovine ICM explants may be more similar to hESCs than to mESCs. However, it did not effectively maintain the expression of POU5F1 or SOX2. As Noggin had a positive effect on NANOG expression, it was surprising to see the down regulation of POU5F1 and SOX2, since it is well known that these three transcription factors are tightly regulated and influence each other's expression as well. Studies in hESCs have shown a beneficial effect of using FGF2 in combination with Noggin (Wang et al., 2005; Xu et al., 2005). It was anticipated that the combination of Noggin and FGF2 would help maintain the expression of pluripotency better than Noggin alone. Although the combination of Noggin+FGF2 did not increase the expression of NANOG expression significantly, there was high variability in the expression of the transcription factors in the ICM explants cultured in the presence of the cytokines; this variability was not seen in the controls. This indicates that individual colonies may be responding differently to the supplementation. In the light of this observation it may be speculated that repeating the study with larger numbers or with embryos of a consistent quality may divulge the true effect of these cytokines.

Numerous attempts have been made to isolate ESCs from bovine embryos, but more often than not the primary colonies undergo spontaneous differentiation. Results from the culture of bovine ICM explants also showed a mixed population of cell types very early on in the culture. In mESC derivation, it is critical that the primary explant

be cultured for a sufficient time to allow multiplication of ESC progenitors yet without allowing extensive differentiation (Abbondanzo et al., 1993). The differentiation signals sent out by the differentiating cells in cultures of mESCs and hESCs can cause the pluripotent cells to differentiate as well. It is well known that the higher the percentage of pluripotent cells in a population, the greater is the chance of survival and propagation of the ESCs. In order to circumvent this problem the strategy of selectively ablating differentiating cells was adopted. This strategy has previously been implemented for derivation of ESCs from refractory strains of mice such as CBA (Gallagher et al., 2003; Mc Whir et al., 1996). Among mouse strains, genetic background strongly affects the efficiency of ESC isolation, and almost all ESC lines used for research are derived from strain 129 (Evans and Martin, 1981; Magin et al., 1992) and some from strain C57BL/6 (Kawase et al., 1994). McWhir et al. (1996) utilized a Neomycin cassette driven by the POU5F1 promoter to isolate pluripotent cells. Using this strategy they were able to generate germline pups from CBA ESCs. Prior to the above mentioned study it had not been possible to derive ESCs from the CBA strain, let alone generate germline chimeras. In the current study, the NANOG promoter was chosen instead of the POU5F1, because the expression of the latter is increased on differentiation into primitive endoderm and mesoderm cells as well (Niwa et al., 2000). Moreover, in bovine embryos the expression of POU5F1 is not restricted to the pluripotent cells of the ICM but is expressed in the trophectoderm as well (van Eijk et al., 1999). Furthermore, NANOG not only inhibits the differentiation of stem cells into endoderm but also actively maintains

pluripotency, in contrast to the role of POU5F1 as a blocker of differentiation of ICM and ESC into trophectoderm (Cavaleri and Scholer, 2003; Niwa et al., 2000).

Transgenic embryos were generated via Hand-made cloning that carried the hygromycin phosphotransferase gene under the control of the bovine NANOG promoter. It was hypothesized that since pluripotent blastomeres of the pre-implantation embryo and progenitors of ES-like cells potentially have the NANOG promoter active, all cells in which the gene is silenced following differentiation would be susceptible to the toxic effects of the antibiotic. This would encourage the survival of pluripotent cells. It has previously been shown that these 446 bases of the NANOG promoter include the regions important for the transcription of NANOG gene in mice (Wu and Zhen, 2005). Previous experiments using the GFP reporter have shown the bovine NANOG promoter to be active in mESCs. On transfecting the construct into mESCs, the cells survived in the presence of hygromycin in culture thereby proving the efficacy of the vector construct to confer resistance to hygromycin. Loss of resistance to hygromycin following LIF removal confirmed the specificity of the NANOG promoter. Bovine fetal fibroblasts stably transfected with the pNANhygro vector construct were used as donor cells for creating embryos. These transgenic blastomeres formed colonies when grown on feeder cells and were able to proliferate when the medium was supplemented with Noggin. However, they failed to grow on passaging indicating that the culture conditions were not optimal for the NANOG promoter to remain active over extended culture.

Recently published research by Chambers et al. (2007) suggests that mESCs can maintain most of their pluripotent characteristics in the absence of NANOG.

Moreover, cells not expressing NANOG can generate NANOG expressing colonies and vice versa. In the light of these findings, it is probable that the expression of NANOG or the activity its promoter may not be a prerequisite for the maintenance of ES-like cells. This also raises questions regarding our strategy of selective ablation. The colonies generated by the HMC blastomeres succumbed to the effects of hygromycin when passaged. It is likely that our initial colonies were pluripotent but the NANOG promoter was silenced at passaging. This suggests that adoption of a different selection strategy or the removal of hygromycin at passaging may have permitted the survival and proliferation of pluripotent cells. However, other reports studying the over-expression of NANOG in ESCs have demonstrated it to be important for the maintenance of pluripotency and its utility as a marker of pluripotency (Darr et al., 2006; Hatano et al., 2005; Hyslop et al., 2005). Furthermore in order to truly define the role played by NANOG, further investigations are needed.

In summary, this research has shown that

- Bovine blastocysts express NANOG and POU5F1.
- ICM explant cultures express the established molecular markers of pluripotency (NANOG, POU5F1, and SOX2) during term culture, and
- ICM explants express components of pathways known to be involved in maintenance of mouse and human ESCs.
- Culture of ICM explants in Noggin helps maintain the expression of NANOG.
- Selective ablation of differentiating cells in ICM explants can potentially facilitate survival and proliferation of pluripotent cells under optimized conditions.

Future Directions

The findings of this research showed that the transcripts for the pluripotency determining transcription factors, NANOG, POU5F1 and SOX2 are expressed in the ICM explants in the initial days of the culture. However, they disappear early in culture and at the same time there is a prominent presence of the markers of differentiation of all the three lineages as well of the trophectoderm. This indicates that though the cells are pluripotent for a while, there is spontaneous differentiation as the culture system is not optimal for sustenance of pluripotency. We were successful in improving the culture condition by supplementing the culture medium with the growth factor Noggin, which aided in maintaining the expression of NANOG. However, the culture conditions remained sub-optimal for the maintenance of the other two transcription factors (POU5F1 and SOX2). In the future, identification of a combination of cytokines might be able to improve the expression of all three transcription factors.

Most of the research in this study was conducted with embryos derived from slaughter house oocytes. The embryos thus obtained are highly variable in their quality and gene expression patterns. The possibility cannot be ruled out that using a more consistent source of such as embryos or embryos from similar genetic background might have helped alleviated the variation in the responses to the cytokine treatments. At the same time performing the experiments with a larger number of embryos might assist in mitigating the variation and other cytokines than

that were investigated in this research might prove to be effective in supporting the expression of the pluripotency-determining transcription factors.

The candidate gene expression demonstrated that the transcripts for representatives of the various signaling pathways were expressed in the ICM explants. It is important to pursue this further and investigate which pathways are actually functional by evaluating for the presence of activated secondary messengers of the pathways such as the Smad 1/5/8 in the BMP4 pathway, Smad 2/3 in the Activin pathway. Once it is established that the members of the respective pathways are not merely transcribed, but are actually functional steps can be taken to activate the concerned signaling pathway. This knowledge might be helpful in optimizing the culture conditions as well.

Several experiments that can be attempted to follow up this line of research demand a source of self-renewing cells. One approach to serve this purpose would be to over-express the NANOG gene in early ICM explants or create bovine embryos over-expressing NANOG for the purpose of deriving cells lines from them. The colonies thus obtained could then provide for a source of cells that could be utilized for performing experiments that require a large number of cells such as for examining the presence of signaling pathways and performing experiments like protein analysis by Western blots.

A similar approach that could be adopted would be to use bovine fetal fibroblasts for the purpose of reviving their pluripotent nature by introducing a set of transcription factors that have been proven to turn differentiated cells into pluripotent cells. This technique has been demonstrated by Yamanaka's group (Okita et al., 2007;

Takahashi et al., 2006) where they generated cell lines resembling pluripotent stem cells by the retroviral introduction of Pou5f1, Sox2, c-Myc and Klf4. A similar study conducted in human somatic cells by Thomson's group (Yu et al., 2007) using a similar set of transcription factors (Pou5f1, Sox2, c-Myc and Lin28) showed that this procedure is applicable in other species as well. Cells obtained by implementing this procedure in bovine cells should provide sufficient material for conducting further research.

The present research involved inserting of the NANOG promoter-hygromycin phosphotransferase construct into bovine oocytes via Hand–made cloning. The vector construct has been demonstrated to be functional in pluripotent cells of other species as well (pilot study done with mESCs). The same could be attempted with other species as well. The derivation of ESCs is governed by a strong genetic component. It is difficult to speculate which species would be more amenable to the procedure of selective ablation for the self-renewal of pluripotent cells. We already have frozen stocks of in vivo derived caprine blastocysts and also early passages (P0 and P1) of colonies derived from these blastocysts and this research could be replicated using them.

This study has been successful in identifying the key core transcription factors in bovine ICM explants. It has been shown for the first time that NANOG, POU5F1 and SOX2 are expressed in cultures derived from the bovine embryos and that this expression is maintained in basal ESC medium, albeit for only a short time. We successfully identified a potential ligand (Noggin) that enables the maintenance of NANOG and might be the key to maintenance of pluripotency in bovine ES-like

cells. Noggin blocks the BMP4 pathway from being activated by binding to its receptors. Further exploration of this pathway and the role activated Smad 1/5/8 and their target genes play in the regulation of pluripotency in bovine ICM explants and ES-like cells is likely to unravel the nature of these cells further.

Embryos generated via Hand-made cloning generated colonies in the presence of Noggin. It is likely that these colonies would have survived for longer periods of time if the culture system or passaging could be optimized. Once optimal conditions for maintenance of pluripotency are established, the culture system should allow the NANOG promoter to remain functional and the pluripotency maintaining factors would continue to be expressed.

The current research demonstrated that the molecular markers of pluripotency (NANOG, POU5F1, and SOX2) are expressed in bovine ICM explant cultures and can potentially be used for monitoring ES-like cells derived from bovine embryos. At the same time candidate genes representing the signaling pathways implicated in maintenance of pluripotency are also expressed in ICM explant cultures and these pathways can potentially be stimulated to sustain pluripotency. We were also able to identify a potential ligand (Noggin) that might be critical in self-renewal of bovine ES-like cells. The tools for selective ablation of differentiating cells developed as part of this research also provide a means for creating an environment supportive of pluripotency. We are hopeful that future experiments built on the findings of this study will lead to the derivation of ESCs in domestic animals in the near future.

Appendix

Bovine RT-PCR primers

Table 7: List of bovine RT-PCR primers along with the product length, melting temperatures and the tissues that are positive and negative for the expression of the respective genes.

Gene		Sequence	Product length	Melting temp	Positive tissue	Negative tissue
LIFR	fwd	ATCATCAGTGTGGTGGCAAA	573	67.9	trophectoderm	heart
	rev	CGCAAGACCAGGTGGTAACT		60.1		
GP130	fwd	AGAAGCAGAGAATGCCCTTG	298	63.4	adipose	pancreas
	rev	TCACTCCAGTCACTCCAGTA		58.3		
BMPR1A	fwd	AGCCTCCAGACTCACAGCAT	387	64	lung	lymph node
	rev	ACCCAGAGCTTGACTGGAGA		64		
BMPR2	fwd	CTGGACAGCAGGACTTCACA	381	64.2	lung	muscle
	rev	CTTGGGCCCTATGTGTCACT		63.9		
ID1	fwd	CTCCAGCACGTCATCGACTA	486	64.1	adipose	lymph node
	rev	CTGAGAAGCACCAAACGTGA		64.1		
ID3	fwd	ACTCACTCCCCAGCATGAAG	242	64.3	kidney	lymph node
	rev	GTAGTCGATGACGCGCTGTA		64		
FZLD	fwd	CATTTGGTCAGTGCTGTGCT	632	64	heart	uterus
	rev	CCATGAGCTTCTCCAGCTTC		64		
B-CATENIN	fwd	GCTTGGTTCACCAATGGATT	500	63.6	muscle	uterus
	rev	TGATGTCTTCCCTGTCACCA		64.3		
ALK4	fwd	TGACATGGAACGCATCTGGCAGTA	478	60	brain	pancreas
	rev	AAGGGTGTACTGCACCTTCTCAGT		60		
ACTR2B	fwd	TCATGAACGACTTCGTGGCTGTCA	380	60	brain	pancreas
	rev	AGCAGTGAGGTCACTCTTCAGCAA		60		
FGFR1	fwd	ACACCTGCATCGTGGGAGAATGAGT	532	60	lung	spleen
	rev	TCTTCTTGGTGCCGCTCTTCATCT		60		
ACTB	fwd	CTCTTCCAGCCTTCCTTCCT	513	63.6	heart	
	rev	CACCTTCACCGTTCCAGTTT		63.8		
NANOG	fwd	CAGTCCTGATTCTTCCACAA	696	60.25	blastocyst	muscle
	rev	TTACAAATCTTCAGGCTGTATGTT		61.8		
POU5F1	fwd	GGTTCTCTTTGGAAAGGTGTTC	498	62.6	blastocyst	muscle
	rev	ACACTCGGACCACGTCTTTC		64		
SOX2	fwd	CAAAACCATCTCCGTGGTCT	300	63.8	brain	kidney
	rev	ACATGTATTCTCGGCAGACT		63.6		
CDX2	fwd	GACTACGGCGGATACCATGT	599	63.6	trophectoderm	heart
	rev	CTGCGGTTCTGAAACCAAAT		63.8		
NCAM	fwd	CCGGCATTTACAAGTGTGT	595	61	kidney	lymph node
	rev	ATTCCATGGCAGTCTGGTTC		63.9		

Bovine qRT-PCR primers

Table 8: List of bovine quantitative RT-PCR primers along with the product length and the melting temperatures.

Gene		Sequence	Product length	Melting temp
ACTB	fwd	TTGCTGACAGGATGCAGAAG	142	60.14
	rev	TGATCCACATCTGCTGGAAG		60
NANOG	fwd	GTCCCGGTCAAGAAACAAAA	107	63.7
	rev	TGCATTTGCTGGAGACTGAG		64.2
POU5F1	fwd	TGCAGCAAATTAGCCACATC	123	63.7
	rev	AATCCTCACGTTGGGAGTTG		63.8
SOX2	fwd	ACAGTTGCAAACGTGCAAAG	114	63.8
	rev	AGACCACGGAGATGGTTTTG		63.8
GATA6	fwd	ATACTTCCCCCACCACACAA	118	64
	rev	AGCCCGTCTTGACCTGAGTA		63.7
CDX2	fwd	CTCCTGGACAAGGACGTGAG	119	60
	rev	ACATGGTATCCGCCGTAGTC		60
HNF4	fwd	GGAGGATCCGGAATGAAAAAAGAAGCTGT	200	62.9
	rev	GAAGAATTCACAGACATCACCAAT		64.1
NCAM	fwd	ACAAAGGCCGAGATGTCATCCTGA	108	60.2
	rev	AGCGGTAAGTGCCCTCATCTGTTT		60.6
BMP4	fwd	TCAGTGATGTGGGCTGGAATGACT	120	60.2
	rev	CAATGGCGTGGTTGGTTGAGTTGA		60.3

Hygromycin sensitivity in bovine ICM explants

Table 9: Senstivity of the bovine ICM explants to various concentration of hygromycin. Three ICM explants were plated in each well of a 12-well tissue culture dish. Wells (in duplicate) were supplemented with various concentrations of hygromycin. The percentage of cells surviving was qualitatively assessed after 5 days.

Concentration of hygromycin (μg/ml)	% of ICM explant cells surviving after 5 days
0	100
0.5	100
1	100
2.5	100
15	80
25	60-75
50	50
100	0
200	0

Summary of total number of oocytes used and the respective colony formation efficiencies for each study

Table 10: Total number of oocytes used in each study and the respective colony formation efficiencies for each study

Study	Number of blastocysts processed	Colony formation efficiency (%)
Gene expression in in vivo ICM explants (page)	16	100
Gene expression in in vitro ICM explants (page)	64	90
Gene expression in in vitro ICM explants after passaging (page)	40	60
Noggin study (page)	60	73
BMP4 study (page)	40	65
FGF2 study (page)	40	67
Activin A study (page)	40	62
Noggin+FGF2 (page)	40	75

Summary of total number of ICM explants evaluated for the expression of pluripotency determining transcription factors for each of the cytokine studies

Table 11: Total number of ICM explants evaluated for the expression of pluripotency determining transcription factors in each of the cytokine studies

Noggin				
Passage/conc	**0 ng/ml**	**250 ng/ml**	**500 ng/ml**	**750 ng/ml**
P0	11	10	11	12
P1	10	10	9	11
P2	8	10	9	9

BMP4				
Passage/conc	**0 ng/ml**	**5 ng/ml**	**10 ng/ml**	**15 ng/ml**
P0	7	7	6	6
P1	6	5	6	6
P2	6	5	6	6

Activin A				
Passage/conc	**0 ng/ml**	**10 ng/ml**	**25 ng/ml**	**50 ng/ml**
P0	8	7	6	6
P1	8	6	6	6
P2	8	6	6	6

FGF2				
Passage/conc	**0 ng/ml**	**12 ng/ml**	**40 ng/ml**	**100 ng/ml**
P0	6	5	7	7
P1	6	5	7	7
P2	6	5	5	6

Noggin (500 ng/ml)+FGF2 (40 ng/ml)		
Passage/conc	**Control**	**Treatment**
P0	10	20
P1	10	19
P2	9	18

Hygromycin resistance in stably transfected mESC clones

Table 12: mESCs were plated in a 24-well plate at a density of 25,000 cells/well. The cells were cultured in ESCM supplemented with hygromycin (0.1 mg/ml) in the presence or absence of LIF. The resulting colonies were stained for alkaline phosphatase activity 5 days after plating the cells and the number of positive colonies was counted.

	Number of AP positive colonies 5 days in ESCM + LIF + hygromycin	Number of AP positive colonies 5 days in ESCM - LIF + hygromycin
Well 1	676	0
Well 2	712	0
Average	694	0

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