RAT UMBILICAL CORD DERIVED STROMAL CELLS MAINTAIN MARKERS OF PLURIPOTENCY: OCT4, NANOG, SOX2, AND ALKALINE PHOSPHATASE IN MOUSE EMBRYONIC STEM CELLS IN THE ABSENCE OF LIF AND 2-MCE

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Abstract

When mouse embryonic stem cells (ESCs) were grown on mitotically inactivated rat umbilical cord-derived stromal cells (RUCs) in the absence of leukemia inhibitory factor (LIF) and 2-mercaptoethanol (2-MCE), the ESCs showed alkaline phosphatase (AP) staining. ESCs cultured on RUCs maintain expression of the following pluripotency genes, Nanog, Sox2 and Oct4 and grow at a slower rate when compared with ESCs grown on mitotically inactivated mouse embryonic fibroblasts (MEFs). Differences in gene expression for the markers of pluripotency Oct4, Sox2 and Nanog, AP staining and ESC growth rate were also observed after LIF and 2-MCE were removed from the co-cultures. Reverse transcriptase polymerase chain reaction (RT-PCR) suggested differences in Sox2 and Nanog mRNA expression, with both genes being expressed at higher levels in the ESCs cultured on RUCs in the absence of LIF/2-MCE as compared to ESCs cultured on MEFs. Semi-quantitative RT-PCR indicated that Nanog expression was higher when ESCs were grown on RUCs in the absence of LIF and 2-MCE as compared to MEFs in the same treatment conditions. Bisulfite-mediated methylation analysis of the Nanog proximal promoter suggested that the maintenance of Nanog gene expression found in ESCs grown on RUCs after culture for 96 hours in the absence of LIF/2-MCE may be due to prevention of methylation of the CpG dinucleotides in the Nanog proximal promoter as compared to ESCs grown on MEFs. Thus, RUCs may release factors into the medium that maintain the pluripotent state of mouse ESCs in the absence of LIF and 2-MCE.

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Dedication

The four and a half years spent here are dedicated to those afflicted with physical ailments, who cannot be with their loved ones. I thank my parents, brother and sister-in-law, family and friends and the community of Manhattan, Kansas for all their support, in my pursuit of happiness.

Preface

CHAPTER 1 - Literature Review

Introduction: Embryonic Stem Cells

This review will focus on embryonic stem cells (ESCs) derived from the mouse. Unless otherwise stated, all references are to mouse ESCs. The ability to maintain the pluripotent state in ESCs allows homologous recombination-mediated targeted genetic manipulation and the creation of gene-specific disease models (Evans et al., 2001;Zwaka and Thomson, 2003). The first hint of ESCs came with the description of serially transplantable teratoma cells in the 129 strain mouse (Solter, 2006;Stevens and Little, 1954). Some teratocarcinoma cell lines resemble closely ESCs in that among others things, they were able to form embryoid bodies and contribute to chimera formation (Martin and Evans, 1975). The stem cells of teratocarcinomas are known as embryonal carcinoma (EC) cells which contribute inefficiently to the germ line (Bradley et al., 1984). Martin Evans' lab reported the first ESCs from the 129 SvE mouse strain in the same year as Gail Martin (Evans and Kaufman, 1981; Martin, 1981). ESCs were limited to the mouse species until 1998 (Thomson et al., 1998). Even within the mouse species, derivation of ESCs from strains other than the Sv129 strain was difficult until the importance of mitogen-activated protein kinase kinase (MEK) and glycogen synthase kinase 3 beta (GSK3B) inhibition for maintaining the pluripotent state was recognized (Sato et al., 2004;Smith, 2001). ESCs have been derived from different mouse strains by the combination of MEK and GSK3B inhibition (Buehr et al., 2008;Ying et al., 2008).

Oct4, Sox2 and Nanog Expression and Alkaline Phosphatase (AP) Activity in Mouse ESC Pluripotency

The importance of the transcription factors Pit-Oct-Unc (POU) domain, class 5, transcription factor 1 (POU5F1 or Oct4), SRY (sex determining region Y)-box 2 (Sox2) and Nanog in maintaining the pluripotent state of ESCs can be seen in their ability to cause a change in the expression program in, or reprogram, non-pluripotent cells to the pluripotent state (Nakagawa et al., 2008;Yu et al., 2007;Zhou et al., 2009). Expression of Oct4, Sox2 and Nanog are required for the maintenance of mouse and human ESCs in culture (Boyer et al., 2005; Ivanova et al., 2006). MicroRNA (miRNA) that causes the downregulation of Oct4, Sox2 and

Nanog mRNA modulate ESC differentiation (Tay et al., 2008). Short hairpin RNA (shRNA) knockdown of Oct4, Sox2 and Nanog expression causes ESC differentiation, and overexpression of Oct4 and Sox2 also causes differentiation (Boer et al., 2007; Niwa et al., 2000).

ESCs also show heterogeneity within a single colony where the "less" differentiated zinc finger protein 42-positive (Zfp42 or Rex1) cells express higher levels of Nanog, Sox2 and Oct4 (Toyooka et al., 2008). Oct4 expression at a certain level is required for self-renewal and prevention of differentiation of ESCs (Niwa et al., 2000). Homozygous deficiency of Oct4 *in vitro* prevents the outgrowth of the inner cell mass (ICM) (Nichols et al., 1998). Overexpression of Oct4 reduces Nanog gene expression as shown by reduced Nanog reporter expression following increases in Oct4 transgene dosage (Pan et al., 2006). Oct4 is expressed in the oocyte, in the ectoderm of the egg cylinder stage and restricted to the primordial germ cells (PGC) after 8.5 dpc (Scholer et al., 1990). Oct4 expression can be stimulated in trophoblast stem cells with DNA methyltransferase (DNMT) inhibitor 5-aza-dC and histone deacetylase 1 (HDAC1) inhibitor trichostatin A (TSA) (Hattori et al., 2004).

Sox2 is expressed in embryonal carcinoma (EC) cells and ESCs (Yuan and Dailey, 1995). Sox2 null mice, such as homozygous knockouts, did not show outgrowth of the ICM when blastocysts were cultured *in vitro* (Avilion et al., 2003). The absence of ICM outgrowths can be explained by the differentiation of the ICM to trophectoderm-like cells (Masui et al., 2007). Transgene electroporation of exogenous Oct4 rescued the proliferation of the Sox2 null ESCs, but Nanog did not show a rescue effect, suggesting that Oct4 can substitute for Sox2 and that a functional Sox2 is required for the Nanog-mediated prevention of differentiation mentioned above. shRNA knockdown of Sox2 results in differentiation of mouse ESCs (Ivanova et al., 2006). Overexpression of Sox2 reduces the expression of Oct4-Sox2 target genes, including Oct4, Sox2 and Nanog (Boer et al., 2007). Sox2 is expressed widely in the 6.5 to 8.0 dpc embryo and is also strongly expressed in neural stem cells (Kim et al., 2008; Wood and Episkopou, 1999). Sox2 is expressed in the brain, retina, lung and stomach (Yuan et al., 1995). Oct4 and Sox2 are expressed in the regenerating rat epithelium (Song et al., 2009).

The Nanog gene is expressed in embryonic tissues from 3.5 - 8.5 dpc and is relatively strongly expressed in the ovary, testis, kidney liver and spleen (Hart et al., 2004). It is also expressed in ESCs, embryonic germ cells and EC cells and decreases during ESC differentiation (Chambers et al., 2003;Mitsui et al., 2003). It is required for the maintenance of pluripotency.

Nanog deficiency *in vitro* results in differentiation (Mitsui et al., 2003). Overexpression of Nanog can maintain ESC pluripotency in the absence of other pluripotency associated factors (Ivanova et al., 2006). In fact, Nanog overexpression alone has been found to reduce differentiation of ESCs under differentiation-inducing conditions (Chambers et al., 2003;Ivanova et al., 2006). This effect is also seen in the absence of leukemia inhibitory factor (LIF) (Mitsui et al., 2003). Nanog maintains pluripotency through activation of signal transducer and activator of transcription 3 (Stat3) and repression of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-Kb) (Torres and Watt, 2008).

Two forms of alkaline phosphatase (AP) are expressed during early mouse development, alkaline phosphatase placental-like 2 (Alppl2) and alkaline phosphatase liver/bone/kidney (Alpl), the latter also known as tissue-non-specific AP (TNAP) (Hahnel et al., 1990). Alppl2 is found in 2-cell and 8-cell embryos, blastocysts, adult testis and thymus. Disruption of Alppl2 causes a slight delay in the cellular proliferation of the pre-implantation embryo (Dehghani et al., 2000). The Alpl gene is not expressed in the mouse 3.5 days *post-coitum* (dpc) blastocyst; the protein is primarily restricted to the mural trophectoderm in 4.5 dpc blastocyst, the extraembryonic ectoderm in 5.5 and 6.5 dpc embryos, and is found in primordial germ cells (PGCs), the adult testis and the thymus (Hahnel et al., 1990;MacGregor et al., 1995). In the MacGregor study, Alpl or TNAP was observed in the inner cell mass (ICM) outgrowths that were in contact with the trophectoderm-derived cells and was never observed in blastocysts. The above suggests that Alppl2 is expressed in the ESCs.

Oct4, Sox2 and Nanog Expression, and Alkaline Phosphatase (AP) Activity during Differentiation of Mouse ESCs

The transcription factors Oct4, Sox2 and Nanog play important roles in ESC self-renewal. Oct4 gene expression is not downregulated even after culture of ESCs in the absence of LIF for 4 weeks in a feeder-free ESC culture system (Berrill et al., 2004). Other studies have also found that ESC expression of the Oct4 gene was not completely downregulated after four or five days of culture in the absence of LIF (Faherty et al., 2007; Hamazaki et al., 2004). In the Hamazaki et al. study, Nanog gene expression, as determined by gel electrophoresis RT-PCR, was found to be downregulated to a greater extent, though not completely abolished, as compared to the downregulation of Oct4 gene expression (Kaji et al., 2006). Another study

found that ESCs lose Nanog and Oct4 gene expression after culture in the absence of LIF for two passages (Kawazoe et al., 2009). These studies suggest that the expression of the Oct4 gene is maintained for up to four days of culture in the absence of LIF and that the expression of the Nanog gene is also maintained, or possibly partially downregulated, for up to four days of culture in the absence of LIF. Sox2 expression was found to be downregulated to 40% of control after 10 days of embryoid body culture in the absence of LIF (Wu et al., 2009).

The expression of Oct4, Sox2 and Nanog genes may be downregulated at an accelerated rate after RA treatment. In ESCs, Nanog gene expression decreases nearly 10 fold and Oct4 gene expression decreases >10 fold after only 120 hours of RA treatment (Lin et al., 2005). Sox2 gene expression is completely abolished in ESCs after five days of retinoic acid (RA)-induced differentiation (Maruyama et al., 2005). RA-induced differentiation of ESCs is also accompanied by downregulation of the Oct4 and Sox2 protein levels (Chew et al., 2005). In the Chew et al. study, after 3 days of RA treatment, there was downregulation of Oct4 and Sox2 protein levels and after 6 days of RA treatment, Oct4 protein was undetectable and Sox2 protein was nearly abolished. This is in line with chromatin immunoprecipitation studies in ESCs that analyzed the binding of the Oct4 and Sox2 proteins to the oct-sox binding element within the Nanog gene promoter. This study showed that after 3 days of RA treatment, the binding of both Oct4 and Sox2 proteins to the oct-sox binding element was reduced by 75% and after 6 days of RA treatment, binding was reduced to <10% (Rodda et al., 2005). RA treatment for 3 days nearly abolishes Nanog protein levels (Kuroda et al., 2005).

LIF and Feeder Cells in Pluripotency and Differentiation

Fibroblasts were first used as a feeder layer to support embryonal carcinoma (EC) cells which die after two or three passages in the absence of fibroblasts. EC grow indefinitely when plated on a feeder layer made from mitotically inactivated STO fibroblasts (Martin and Evans, 1975). STO is a thioguanine and ouabain resistant fibroblast cell line derived from the SIM mouse (ATCC). A STO feeder layer alone was found to be insufficient to maintain ESCs in the undifferentiated state in the absence of conditioned media from teratocarcinoma cultures (Martin, 1981). Martin speculated that there was a secreted factor in the teratocarcinoma cultures. The conditioned medium from buffalo rat liver cell cultures also prevents the differentiation of ESCs in the absence of feeders (Smith and Hooper, 1987). The conditioned medium contained a

molecule termed polypeptide with differentiation inhibiting activity (DIA). Leukemia inhibitory factor (LIF) was discovered in 1988 through analysis of homology to DIA (Smith et al., 1988;Williams et al., 1988).

LIF belongs to the interleukin 6 (IL-6) family of cytokines (Kristensen et al., 2005;Rose and Bruce, 1991). An autocrine loop is involved in LIF maintenance of pluripotency (Davey et al., 2007). LIF stimulates the cell surface receptor gp130 and signals through the intracellular Janus kinase-signal transducer and activator of transcription (JAK-STAT) and rat sarcoma viral oncogene homolog-mitogen activated protein kinase (RAS-MAPK) pathways (Ernst et al., 1996). Loss of signaling downstream of JAK-STAT reduces proliferation, and hyper-activation of JAK-STAT results in tumors (Harrison et al., 1995). In ESCs, decrease in alkaline phosphatase (AP) staining begins at concentrations of LIF less than 250 IU/mL (Zandstra et al., 2000). With complete LIF removal, associated phosphorylated STAT3 levels drop to ~50% by 24 hours and Oct4 expression decreases to less than 60% by 60 hours (Davey et al., 2007). Of the cells found to express Oct4, less than 65% were positive for Nanog expression.

The conditioned medium from serum-free cultured STOs contains cell trophic factors including insulin growth factor binding protein 4 (IGFBP-4), pigment epithelium derived factor (PEDF) and secreted acidic cysteine rich glycoprotein (SPARC) and the antioxidant peroxiredoxins (Lim and Bodnar, 2002). MEFs derived from the CF-1 mouse strain have been shown to secrete transforming growth factor, beta 1 (TGFβ1) and inhibin beta dimers (activin A) and low levels of bone morphogenetic protein 4 (BMP-4) (Eiselleova et al., 2008). Human umbilical cord-derived stromal cells (HUCs) express various trophic factors and cytokines, and have been used support the maintenance primate ESCs (Hiroyama et al., 2008;Weiss et al., 2008). Bovine umbilical cord-derived stromal cells have used to derive ESCs from the equine blastocysts (Saito et al., 2002). Here, I will evaluate whether rat umbilical cord-derived stromal cells (RUCs) can maintain mouse ESCs in the pluripotent state.

2-Mercaptoethanol (2-MCE) Effects on Proliferation and Apoptosis

In the culture of *in vitro* fertilized porcine embryos, the average number of cells per blastocyst was increased in cultures with 50 uM 2-MCE as compared to controls, though the percentage of embryos developing to the blastocyst stages was lower (Funahashi, 2005). This increase in average number of cells per blastocyst was also seen in culture after thaw of vitrified

bovine blastocysts, and the viability of post-thaw bovine blastocysts was higher in the presence of 2-MCE (Nedambale et al., 2006). The average number of cells in the ICM is also increased in the presence of 2-MCE as well as a decrease in the number of apoptotic blastomeres in bovine embryos (Park et al., 2004). Withdrawal of 2-MCE promotes apoptosis in a B-cell line via upregulation of TNF receptor superfamily member 6 (Fas) and Fas ligand and increases the percentage of cells arrested in G_0/G_1 of the cell cycle (Neumann et al., 1998). 2-MCE also induces increases in DNA content in cultures of primary human bone marrow adherent cells which may be associated with increased proliferation as determined by ^3H thymidine incorporation assays (Inui et al., 1997).

Promoter Methylation

CpG islands were coincident with ~60% of promoters for genes transcribed by human RNA polymerase II (Antequera and Bird, 1993). These islands are protected from CpG suppression characterized by high CpG content (65% G+C). Methylated cytosines were recognized and bound by a family of methylation dependent DNA binding proteins (MBPs) whose binding can initiate transcriptional repression (Chandler et al., 1999;Horike et al., 2005;Ng et al., 1999). Prior *in vitro* methylation of specific cytosines in a reporter transgene results in its reduced promoter activity (Martinowich et al., 2003). These studies suggest that methylation of the promoters is not an all-or-nothing process (Boyes and Bird, 1992). Methylation of cytosines occurs in the Oct4 proximal promoter after differentiation (He et al., 2009; Gu et al., 2006). For Nanog, the percentage of methylated CpG dinucleotides within a 526 bp stretch of the proximal promoter is decreased from 27% in the one-cell zygote to 2.2% in the blastocyst (Farthing et al., 2008). In the same study, it was shown that the increased methylation of the Nanog upstream activation sequence (UAS) to 40.9% in a reporter transgene was associated with reduced expression of the transgene.

RT-PCR and Semi-quantitative RT-PCR

Polymerase chain reaction (PCR) allows the amplification of cDNA which is representative of the mRNA for a gene present in a cell. RNA is collected from a cell pellet using various commercial kits. These kits are based on RNA-specific binding to a silica column and contain RNase inhibitors in proprietary buffers (Qiagen). Purified RNA is comprised of

various species of RNA molecules with ribosomal RNA accounting for the majority. mRNA comprise 1-5% of the total RNA (Sambrook et al., 2001). cDNA synthesis of RNA is performed with reverse transcriptases. Reverse transcriptases require Mg2+ cofactor and primers. Oligo-dT oligonucleotides, which bind the poly-A tail of mRNA, or random hexamer oligonucleotides, serve as the primer for the reverse transcription.

The calculation of band pixel numbers, or densitometry, of agarose gel photographs can provide semi-quantitative data of cDNA mass by reference to known quantity DNA ladders (Bautista et al., 2009). Semi-quantitative PCR can quantify the relative differences in starting cDNA copy number which can be automatically calculated (Peirson et al., 2003;Stahlberg et al., 2004;Tichopad et al., 2003). For absolute quantification of cDNA, this must be performed after primer efficiency analysis, validated control genes and with reference dye loading controls (Bustin et al., 2009;Reece et al., 2009).

Summary

Stem cell research is an exciting area. The FDA has approved a clinical trial for a human ESC derivative for the treatment of complete thoracic spinal cord injury (www.geron.com/media/pressview.aspx?id=1195). Researchers are forging ahead in this stem cell transplantation approach to disease therapy, but progress into the clinic is painstakingly meticulous due to the potential for tumor formation (Lensch et al., 2007).

Mouse ESCs must be pluripotent in order to contribute to the germline after blastocyst injection. Pluripotency can be maintained *in vitro* when ESCs are cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) with the supplementation of leukemia inhibitory factor (LIF). Previous work has shown that removal of LIF and associated loss of JAK-STAT signaling reduces proliferation and reduces Oct4 expression to less than 60% by 60 hours.

Expression analysis of the transcription factors Oct4, Sox2 and Nanog, along with alkaline phosphatase staining, can identify putative pluripotent stem cells. Oct4, Sox2 and Nanog are each required for the maintenance of ESCs pluripotency *in vitro*. Oct4 and Sox2 deficiency prevents the outgrowth of the ICM and Nanog deficiency results in differentiation. Overexpression of Oct4 results in differentiation of mouse ESCs whereas overexpression of a Nanog transgene is sufficient to maintain mouse ESCs in the absence of LIF. The culture of ESCs in the absence of LIF for four days does not downregulate Oct4 gene expression. This is

also true for Nanog gene expression as RT-PCR has shown continued Nanog gene expression after culture of ESCs in the absence of LIF for our days. Sox2 gene expression in ESCs cultures in the absence of LIF for 10 days in an embryoid body assay is reduced to 40%.

Subtle changes in expression and crosstalk affect cell fate, such as the pluripotent state. In mouse ESCs, increasing Oct4 gene expression reduced Nanog reporter expression. Overexpression of Sox2 decreases the expression of Oct4, Sox2 and Nanog genes and increasing the Sox2 transgene 3-fold caused further reduction in expression. These effects of gene dosage can be seen in the heterogeneity of ESCs with the Rex1-positive cells showing relatively higher levels of Nanog, Sox2 and Oct4 gene expression (Toyooka et al., 2008). Detailed characterization of the optimal levels of Oct4, Sox2 and Nanog expression would have applications in increasing the efficiency of selecting pluripotent embryonic as well as induced pluripotent stem cells for transgenesis or differentiation to adult tissue.

Methylated DNA is generally thought to represent heterochromatin which is considered silent or transcriptionally repressed. The downregulation of Oct4 and Nanog gene expression during differentiation from the pluripotent state is accompanied by methylation of CpGs in their respective proximal promoters. This silencing can be initiated by binding of methylation dependent DNA binding proteins (MBPs) to symmetrically methylated CpG dinucleotides and may not necessitate the complete methylation of all cytosines in the regulatory region of the genes being silenced.

CHAPTER 2 - Introduction

Mouse embryonic stem cells (ESCs) can be maintained indefinitely in the undifferentiated state when cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) with leukemia inhibitor factor (LIF) supplementation (Martin and Evans, 1975;Smith et al., 1988;Williams et al., 1988). The combination of alkaline phosphatase (AP) histochemical staining and Oct4, Sox2 and Nanog expression can be used to distinguish putative pluripotent stem cells (Masui et al., 2007;Nakagawa et al., 2008;O'Connor et al., 2008).

Recently, it has been suggested that the ESC colonies are heterogeneous (Hayashi et al., 2008). This heterogeneity is affected by the culture microenvironment (Chou et al., 2008). This microenvironment includes the feeder layers or feeder cells that support the growth of ESCs. The use of cells to support the growth of ESCs has evolved from the use of a fibroblast cell line derived from the SIM mouse (STO) and the addition of conditioned media from embryonal carcinoma cultures to the invention of feeder-free and serum-free cultures under defined conditions (Ludwig et al., 2006;Xu et al., 2001). Different feeder types have been used to support the growth of ESCs. These include stromal cells from the umbilical cord blood, buffalo rat liver cells and immortalized human foreskin fibroblasts, which all share a mesodermal lineage (Eiselleova et al., 2008;Smith and Hooper, 1987;Ye et al., 1994).

Our lab works with human umbilical cord matrix stromal cells (HUC) which have been shown to secrete cytokines *in vitro* (Weiss et al., 2008). Here, the rat umbilical cord matrix-derived stromal cells (RUCs) were compared to MEFs for their ability to support the undifferentiated state and proliferation of two mouse ESC lines. There is previous work with bovine umbilical cord-derived matrix cells in the derivation of ESC from equine blastocysts (Saito et al., 2002).

To analyze the effects of LIF/2-MCE removal on the pluripotent state of ESCs cultured on RUCs as compared to ESCs cultured on MEFs, AP histochemical staining, ESC growth and Oct4, Sox2 and Nanog expression were evaluated. When ESCs were co-cultured with mitotically inactivated RUCs, they grew as 3-dimensional, phase-bright colonies resembling the morphology of ESCs grown on MEFs. AP histochemical staining indicated RUCs were able to maintain the *in vitro* pluripotent state of two different mouse ESCs, the D3 and LC3 cell lines, in contrast to MEFs which showed reduced AP staining after removal of LIF/2-MCE. Gel

electrophoresis of RT-PCR showed bands for Oct4, Sox2 and Nanog during RUC co-culture and semi-quantitative RT-PCR suggested the Nanog and Sox2 genes were downregulated after LIF/2-MCE removal when ESCs were cultured on MEFs. Analysis of the methylation of the Nanog proximal promoter supported the hypothesis that the Nanog gene was being expressed when grown on RUCs in the absence of LIF/2-MCE and indicated increased methylation when ESCs were cultured on MEFs in the absence of LIF/2-MCE. It is concluded that RUCs were able to maintain mouse ESCs in a pluripotent state *in vitro*, perhaps by the release of cytokines such as LIF, and that this model may prove useful in the characterization of the timing of the methylation of the proximal Nanog promoter in mouse ESCs during differentiation.

CHAPTER 3 - Methods

Cell Culture

Mouse embryonic fibroblasts (MEFs) (ATCC SCRC 1008, day 15, lot 7379965) from passages 1 through 4 were mitotically inactivated by exposure to 10 ug/mL of mitomycin C (MMC, Sigma-Aldrich, M4287-2MG) for 2.5 hours. Mitotically inactivated MEFs were rinsed with PBS, lifted with 0.25% Trypsin with 0.38 g/L of EDTA (Invitrogen, 25200-106) and frozen in 10% DMSO and 90% MEF medium in a controlled freezing device at >500,000 cells per vial to -80^{o}C, then moved to liquid Nitrogen at -196^{o}C until use. To use, the cryovials were thawed rapidly in a water bath at 37^{o}C until a pea-sized ice crystal remained and the contents diluted with 3 volumes of culture medium and plated in MEF medium pre-warmed to 37^{o}C and pre-equilibrated in 5% CO_2. Rat umbilical cord-derived stromal cells (RUCs) were isolated from the gestation day 21 rat fetal umbilical cord in our lab after hysterectomy. RUCs were grown in MEF medium prior to inactivation. RUCs were inactivated at passage 33 and 34 by exposure to MMC for 5 ug per mL for 1 hour, rinsed with PBS and frozen as described above for MEFs. Mouse embryonic stem cells (ESCs) (D3, ATCC, CRL-11632 at passage 5 and LC3, GlobalStem, GSC-5002 at passage 11) were grown in ESC medium: high glucose DMEM (Gibco, Invitrogen, 11965-073) with 10% heat-inactivated Fetal Bovine Serum (HyClone, 300071-03, SH30071.03), 1% Non-essential amino acids (Gibco, Invitrogen, 11140-050), 2 mM Glutamax (Invitrogen, 35050-061), 86 uM 2-mercaptoethanol (Sigma-Aldrich, M7522, Nedambale et al., 2006) and 500 IU/mL mouse leukemia inhibitory factor (Chemicon, ESG1106). MEFs were grown in MEF medium: high glucose DMEM (Gibco, Invitrogen, 11965-073) with 10% heat-inactivated Fetal Bovine Serum (HyClone, 300071-03, SH30071.03), 1% Non-essential amino acids (Gibco, Invitrogen, 11140-050), and 2 mM Glutamax (Invitrogen, 35050-061).

Plating MEF or RUC feeder layers

6-well (BD Falcon, ThermoFisher, 08-772-1B) and 12-well plates (Corning Costar, ThermoFisher, 07-200-82) were coated with 0.1% bovine gelatin (Sigma-Aldrich, G2500-500) in single distilled water by adding 1 mL of the gelatin solution per well. Gelatin was aspirated immediately, 1500 uL per well of 6-well plates and 750 uL per well of 12-well plates of MEF

medium was added immediately and the plate was placed into the incubator to equilibrate at 5% CO2, 37^{o}C and 90% humidity for 15 minutes. Both mitotically inactivated MEFs and RUCs were plated at either one of two densities in MEF medium on gelatin-coated plates (12,500 or 25,000 cells per cm^2, figure 3-1). Twenty four (24) hours after plating the feeder layer, MEF medium was replaced with ESC medium. Twenty four (24) hours after replacing with ESC medium, ESCs were plated at 500 cells per cm^2 and cultured for a total of five days. Medium changes (complete replacement) were at 24 hours and 72 hours after plating ESCs. In some cases, ESC medium was replaced with ESC medium without LIF and 2-MCE (figure 3-1). All cells were harvested at 120 hr. Thus, three experimental conditions were made: 1) ESC medium for 120 hr, and 2) 48 and 3) 96 hour LIF-2-MCE deprivation conditions.

Alkaline Phosphatase (AP) Staining

At the end of the experiment (120 hr after plating ESCs), wells were fixed with 4% paraformaldehyde (ThermoFisher Scientific, 04042-500) in 0.05M Sorenson's phosphate Buffer, pH 7.2-7.4, for <2 minutes at room temperature, rinsed with PBS and stained with an AP staining kit according to the manufacturer's instructions (Millipore, SCR004). All plates were photographed using a 55 mm Nikkor macro lens from a distance of 0.25 meters and these photographs were used to count colonies using a grid method as illustrated (Figure 3-2). Intensely stained colonies within the grid were counted. Diffusely staining colonies were not included in the analysis. Counting was done by two investigators (one investigator was blind to the experimental conditions; there was no significant differences between the counts of the two investigators, data not shown) and their counts were averaged. For all experiments, an n of 1 is comprised of three wells, one well each for control conditions (supplemented continuously with LIF/2-MCE) and two treatment conditions where ESCS were grown in the absence of LIF/2-MCE for 48 hours or 96 hours. n = 5 for D3 cultured on MEFs plated at 12,500/cm^2, n = 4 for D3 cultured on MEFs plated at 25,000/cm^2, n = 4 for D3 cultured on RUCs plated at 12,500/cm^2, n = 5 for D3 cultured on RUCs plated at 25,000/cm^2, n = 3 each for LC3 cultured on MEFs plated at 12,500/cm^2 and 25,000/cm^2 and n = 3 each for LC3 cultured on RUCs plated at 12,500/cm^2 and 25,000/cm^2.

Cell Counts

At the end of the experiment (120 hr after plating ESCs), wells were washed with PBS, and the cells were lifted using 0.25% Trypsin (Invitrogen, 25200-106). Trypsin was inactivated by rinsing with 3 volumes of medium and the cells from all conditions were resuspended in ESC medium with LIF and 2-MCE. D3 ESCs were counted using a hemocytometer and 0.2% Trypan Blue stain (Sigma-Aldrich, T8154). LC3 ESCs were counted using a Guava EasyCyte Plus and the Guava Viacount assay according to the manufacturer's instructions (Millipore, 4000-0041). n = 3 each for D3/LC3 cultured on MEFs plated at 12,500/cm^2 and 25,000/cm^2 and n = 3 each for D3/LC3 cultured on RUCs plated at 12,500/cm^2 and 25,000/cm^2.

Gel Electrophoresis and Reverse transcriptase polymerase chain reaction (RT-PCR)

At the end of the experiment (120 hr after plating ESCs), to lift cells, wells were washed with PBS and treated with 0.05% Trypsin with 0.08 g/L of EDTA (this concentration of Trypsin and EDTA minimizes the lifting of feeders; not shown), and Trypsin inactivated with 3 volumes of ESC medium. The cells were spun at 1000 rpm for 3 minutes. The resulting cell pellet was used for RNA isolation. Total RNA was collected using the RNeasy Midi Kit (Qiagen, 75144). RNA was treated with DNase (Qiagen, 79254) on column for 15 minutes at room temperature to remove genomic DNA. The RNA was quantified using Nanodrop 8000. At least 5 ug of total cDNA for each RNA sample was made using Superscript III First-Strand Synthesis Supermix (Invitrogen, 18080-400) primed with oligo-dT using a BioRad iCycler: 65^oC incubation for 5 minutes, 50^oC cDNA synthesis for 50 minutes and 85^oC termination for 5 minutes in a total reaction volume of 22 uL in MicroAmp 8-tube strips (Applied Biosystems, N8010580). cDNA samples were stored at -20^oC. RT-PCR was carried out using a BioRad iCycler with a 96-well block as per the following using 4 ng of template DNA and 0.5 units of REDTaq polymerase and 2 uL REDTaq PCR Reaction Buffer containing 11 mM $MgCl_2$ (Sigma, D4309) with 0.5 uM primer mix and 0.25 mM dNTP in a total reaction volume of 20 uL: 95^oC denaturing for 3 minute and then cycled between 95^oC denature for 30 seconds, 55^oC annealing for 30 seconds and 72^oC 30 seconds for extension for a total of 35 cycles, followed by 72^oC for 10 minutes for final extension in MicroAmp 8-tube strips (Applied Biosystems, N8010580). The RT-PCR products were held at 4^oC prior to resolving on 1.0% agarose gels containing 0.75 ug/mL solution of Ethidium Bromide (Sigma-Aldrich, E8751). Nine (9) uL of the RT-PCR products for

each sample were loaded into the agarose gels along with 6 uL of DNA ladder (Promega, G2101). For RNA collection, n = 4 for D3s cultured on MEFs and n = 3 for D3s cultured on RUCs. For RT-PCR, n = 1 for D3s cultured on MEFs plated at 12,500 cells/cm^2, n = 1 for D3s cultured on MEFs plated at 25,000 cells/cm^2, n =1 for D3s cultured on RUCs plated at 12,500 cells/cm^2 and n = 1 for D3s cultured on RUCs plated at 25,000 cells/cm^2.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR for Nanog gene expression was performed using 4 ng and 0.4 ng of cDNA per reaction tube containing reference dye and performed in duplicate using the RT2 SYBR® Green qPCR Master Mix according to the manufacturer's recommendation (SABiosciences, PA-011) using the BioRad iCycler and the cycle threshold (Ct) method. Semi-quantitative RT-PCR for Sox2 gene expression was performed using 9.4 ng of cDNA per reaction tube containing reference dye using the RT2 SYBR® Green qPCR Master Mix according to the manufacturer's recommendation (SABiosciences, PA-010) using the BioRad iCycler and the cycle threshold (Ct) method. Ct values represent the cycle at which the fluorescence of the sample exceeds the background fluorescence of the reference dye determined prior to the first amplification cycle as measured by the BioRad iCycler. delta Ct was calculated by subtracting the average Ct value of the sample from the average Ct value of the control gene. Semi-quantitative RT-PCR was carried out with an initial denature step at 95^{o}C for 15 minutes, 45 cycles of denature at 95^{o}C for 15 sec, annealing at 52^{o}C for 15 sec and extension at 60^{o}C for 1 minute in a total reaction volume of 25 uL in MicroAmp 8-tube strips (Applied Biosystems, N8010580). The expression of three different genes was evaluated to determine which would be appropriate to use as a control gene here: 18S ribosomal RNA (X00686), hydroxymethylbilane synthase (PBGD or HMBS) (NM_013551.2) and aminolevulinic acid synthase 1 (ALAS) (NM_020559). The standard deviation for 18S expression was lowest across all experimental conditions (figure 4-6B). Based upon this criterion, 18S was selected as the control gene for semi-quantitative RT-PCR. The primers used in these experiments are shown in Table 3-1.

Nanog proximal promoter methylation analysis

The methylation protocol was as described previously for the rat Oct4 promoter (He et al., 2009). The primer set used for the amplification of the Nanog proximal promoter produced a

630 bp fragment referenced to Genebank number: NT_039353.7|Mm6_39393_37. The primers are shown in Table 3-1. n = 1 for D3 cultured on MEFs and n = 1 for D3 cultured on RUCs. Plasmid DNA from 10 clones collected using QIAprep DNA isolation kit was used for sequencing (Qiagen, 27106). Sequencing was completed using the GenomeLab Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, 608120) in a total reaction volume of 20 uL using Beckman Coulter's CEQ 8000 GeXP: 97^{o}C for 3 minutes, 35 cycles of 96^{o}C denature for 20 seconds, 50^{o}C annealing for 20 seconds and 60^{o}C extension for 4 minutes. Sequencing reactions were carried out using V-bottom, 96-well plates (Beckman Coulter, 609801). Samples were overlayed with light mineral oil (Sigma-Aldrich M5904).

Statistical analysis

For AP and cell count assays, at least three replicas were completed and compared using a two-tailed, repeated measures ANOVA. Post hoc testing with a two-tailed, unpaired T-test was done to compare cell counts for ESCs cultured on RUCs as compared to ESCs cultured on MEFs in the control and 96 hours deprivation conditions. P values of <0.05 were considered significant.

Figure 3-1

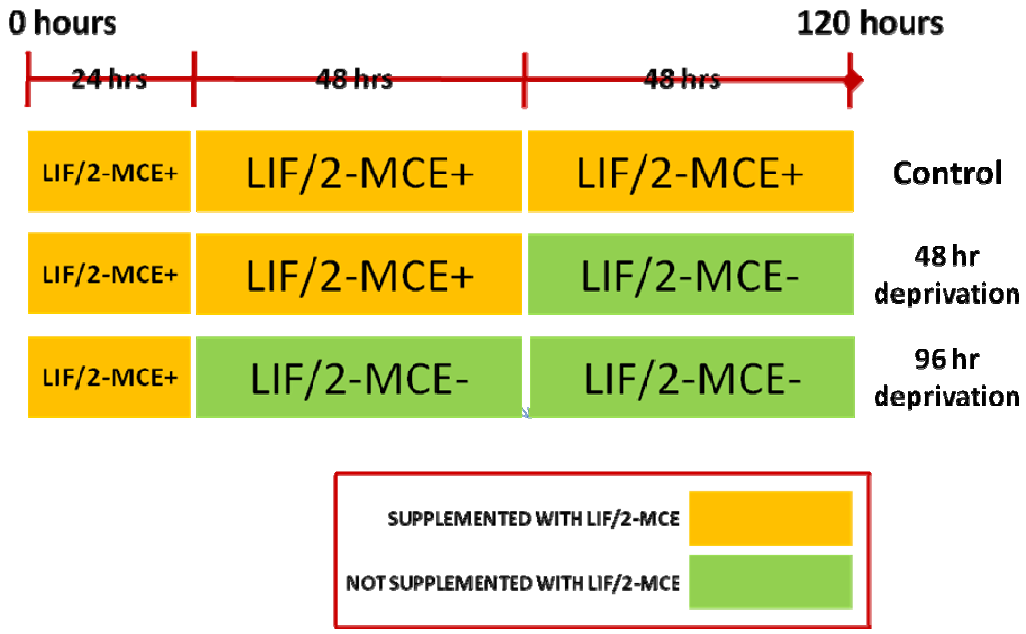

Figure 3-1. ESCs treatments were divided into three groups.

ESCs in control wells (top row) were continuously supplemented with LIF/2-MCE (orange bars). For the 48 hours deprivation treatment (middle row), ESCs were cultured for 72 hours supplemented with LIF/2-MCE and for an additional 48 hours after removal of LIF/2-MCE (green bars). For the 96 hours deprivation (bottom row), ESCs were cultured for 24 hours supplemented with LIF/2-MCE and for an additional 96 hours after removal of LIF/2-MCE. All feeder cells were plated 48 hours prior to plating ESCs. Time point where ESCs were plated is indicated here as 0 hours.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol

Figure 3-2

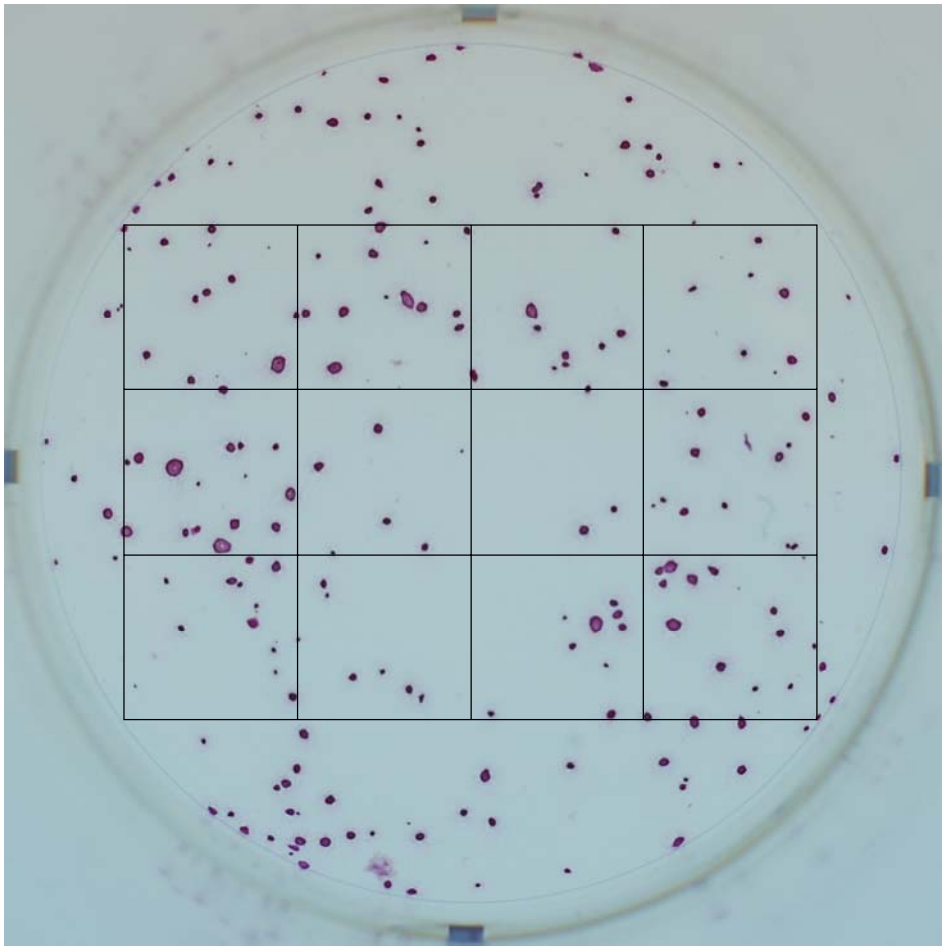

Figure 3-2. Grid for counts of Alkaline Phosphatase-stained colonies.

Alkaline phosphatase-stained colonies within the 2.4 cm^2 area represented by the total surface area of all 12 boxes depicted were counted.

Table 3-1

	Semi quantitative RT-PCR		Product				
Species	Gene	Accession	Size		Primer sequence		Position
RAT	OCT4-4F	NM_001009178	132	5'	AGA ACC GTG TGA GGT GGA AC	3'	692-711
RAT	OCT4-4R	NM_001009178	132	5'	GCC GGT TAC AGA ACC ACA CT	3'	823-804
RAT	NANOG-4F	XM_575662	140	5'	TAC CTC AGC CTC CAG CAG AT	3'	364-383
RAT	NANOG-4R	XM_575662	140	5'	AGG CCG TTG CTA GTC TTC AA	3'	503-484
MOUSE	M.SOX2-1F	U31967	181	5'	AGG GGT GCA AAA AGA GGA GA	3'	1381-1400
MOUSE	M.SOX2-1R	U31967	181	5'	GAA GCG CCT AAC GTA CCA CT	3'	1561-1542
MOUSE	M.18s-F1	X00686	155	5'	AAA CGG CTA CCA CAT CCA AG	3'	448-467
MOUSE	M.18s-R1	X00686	155	5'	CCT CCA ATG GAT CCT CGT TA	3'	602-583
MOUSE	M.ALAS1-3F	NM_020559	179	5'	CTC CAT GAT CCA AGG GAT TC	3'	1217-1236
MOUSE	M.ALAS1-3R	NM_020559	179	5'	CGT CAC ACA GCT CTT CCA GT	3'	1395-1376
RAT	PBGD-1F	X06827	163	5'	GCA CGG CAG CTT AAT GAT GT	3'	1065-1084
RAT	PBGD-1R	X06827	163	5'	CAA GGC CGA AGT CTC AAC AA	3'	1228-1209

	RT-PCR Primers		Product				
Species	Gene	Accession	Size		Primer sequence		Position
RAT	OCT4-F	XM_228354.1	238	5'	ACA ACA ACG AGA ACC TTC AG	3'	614-633
RAT	OCT4-R	XM_228354.1	238	5'	TCA ATG CTC GAT CTT TTC CC	3'	851-832
RAT	OCT4-5F	NM_001009178	446	5'	CGA GGA GTC CCA GGA TAT GA	3'	378-397
RAT	OCT4-5R	NM_001009178	446	5'	GCC GGT TAC AGA ACC ACA CT	3'	823-804
RAT	NANOG-2F1	NM_001100781	411	5'	TAC CTC AGC CTC CAG CAG AT	3'	567-586
RAT	NANOG-2R1	NM_001100781	411	5'	GAA GTT ATG GAG CGG AGC AG	3'	977-958
RAT	SOX2-F1	NM_001109181	414	5'	GGC GGC AAC CAG AAG AAC AG	3'	460-479
RAT	SOX2-R1	NM_001109181	414	5'	GTT GCT CCA GCC GTT CAT GTG	3'	873-854
RAT	PBGD-4F	X06827	354	5'	TAG CAT GCA AGA GAC CAT GC	3'	872-891
RAT	PBGD-4R	X06827	354	5'	GGC CGA AGT CTC AAC AAC TC	3'	1225-1206

	Methylation Primers						
Species	Gene	Accession	Size		Primer sequence		Position
Mouse	M.NanogPME-3F	ref|NT_03935 3.7|Mm6_3939	630	5'	CCC ACA CTC ATA TCA ATA TAA TAA C	3'	-619
Mouse	M.NanogPME-2R	ref|NT_03935 3.7|Mm6_3939	630	5'	AAT AGA GAT TTT GGT AGT AAG GTT TG	3'	11

Table A-1. Primer log.

Primer sets used for semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), gel electrophoresis RT-PCR and Nanog proximal promoter methylation-specific RT-PCR are shown. The first set of primers was for semi-quantitative RT-PCR. The second set of primers was for gel electrophoresis RT-PCR. The third set of primers was for Nanog DNA methylation analysis. Species indicates the species for which the primers were designed. The accession no is that which is associated with the gene on the National Center for Biotechnology Information website. Size indicates the expected product size after PCR amplification. Position indicates the location of the primer sequence within the associated gene.

CHAPTER 4 - Results

Alkaline phosphatase (AP) staining of ESC colonies

As expected, culture in the absence of LIF and 2-MCE for 96 hours resulted in a decrease in the number of AP staining colonies ($p < 0.01$, figure 4-1A). As shown in figure 4-1B, there was a significant difference between the number of AP stained colonies between the two feeder types: MEFs compared to RUCs ($p < 0.01$), by inspection, it is seen that MEFs have more AP-stained colonies compared to RUCs. Plating density of the feeder layer did not affect the number of AP stained colonies ($p > 0.05$) and the results from the two densities tested here were pooled (data not shown). Culture of ESCs on RUCs under control treatment conditions which were continuously supplemented with LIF/2-MCE showed some irregular morphology (figure 4-1C).

Figure 4-2A shows that there was a significant effect of feeder type x time point on the number of AP- stained colonies ($p < 0.01$). Figure 4-2B (bottom panels) shows RUCs maintained the number of AP-stained colonies after LIF and 2-MCE removal. In contrast, as shown in figure 4-2B (top panels), the number of AP-stained colonies decreased on MEF feeders when LIF and 2-MCE were removed. These results were further evaluated by cell counts (see below).

Cell counts after LIF removal

Two mouse ESC lines were evaluated, D3 and LC3. As shown in figure 4-3A, the total number of cells, live and dead, between the two ESC types was not different ($p > 0.05$) despite the use of different counting methods for the D3 and LC3 (see methods). There was a significant ESC type x feeder type interaction in the number of cells ($p < 0.01$), suggesting that LC3 ESCs grew better on the MEF feeders and as compared to D3s, and D3s grew better on the RUC feeders as compared to LC3s (figure 4-3B). This result was unexpected and not investigated further here.

As shown in figure 4-4A, removal of LIF and 2-MCE resulted in a significant decrease in the number of cells ($p < 0.01$). There was no significant main effect of feeder type ($p > 0.05$), however there was a significant interaction of feeder type x treatment time ($p < 0.01$). This is shown in Figure 4-4B. The number of cells was greater with MEF feeders under control conditions (0 hr) as compared to RUC feeders (post hoc t-test, $p < 0.001$). However, 96 hr of LIF and 2-MCE deprivation resulted in the RUC feeders having significantly more cells than ESCs

with MEF feeders (post hoc t-test, $p<0.005$). In summary, MEF feeders produced more ESCs than RUC feeders when maintained with LIF and 2-MCE, and MEFs were not able to maintain cell numbers as well as RUCs after removal of LIF and 2-MCE.

Gel electrophoresis of RT-PCR for Nanog, Sox2 and Oct4 gene expression

Gel electrophoresis RT-PCR for Oct4, Sox2 and Nanog suggested differences in gene expression after removal of LIF and 2-MCE (figure 4-5). Specifically, Sox2 and Nanog gene expression appeared to be decreased after 96 hours of LIF and 2-MCE deprivation when the ESCs were cultured on MEFs. In contrast, when ESCs were grown on the RUC feeder layer, Nanog, and to a lesser degree, Sox2 bands, appeared brighter as compared to bands from MEF co-cultures (compare lane 6 with lane 3). In addition, when ESCs were cultured on RUC feeders, Nanog and Sox2 bands were present even after culture in the absence of LIF/2-MCE for 96 hours. The RT-PCR results were reevaluated using semi-quantitative RT-PCR (see below).

Semi-quantitative RT-PCR analysis of Nanog and Sox2 gene expression

In ESCs, when hydroxymethylbilane synthase (PBGD or HMBS) was used as the control gene, the delta Ct for ESCs grown on MEF feeders increased and this increase was dependent on the duration of LIF and 2-MCE deprivation with the highest delta Ct values after 96 hours of LIF/2-MCE deprivation (figure 4-6A, left panel). This indicates that the Nanog gene is downregulated by LIF/2-MCE removal when ESCs were grown on MEFs. In contrast, the delta Ct for Nanog gene expression when ESCs were grown on RUC feeders decreased after culture for 96 hours in the absence of LIF/2-MCE. This indicates that Nanog gene expression was maintained following LIF/2-MCE removal when ESCs were grown on RUCs. When Nanog gene expression was evaluated in an independently generated set of biological samples using 18S as the control gene, a similar trend was seen (figure 4-6A, right panel). The delta Ct value for Nanog expression again tended to increase after LIF and 2-MCE deprivation when ESCs were grown on MEFs. In contrast, Nanog gene expression was maintained when ESCs were grown on RUCs. These results suggested that the Nanog gene was downregulated when ESCs were grown on MEFs and not downregulated when ESCs were grown on RUCs. For the Nanog semi-quantitative RT-PCR, three control genes were evaluated: 18S, PBGD and ALAS. 18S was selected for the calculation of the delta Ct value for the Nanog semi-quantitative RT-PCR. 18S

showed the lowest Ct value of three housekeeping genes tested. It had an average Ct value of 13.4 across 19 different samples tested (figure 4-6B). The standard deviation was 1.39 and the standard error of the mean was 0.29.

Sox2 gene expression was also analyzed using the housekeeping gene PBGD with respect to feeder cell density and LIF/2-MCE treatment (figure 4-7, top). The gene expression relative to PBGD shows Sox2 is downregulated in ESCs cultured on MEFs at 25,000 cells/cm^2 (blue bars). In contract, ESCs cultured on RUCs show an upregulation of Sox2 when ESCs were cultured on feeders at 12,500 cells/cm^2 (red bars) and no change in Sox2 gene expression when feeders were plated at 25,000 cells/cm^2 (green bars). The results suggest RUCs may maintain expression of Sox2 in the absence of LIF/2-MCE and that Sox2 was downregulated when ESCs were cultured on MEFs in the absence of LIF/2-MCE. There was no effect of density on the downregulation of Nanog gene expression when ESCs were cultured on MEF feeders (figure 4-7, bottom).

Methylation of the Nanog promoter

To understand better what was happening to Nanog gene expression when ESCs were grown on the different feeders, the methylation status of the Nanog promoter was evaluated, since promoter methylation is known to correspond to loss of Nanog gene expression (Farthing et al., 2008). The methylation of all the possible CpG dinucleotides in the proximal promoter from -619 bp upstream of the start site at positions -517, -495, -301, -292, -210, -200, -142 and -15 bp were analyzed. There was a trend for this region of the Nanog promoter to be more highly methylated after removal of LIF/2-MCE when ESCs were cultured on MEFs as compared to RUCs (figure 4-8). When ESCs were cultured on MEFs, methylation was 5% in the control wells supplemented with LIF/2-MCE for the duration of the assay. After 48 hours of LIF/2-MCE deprivation, methylation was 9% and after 96 hours of LIF/2-MCE deprivation, methylation was 31% when ESCs were cultured on MEFs. When ESCs were cultured on RUCs, methylation was 4% in the controls. After 48 hours of LIF/2-MCE deprivation, methylation was found to be 2% and after 96 hours of LIF/2-MCE deprivation, methylation was 9% when ESCs were cultured on RUCs. The analysis of the Nanog promoter supports the decrease in Nanog gene expression when ESCs were cultured on MEFs and the maintenance of Nanog gene expression when ESCs were cultured on RUCs, as seen in both RT-PCR and q RT-PCR.

Figure 4-1

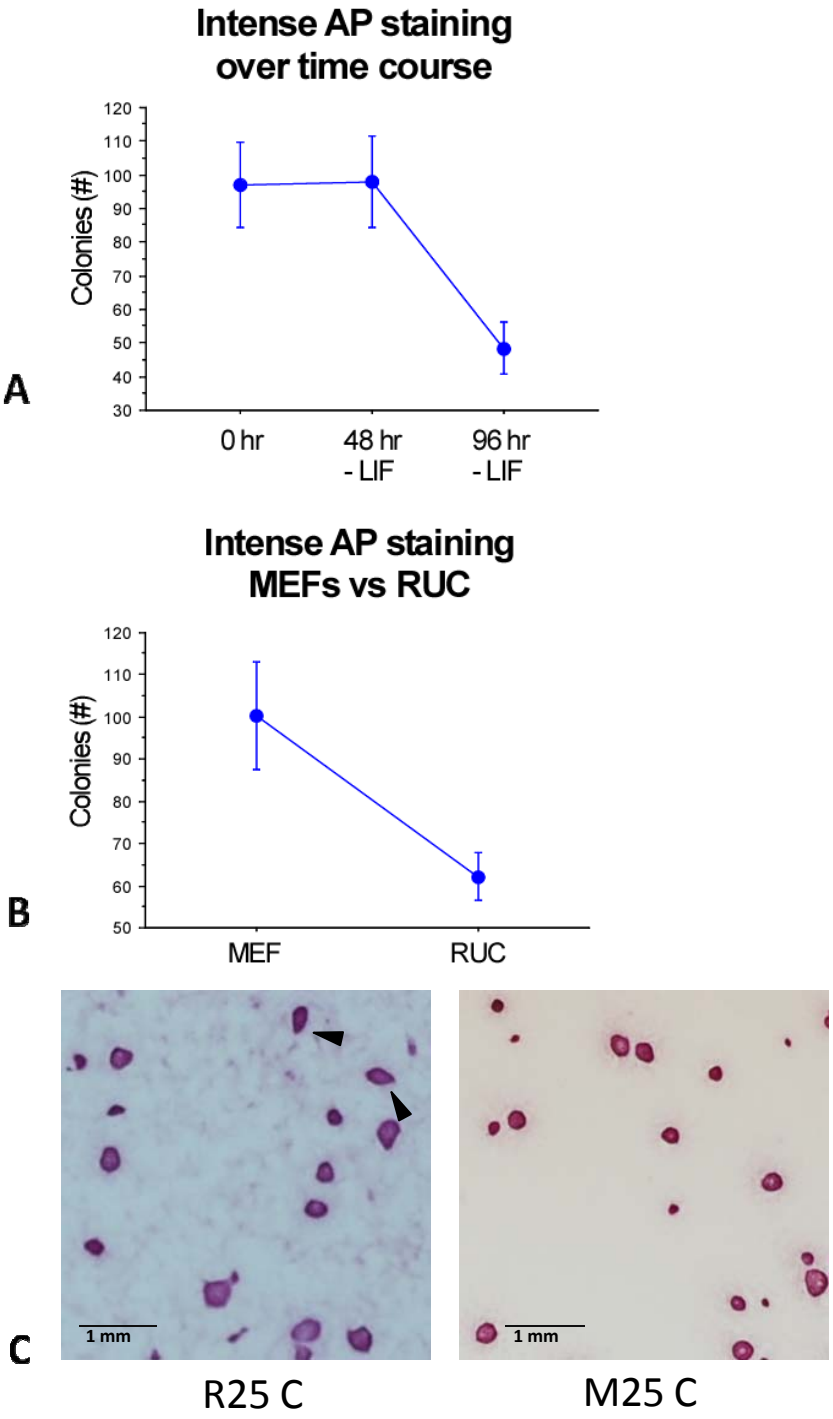

Figure 4-1. The average number of AP-stained colonies.

A) To analyze the effect of LIF/2-MCE removal, the ESCs were treated under three conditions: control, 48 hour deprivation and 96 hour deprivation. All data were combined irrespective of feeder cell plating density or feeder cell type and analyzed for the effects of

differential LIF/2-MCE treatment. There is a decrease in the number of AP-stained colonies after removal of LIF/2-MCE when ESCs were cultured on a feeder layer (p-value < 0.01).

B) To analyze the effect of feeder type, two feeder types were tested, mouse embryonic fibroblasts (MEFs) and rat umbilical cord matrix-derived stromal cells (RUCs). All data were combined and analyzed for the effects of feeder type. The two feeder cell types tested differ in the number of AP-stained colonies (p-value < 0.01).

C) Representative photographs of AP-stained ESCs on RUCs (left) and MEFs (right). The AP staining of the ESCs on RUCs showed some irregular morphology (arrow heads) as compared to the consistent circular to oval morphology of the ESCs cultured on MEFs.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; AP, alkaline phosphatase; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-2

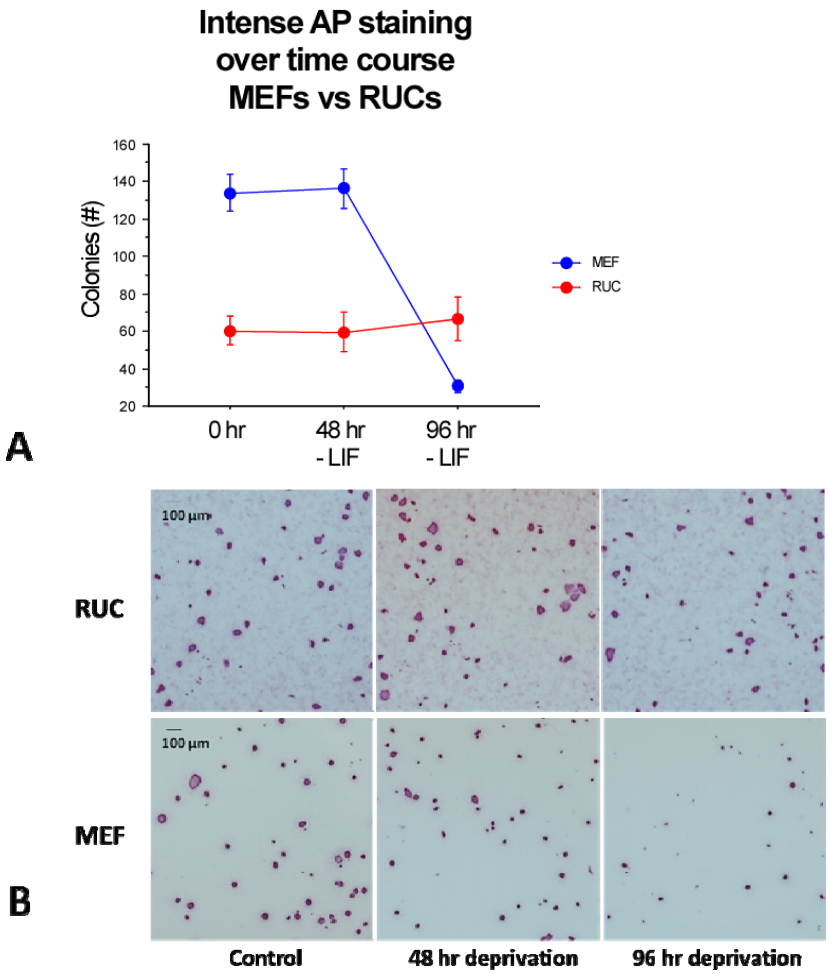

Figure 4-2. Interaction effect of feeder type and time.

A) To analyze the interaction effect of feeder type and LIF/2-MCE treatment, the ESCs were treated with LIF/2-MCE under three conditions and two feeder types were evaluated, MEFs and RUCs. All data were combined irrespective of feeder cell density and analyzed for the interaction effect of feeder cell type and LIF/2-MCE treatment. ESCs cultured on MEFs (blue line) showed a loss of AP-staining after LIF/2-MCE removal, whereas the RUCs (red line) did not (p-value < 0.01).

B) Representative photographs taken using a 55 mm Nikkor macro lens. In contrast to ESCs cultured on MEFs (bottom panels), ESCs cultured on RUCs (top panels) maintained the average number of AP-stained colonies even after LIF/2-MCE removal for 96 hours (third column).

Control (left), 48 hour deprivation (middle), 96 hours deprivation (right).

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; AP, alkaline phosphatase; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-3

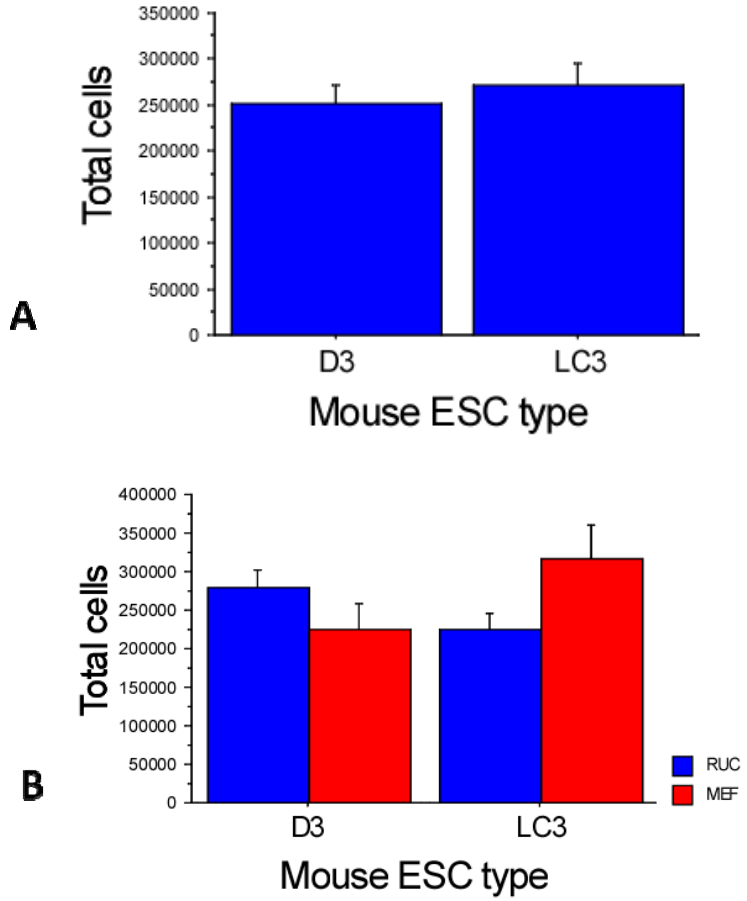

Figure 4-3. Average total cells for D3 or LC3 grown on MEFs or RUCs.

A) To analyze the effect of ESC type, two mouse ESC types were tested, D3 and LC3. All data were combined irrespective of LIF/2-MCE treatment and analyzed for differences in average total cell counts dependent on ESC type. There was no difference in the average total number of cells between the D3s and the LC3s (p-value > 0.05).

B) The interaction effect of ESC type and feeder cell type was analyzed. D3s (left) showed higher average total cell numbers when cultured on RUCs (blue bars) and LC3s (right) showed higher average total cell numbers when cultured on MEFs (red bars) (p-value < 0.01).

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-4

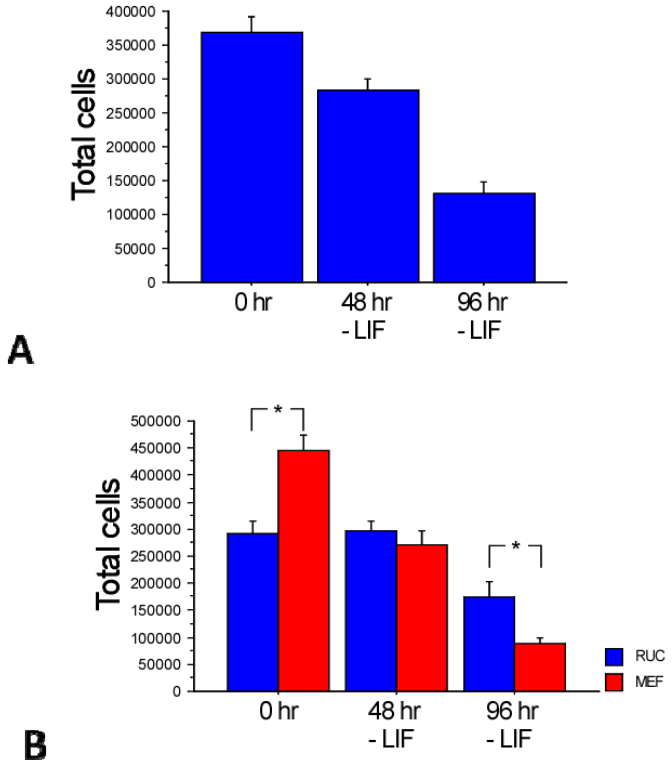

Figure 4-4. Effect of LIF/2-MCE treatment on average total cell numbers.

A) To analyze the effect of LIF/2-MCE removal, the ESCs were treated under three conditions: control, 48 hour deprivation and 96 hour deprivation. All data were combined and analyzed for the effect of LIF/2-MCE removal. LIF/2-MCE removal causes a decrease in the average total number of cells (p-value < 0.01). Data shown were combined data from all samples irrespective of ESC type, feeder cell type or feeder cell density and segregated only by treatment time points.

B) To analyze the interaction effect of LIF/2-MCE treatment and feeder type, the ESCs were treated under three conditions as above and two feeder types were tested, MEFs and RUCs. The average total number of ESCs was higher when continuously supplemented with LIF/2-MCE (control) when cultured on MEFs (red bars) (p-value < 0.005). The average total number of ESCs was higher in 96 hour deprivation when cultured on RUCs (blue bars, p-value < 0.004). Post hoc unpaired T-test was completed for the p values indicated.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-5

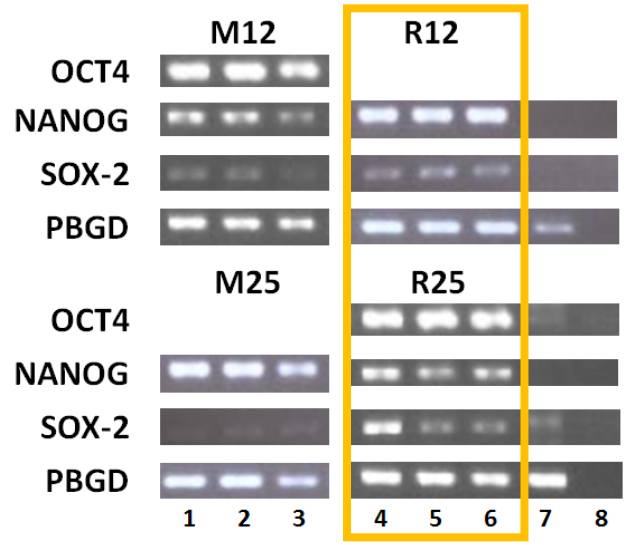

Figure 4-5. Gel electrophoresis RT-PCR for Oct4, Nanog and Sox2

To analyze the interaction effect of LIF/2-MCE treatment and feeder type, the ESCs were treated under three conditions and two feeder types were tested, MEFs and RUCs, as above and mRNA was collected. Nanog gene expression when ESCs were cultured on MEFs at 12,500 cells/cm^2 (M12) appears to be downregulated after 96 hours of LIF/2-MCE deprivation. Sox2 gene expression appears to be expressed at a lower level when ESCs were cultured on MEFs at either density (M25 and M12) as compared to RUCs (orange box). When ESCs were cultured on RUCs, Nanog and Sox2 gene expression was maintained. Oct4 gene expression does not appear to be affected even 96 hours after LIF/2-MCE removal.

M25 and M12 are MEFs at 25,000 and 12,500 cells/cm^2, respectively.

R12 and R25 are RUCs at 12,500 and 25,000 cells/cm^2, respectively.

Lanes 1 and 4, control conditions, supplemented continuously with LIF/2-MCE

Lanes 2 and 5, 48 hour deprivation of LIF/2-MCE

Lanes 3 and 6, 96 hour deprivation of LIF/2-MCE

Lane 7, negative control, either RUCs or MEFs only, absent ESCs and lane 8, water control; housekeeping gene PBGD was used as the DNA loading control.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; Oct4 or POU5F1, Pit-Oct-Unc (POU) domain, class 5, transcription factor 1; Sox2, SRY (sex determining region Y)-box 2; PBGD or HMBS, hydroxymethylbilane synthase; cm^2, square centimeters; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-6

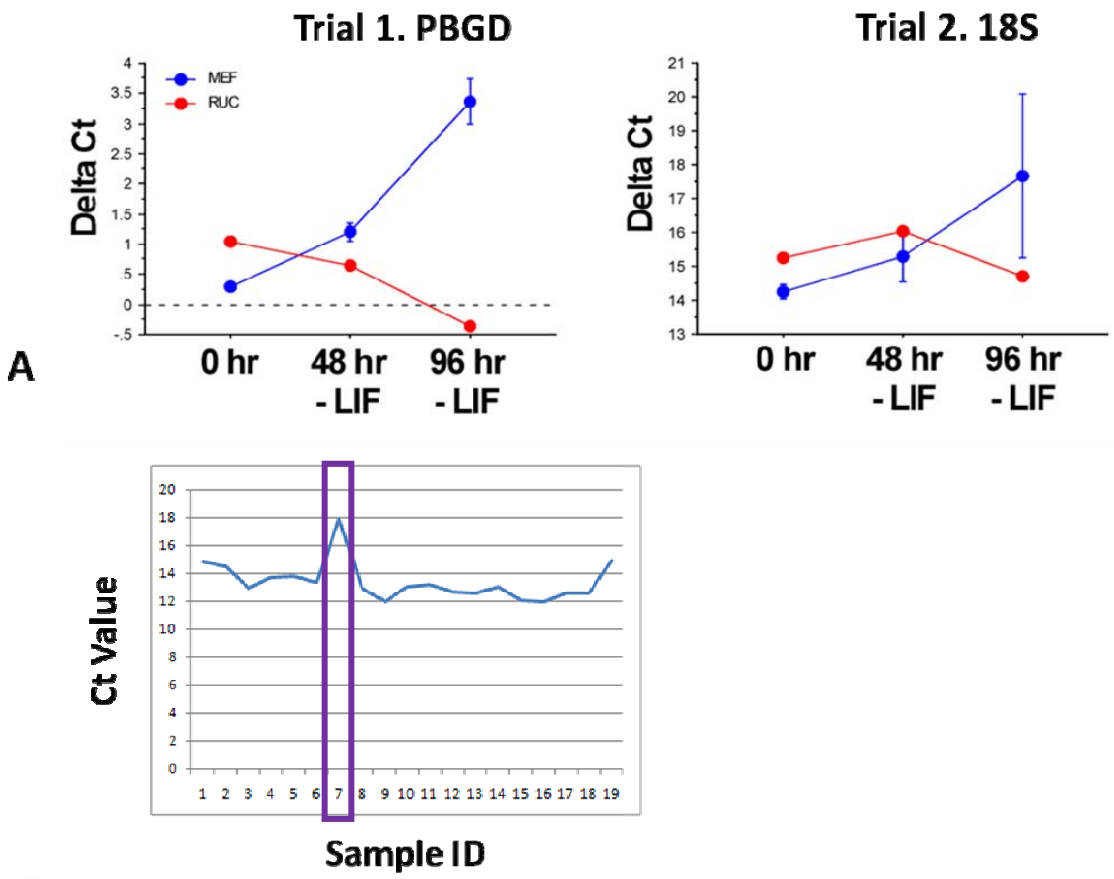

Figure 4-6. Semi-quantitative RT-PCR for Nanog gene expression

A) Nanog gene expression was analyzed relative to two housekeeping genes, PBGD (left) and 18S (right) using delta Ct value. The delta Ct values calculated using both housekeeping genes showed similar trends for Nanog gene expression from the cultures of ESCs on both MEFs (blue lines) and RUCs (red lines). The trends show Nanog gene expression is downregulated after culture in MEFs in the absence of LIF/2-MCE for 96 hours as compared to the control treatment, and Nanog gene expression was upregulated when ESCs were cultured on RUCs in the absence of LIF/2-MCE for 96 hours as compared to the control treatment.

B) Ct values from semi-quantitative RT-PCR for 18S for all cDNA samples used in this study. As shown, the average Ct value was 13.4, the standard deviation was 1.39 and the standard error of the mean was 0.29. If Sample 7 in not included, the standard deviation drops to 0.9.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; Oct4 or POU5F1, Pit-Oct-Unc (POU) domain, class 5, transcription factor 1; Sox2, SRY (sex

determining region Y)-box 2; RT-PCR, reverse transcriptase polymerase chain reaction; PBGD or HMBS, hydroxymethylbilane synthase; 18S, 18S ribosomal RNA; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-7

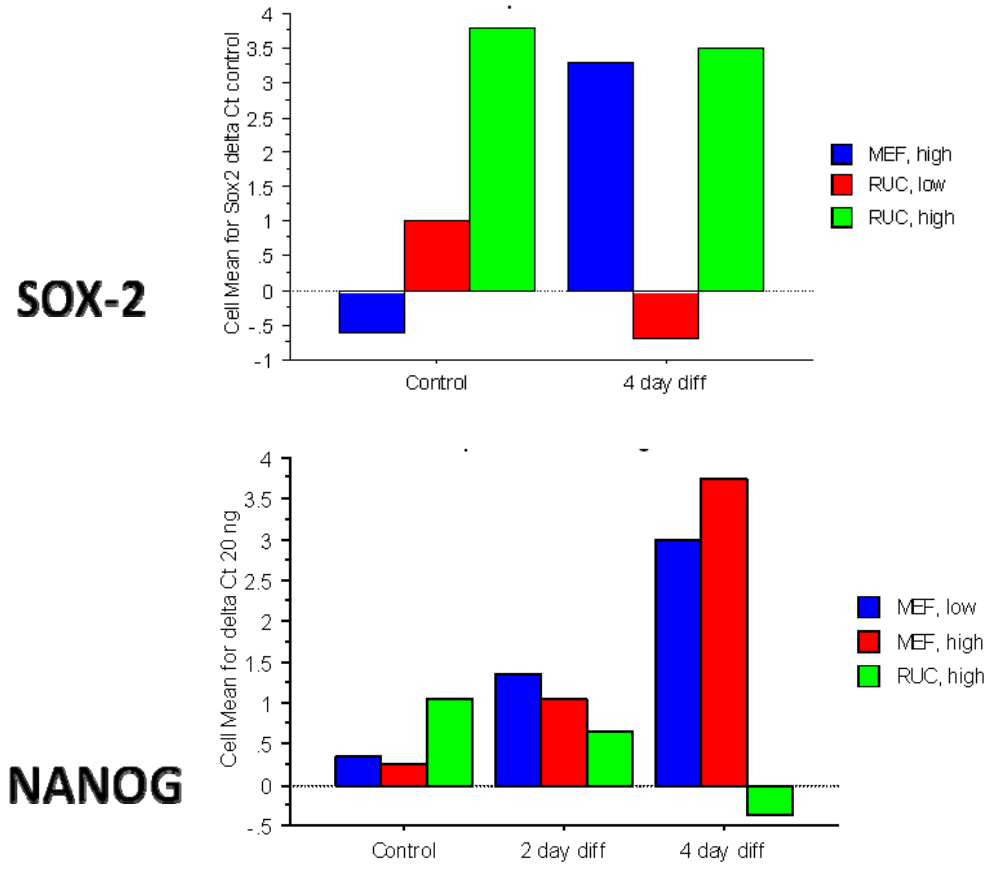

Figure 4-7. Semi-quantitative RT-PCR analyzed with respect to feeder cell density and LIF/2-MCE treatment for Sox2 (top) and Nanog (bottom) gene expression.

delta Ct values for Sox2 were calculated using Ct values for the housekeeping gene PBGD. The relative gene expression analysis shows Sox2 was downregulated in ESCs cultured on MEFs plated at 25,000 cells/cm^2 in the absence of LIF/-2MCE (blue bars). In contract, ESCs show an upregulation of Sox2 in the absence of LIF/-2MCE for 96 hours when cultured on feeders plated at 12,500 cells/cm^2 (red bars) and no change when feeders were plated at 25,000 cells/cm^2 (green bars).

delta Ct values for Nanog were calculated using Ct values for the housekeeping gene 18S. Nanog gene expression analysis with respect to feeder cell density and LIF/2-MCE treatment showed a progressive downregulation of Nanog gene expression in the ESCs when cultured on MEFs plated at both 12,500 and 25,000 cells/cm^2 (blue and red bars, respectively) in the absence of LIF/2-MCE as compared to the control treatment conditions where the culture of

ESCs was continusously supplemented with LIF/2-MCE. Nanog gene expression was progressively upregulated when ESCs were cultured on RUCs at 25,000 cells/cm^2 in the absence of LIF/2-MCE as compared to the control treatment.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; Sox2, SRY (sex determining region Y)-box 2; RT-PCR, reverse transcriptase polymerase chain reaction; PBGD or HMBS, hydroxymethylbilane synthase; 18S, 18S ribosomal RNA; cm^2, square centimeters; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

Figure 4-8

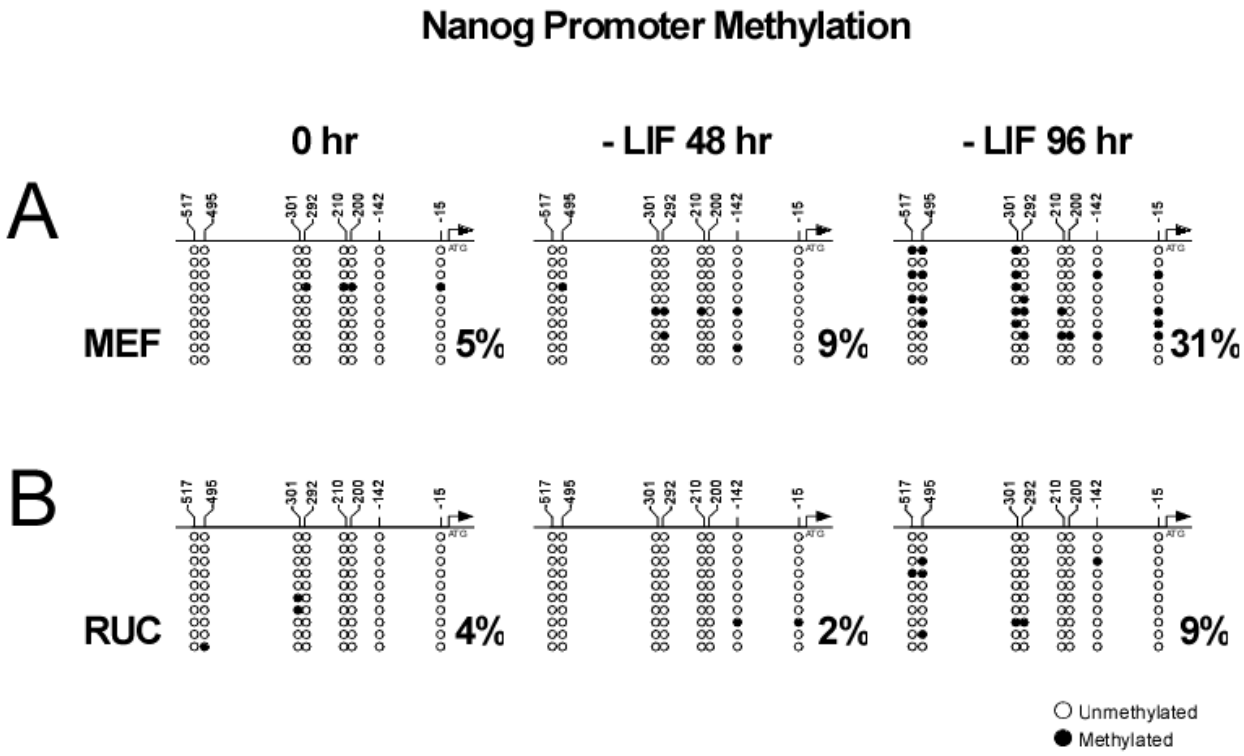

Figure 4-8. Methylation analysis of the Nanog proximal promoter region.

To analyze the effect of LIF/2-MCE treatment on DNA methylation of the Nanog proximal promoter, ESCs were treated under three treatment conditions, control supplemented continuously with LIF/2-MCE and 48 and 96 hours deprivation conditions where ESCs were cultured for 48 and 96 hours, respectively, in the absence of LIF/2-MCE. Two feeder types were tested as above, RUCs and MEFs. The CpG dinucleotides in the Nanog proximal promoter region spanning 619 bp upstream from the start site were analyzed. ESCs cultured on MEFs are shown in (A) and ESCs cultured on RUCs are shown in (B). Nanog promoter methylation appears to be highest when ESCs were cultured on MEFs for 96 hours after LIF/2-MCE removal. MEFs and RUCs were plated at 25,000 cells/cm^2.

LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or beta-mercaptoethanol; cm^2, square centimeters; MEF, mouse embryonic fibroblast; RUC, rat umbilical cord-derived stromal cell

CHAPTER 5 - Discussion

Mouse embryonic stem cells (ESCs) were grown *in vitro* on rat umbilical cord derived stromal cells (RUCs) and mouse embryonic fibroblasts (MEFs) to evaluate the effects of ESC culture in the absence of LIF/2-MCE for 48 and 96 hours. Previous studies showed the culture of ESCs in the absence of LIF resulted in the downregulation of Oct4, Sox2 and Nanog and a loss of alkaline phosphatase (AP) staining (Mitsui et al., 2003; Maruyama et al., 2005; Zandstra et al., 2000). The effects of LIF/2-MCE removal and feeder type were assessed using AP staining, cell counts, RT-PCR for Oct4, Sox2 and Nanog gene expression and methylation analysis of the Nanog gene promoter. Here, it was shown that when mouse ESCs were cultured on RUC feeders in the absence of LIF, the number of AP-stained ESC colonies was not reduced. Cell counts showed that the number of ESCs was higher when cultured for 96 hours in the absence of LIF/2-MCE on RUCs as compared to culture of ESCs on MEFs in the same conditions. It was also shown that when ESCs were cultured on RUCs in the absence of LIF/2-MCE, the ESCs showed continued expression of Oct4, Sox2 and Nanog genes and reduced methylation of the proximal promoter of the Nanog gene. This suggests that RUCs, through undefined mechanisms, support ESC self-renewal and maintain the undifferentiated state in the absence of LIF/2-MCE whereas MEFs are not able to do so.

ESCs cultured on MEFs in the control conditions where the ESCs were supplemented continuously with LIF/2-MCE for the duration of the 120 hour experiment showed, overall, higher numbers of AP-stained colonies as compared to when ESCs were cultured on RUCs under the same conditions. In contrast, ESCs grown on MEFs showed the lowest number of AP-stained colonies in the 96 hours deprivation conditions where the ESCs were cultured for 96 of the 120 hours in the absence of LIF/2-MCE. In contrast, ESCs cultured on RUCs showed no change in the numbers of AP-stained colonies in both LIF/2-MCE treatment conditions as compared to the control treatment conditions. The results from the culture of ESCs on RUCs differ from previous work done with MEFs which showed that removal of LIF results in loss of AP staining (Zandstra et al., 2000). In the absence of LIF/2-MCE and with respect to AP-

staining as a barometer for *in vitro* pluripotency, the culture of ESCs on RUCs may be superior to the culture of ESCs on MEFs.

Cell counts of the D3 and LC3 mouse ESCs showed the number of cells was higher when ESCs were cultured on MEFs in the control condition supplemented continuously with LIF/2-MCE as compared to ESCs cultured on RUCs under the same conditions. This showed that with supplementation of LIF/2-MCE, culture of ESCs on RUCs was not equivalent to culture of ESCs on MEFs in terms of total cell numbers. However, the number of cells was lowest when ESCs were cultured on MEFs in the 96 hour deprivation conditions where ESCs were cultured in the absence of LIF/2-MCE for 96 hours. Earlier work states that LIF may not be tightly associated with proliferation. However, the results here were in agreement with previous work that showed deficiency in signaling downstream of LIF results in a decrease in proliferation (Forrai et al., 2006). The cell numbers for ESCs cultured on RUCs after removal of LIF/2-MCE was higher as compared to cell numbers for ESCs cultured on MEFs. It is unknown why RUCs were better able to support self-renewal as compared to MEFs in the absence of LIF/2-MCE. It may be due to gene expression as discussed below.

Gel electrophoresis RT-PCR analysis for Oct4, Sox2 and Nanog gene expression suggested that Sox2 and Nanog were expressed at lower levels when ESCs were cultured on MEFs as compared to expression of these genes when ESCs were cultured on RUCs. Previous work has shown that removal of LIF from the culture of ESCs results in the downregulation of Sox2 and Nanog genes (Mitsui et al., 2003; Maruyama et al., 2005). The continued expression of Oct4 gene after culture in the absence of LIF/2-MCE is in agreement with previous work showing maintenance of Oct4 gene expression after culture for four days in the absence of LIF. Longer time points may show downregulation of Oct4.

Semi-quantitative RT-PCR for Nanog gene expression was also conducted on the same cDNA samples as used in the gel electrophoresis RT-PCR analysis of gene expression. Nanog gene expression was found when ESCs were cultured on RUCs in the controls and treatment conditions and was found to be upregulated when ESCs were cultured in the absence of LIF/2-MCE on RUCs. Nanog gene expression was downregulated when ESCs were cultured on MEFs as compared to the control treatment conditions. Sox2 gene expression was also shown to be downregulated when ESCs were cultured on MEFs, and was not downregulated when ESCs were cultured on RUCs plated at either 12,500 or 25,000 cells/cm^2. The downregulation of

Nanog and Sox2 seen when ESCs were cultured on MEFs is in agreement with previous work that shows downregulation of Nanog and Sox2 after removal of LIF (Mitsui et al., 2003; Maruyama et al., 2005). As will be discussed below, the downregulation of both Sox2 and Nanog found here may have contributed to the reduced cell numbers of the ESCs when they were cultured on MEFs in the absence of LIF/2-MCE.

DNA methylation analysis of the Nanog proximal promoter showed that the percentage of methylated cytosines was highest when ESCs were cultured on MEFs in the absence of LIF/2-MCE for 96 hours. The percentage of methylated cytosines was higher for both treatment conditions when ESCs were cultured on MEFs as compared to ESCs cultured on RUCs. The percentage of methylated cytosines was 9% and 2% for ESCs cultured in the absence of LIF/2-MCE for 48 hours on MEFs and RUCs, respectively.

The above results suggest that RUCs prevent the differentiation of mouse ESCs *in vitro* in the absence of LIF. However, the results also suggest that with continued supplementation of LIF, expansion of ESCs may be superior on the MEFs. The differences in the number of AP-stained colonies and cell numbers when ESCs were cultured for 96 hours in the absence of LIF/2-MCE on RUCs as compared to ESCs cultured on MEFs may be associated with differences in the expression levels of the genes associated with the pluripotent state, Oct4, Sox2 and Nanog.

It should be noted that the undifferentiated state, of which AP-staining is indicative, and proliferation are separable. Nanog and Sox2 are required to prevent the differentiation of mouse ESCs. Nanog and Sox2 have also been found in previous studies to have effects on proliferation (Torres and Watt, 2008;Wang et al., 2008a;Wang et al., 2008b). Here, we found that ESCs cultured on RUCs maintain the level of Nanog and Sox2 gene expression after removal of LIF/2-MCE. Given the previous work and the finding here that Nanog and Sox2 gene expression were not downregulated when ESCs were cultured on RUCs in the absence of LIF/2-MCE could explain the higher numbers of total cells found when ESCs were cultured on RUCs in the absence of LIF/2-MCE. The downregulation of Sox2 and Nanog gene expression seen after culture of ESCs on MEFs in the absence of LIF/2-MCE for 96 hours could also explain the reduced number of AP-stained colonies as well as the reduced number of cells and that the reduction in the number of AP-stained colonies may be due mainly to the reduced proliferation of cells.

It is possible that RUCs produce LIF. Human umbilical cord matrix-derived stromal cells (HUCs) have been shown to secrete LIF (Friedman et al., 2007). Other studies have found contrasting effects of LIF removal on proliferation (Raz et al., 1999; Mitsui et al., 2003). In the Raz et al study, one ESC line showed a two-fold decrease in the proliferation of ESCs cultured for 96 hours in the absence of LIF as compared to ESCs supplemented with LIF.

An important point here is that these studies used ESCs derived from different strains of mice. This is analogous to the predisposition of individuals with a genotype that renders them more susceptible to particular diseases. This should be considered when comparing results here with previous work since the genotype of the ESCs here are different from that used in the Raz et al. study and the effects of LIF on proliferation found in the Raz et al. study was found to be strain dependent. However, this previous work could explain the five-fold difference in the total number of cells observed here between the control conditions and the 96 hour deprivation conditions when ESCs were cultured on MEFs. It was also interesting that the cell numbers when ESCs were cultured on RUCs was nearly two-fold higher in the control conditions as compared to the 96-hour deprivation conditions suggesting a similar effect of LIF withdrawal here as that found in the Raz et al. study.

The downregulation in Nanog gene expression after LIF/2-MCE removal may be associated with methylation of the Nanog proximal promoter. As shown in previous studies with Oct4 where the proximal promoter of the Oct4 gene was shown to be progressively methylated with significant methylation after 4 days of retinoic acid treatment, the proximal promoter for Nanog in the ESCs cultured on MEFs in the absence of LIF/2-MCE for 96 hours was more methylated as compared to the proximal promoter for ESCs cultured on RUCs (Gu et al., 2006). The highest level of methylation seen in the ESCs cultured on RUCs was 9% as compared to 31% for ESCs cultured on MEFs. The methylation data was in agreement with the decrease in Nanog gene expression seen here in both the RT-PCR and the semi-quantitative RT-PCR when ESCs were cultured on MEFs for 96 hours after removal of LIF/2-MCE. It was also in agreement with the continued expression of the Sox2 gene in ESCs cultured on RUCs in the absence of LIF/2-MCE at the same or higher levels as compared to control treatment conditions.

The most glaring limitation of the study was the simultaneous removal of LIF and 2-MCE, as 2-MCE has shown effects on proliferation and apoptosis in porcine and bovine embryos (Funahashi 2005; Park et al., 2004). The major limitations of this work include the use of mouse

embryonic fibroblasts (MEFs) rather than rat embryonic fibroblasts. The species differences between the MEFs and RUCs added an unintentional variable. The limitations of this work also include the absence of a mechanistic evaluation of the effects of LIF removal. For example, knocking out the receptor for LIF in the RUCs would help to determine the mechanism by which the removal LIF may have affected the cell numbers when ESCs were cultured on RUCs. Gene expression analysis of the ESCs at time points coincident with the timing of the switch from the ESC medium to the MEF medium would have benefited the study. Such a study would be illustrative of the possible effects of the removal of LIF and 2-MCE on the cell cycle and proliferation. Another limitation of the study was that given the increase in methylation seen in the Nanog proximal promoter after culture on MEFs in the absence of LIF/2-MCE, for the purposes of methylation analysis, longer time points may be helpful since all the cytosines were not methylated here. The Nanog proximal promoter was completely methylated in sperm and 78% methylated in trophoblast stem cells (Farthing et al., 2008; Hattori et al., 2007). Adding longer time points may increase the percentage of methylation of the Nanog proximal promoter in the ESCs cultured on MEFs accompanied by an increased downregulation of the expression of the Nanog gene. The study would have benefited greatly from additional replicates for gel electrophoresis and semi-quantitative RT-PCR and DNA methylation analysis and Sox2 proximal promoter methylation analysis would have been beneficial since gel electrophoresis indicated that the downregulation of Sox2 gene expression may have been greater than downregulation of the Nanog gene expression. Finally, the study would have benefited from a direct measure of pluripotency, i.e., showing germline transmission of the ESCs cultured on RUCs after blastocyst injection.

In future work, it would be interesting to examine how the RUCs were able to maintain both AP staining and ESC numbers in the absence of LIF/2-MCE. As previous work has shown, it may be signaling unassociated with LIF and associated with the Sox2 and Nanog genes. This future work could address the difference between ESC self-renewal, which is the continued propagation of the cells in the pluripotent state, and proliferation which can occur during differentiation from the pluripotent state.

Finally, D3 co-culture on RUCs show some irregularities in morphology. In contrast to D3s cultured on MEFs which show a smooth, circular to oval morphology, D3s cultured on RUCs show a tendency for protrusions, leading to some elongate colonies (not shown).

Preliminary studies of D3 RUC co-culture followed by passage to MEFs show D3s regain the smooth, circular to oval morphology after RUC co-culture (not shown). An extracellular matrix, or basement membrane, encapsulating the colony may be responsible for the smooth, oval morphology (not shown). Sox2 may also be involved as overexpression causes differentiation of ESCs after 72 hours (Kopp et al., 2008). The continued expression of Sox2 when ESCs were cultured on RUCs in the absence of LIF/2-MCE could explain the higher incidence of mixed AP-stained colonies (data not shown).

Overall, this study showed that RUCs can promote the *in vitro* pluripotent state in the absence of LIF/2-MCE, as indicated by the continued gene expression of the pluripotency markers Oct4, Sox2, Nanog and the presence of AP staining after removal. The gene expression for the ESCs cultured on the RUCs also showed a profile distinct from the gene expression profile of the ESCs cultured on the MEFs. Therefore, the culture of ESCs on RUCs represents a simple model for the *in vitro* study of molecules at the core of the pluripotency network, especially with respect to DNA methylation analysis of the Nanog gene proximal promoter. Given the differential gene expression profile found in this study, comparing the downstream effects of Oct4, Sox2 and Nanog differential expression may be possible. Ultimately, this *in vitro* model may reveal threshold levels of transcription factors for the maintenance of the pluripotent state, an active area of current research. As mentioned in the literature review, subtle changes in the levels of gene expression result in changes in the gene expression of Oct4, Sox2 and Nanog (Niwa et al., 2000; Pan et al., 2006; Leonhardt et al., 1992). Understanding how the levels of gene expression of Oct4, Sox2 and Nanog, which have been shown to have complex interactions, can help further delineate that which was described recently as the "ground state of pluripotency" (Medeiros et al., 2009; Ying et al., 2008). Studies such as the co-immunoprecipitation used in the Medeiros study, applied in this *in vitro* model could help in this regard by uncovering Sox2 and Nanog protein-associated molecules integral to the decision to drive or repress a particular fate in the differentiation of ESCs.

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Appendix A - ANOVA Tables

Table A-1

ANOVA Table for Intense

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
conditions	1	11858.000	11858.000	11.918	.0087	11.918	.866
density	1	8.000	8.000	.008	.9308	.008	.051
conditions * density	1	3389.389	3389.389	3.407	.1021	3.407	.359
Subject(Group)	8	7959.417	994.927				
Category for Intense	2	16013.049	8006.524	29.842	<.0001	59.685	1.000
Category for Intense * conditions	2	20792.687	10396.344	38.750	<.0001	77.500	1.000
Category for Intense * density	2	139.521	69.760	.260	.7742	.520	.083
Category for Intense * conditions * density	2	223.215	111.608	.416	.6666	.832	.104
Category for Intense * Subject(Group)	16	4292.708	268.294				

Total Number of AP-stained Colonies ANOVA

ANOVA for main effects are shown. The p-value of 0.0087 is associated with Figure 4-1A indicating that there was an effect of removal of LIF on the number of AP-stained colonies. The p-value 0.9308 indicates that feeder density had no effect on the number of AP-stained colonies. The p-value of 0.1021 indicates that there was no interaction effect between removal of LIF/2-MCE and feeder density.

ANOVA, analysis of variance; AP, alkaline phosphatase; LIF, leukemia inhibitory factor; 2-MCE, 2-mercaptoethanol or β-mercaptoethanol

Table A-2

ANOVA Table for total cells

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
ESC type	1	7344264287.668	7344264287.668	.622	.4386	.622	.113
Cell type	1	6959536268.430	6959536268.430	.590	.4506	.590	.110
ESC type * Cell type	1	105387348268.430	105387348268.430	8.932	.0068	8.932	.828
Subject(Group)	22	259573720528.825	11798805478.583				
Category for total cells	2	749221367688.221	374610683844.111	104.463	<.0001	208.926	1.000
Category for total cells * ESC type	2	10814823585.658	5407411792.829	1.508	.2326	3.016	.293
Category for total cells * Cell type	2	206247593986.889	103123796993.444	28.757	<.0001	57.514	1.000
Category for total cells * ESC type * Cell t...	2	8352056140.735	4176028070.368	1.165	.3215	2.329	.234
Category for total cells * Subject(Group)	44	157786584484.222	3586058738.278				

Total Cell Counts ANOVA

ANOVA for main effects are shown. The p-value of 0.4386 indicates that ESC type had no effect on the average total number of cells. The p-value of 0.4506 indicates that feeder type had no effect on the average total number of cells. The p-value of 0.0068 indicates that there was an interaction effect between ESC type and feeder type (this was not pursued).

ANOVA, analysis of variance; ESC, embryonic stem cell

Appendix B - RNA Log

Table B-1

Sample	conc	ratio	volume	total RNA (ng)
Mouse D3 in 500 IU/mL LIF on MEFs control MEFs at 12,500 per cm2	149.5	2.11	30	4485.0
Mouse D3 in 500 IU/mL LIF on MEFs for 48 hours MEFs at 12,500 per cm2	78.6	2.05	30	2357.6
Mouse D3 in 500 IU/mL LIF on MEFs for 96 hours MEFs at 12,500 per cm2	27.3	2.16	30	819.9
Mouse D3 in 500 IU/mL LIF on RUCs control RUCs at 25,000 per cm2	108.5	2.11	30	3255.0
Mouse D3 in 500 IU/mL LIF on RUCs for 48 hours RUCs at 25,000 per cm2	126.3	2.07	30	3787.5
Mouse D3 in 500 IU/mL LIF on RUCs for 96 hours RUCs at 25,000 per cm2	97.3	2.11	30	2919.2
ATCC MEF scrc 1008 P1 Mitomycin C inactivated at 10 ug/ml for 3 hours	539.1	2.26	50	26952.5
RUC P33 rat umbilical cord stromal cells Mitomycin C inactivated at 10 ug/ml for 3 hours	856.4	2.12	50	42817.5
Mouse D3 in 500 IU/mL LIF on MEFs control MEFs at 25,000 per cm2	227.5	2.09	30	6823.5
Mouse D3 in 500 IU/mL LIF on MEFs for 48 hours MEFs at 25,000 per cm2	97.2	2.05	30	2914.5
Mouse D3 in 500 IU/mL LIF on MEFs for 96 hours MEFs at 25,000 per cm2	25.7	1.95	30	771.2
Mouse D3 in 500 IU/mL LIF on MEFs control MEFs at 25,000 per cm2	207.6	2.07	30	6228.0
Mouse D3 in 500 IU/mL LIF on MEFs for 48 hours MEFs at 25,000 per cm2	213.5	2.08	30	6403.5
Mouse D3 in 500 IU/mL LIF on MEFs for 96 hours MEFs at 25,000 per cm2	77.2	2.05	30	2315.1
Mouse D3 in 500 IU/mL LIF on RUCs control RUCs at 12,500 per cm2	121.8	2.09	30	3652.5
Mouse D3 in 500 IU/mL LIF on RUCs for 48 hours RUCs at 12,500 per cm2	151.4	2.11	30	4542.0
Mouse D3 in 500 IU/mL LIF on RUCs for 96 hours RUCs at 12,500 per cm2	65.6	2.14	30	1966.8
MEFs in 500 IU/mL LIF control both 25k and 12.5K cells combined	38.6	2.14	30	1157.0
MEFs in 500 IU/mL LIF 48 hours in MEF media both 25k and 12.5K cells combined	42.5	2.14	30	1274.1
MEFs in 500 IU/mL LIF 96 hours in MEF media both 25k and 12.5K cells combined	50.9	2.14	30	1526.9
Mouse D3 in 500 IU/mL LIF on MEFs control MEFs at 12,500 per cm2	273.0	2.10	30	8188.5
Mouse D3 in 500 IU/mL LIF on MEFs for 48 hours MEFs at 12,500 per cm2	132.1	2.08	30	3961.5
Mouse D3 in 500 IU/mL LIF on MEFs for 96 hours MEFs at 12,500 per cm2	95.6	2.11	30	2867.3
Mouse D3 in 500 IU/mL LIF on RUCs control RUCs at 25,000 per cm2	55.6	2.04	30	1668.2
Mouse D3 in 500 IU/mL LIF on RUCs for 48 hours RUCs at 25,000 per cm2	106.2	2.06	30	3184.5
Mouse D3 in 500 IU/mL LIF on RUCs for 96 hours RUCs at 25,000 per cm2	101.3	2.11	30	3039.0

RNA sample log showing sample description, sample concentration, 260/280 quality ratio, volume and total amount of RNA in nanograms.

RNA, ribonucleic acid