Evaluation of the TGF-ß Inhibitor RepSox on the Expression of Pluripotency Pathways in Murine and Bovine Cells

Davin M. Larsen

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EVALUATION OF THE TGF-β INHIBITOR REPSOX ON THE EXPRESSION OF
PLURIPOTENCY PATHWAYS IN MURINE AND BOVINE CELLS

by

Davin M. Larsen

A thesis submitted in partial fulfillment of the requirements for the degree
of
MASTER OF SCIENCE
in
Animal, Dairy and Veterinary Sciences
(Animal Molecular Genetics)

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2013
ABSTRACT

Evaluation of the Effects of the Small Molecule RepSox
on the Expression of Pathways Associated with
Pluripotency in Murine and Bovine Cells

by

Davin M. Larsen, Master of Science
Utah State University, 2013

Embryonic stem cells are pluripotent cells isolated from morula stage embryos or the inner cell mass of blastocyst stage embryos. They are capable of differentiating into tissues of all three primary germ layers. In recent years pluripotent cell lines have been created from somatic cell types using various methods, the primary method being viral transduction of exogenous Oct4, Sox2, Klf4, and c-Myc or Oct4, Sox2, Nanog, and Lin28 transgene constructs. The resulting cell lines are termed induced pluripotency stem cells, and are similar to embryonic stem cells in many ways. However, these cell lines are not acceptable for clinical applications due to the use of both modified viral vectors and insertion of exogenous transgenes in their production. Recently the small molecule RepSox, a TGF-β pathway inhibitor, was used to replace Sox2 during cellular reprogramming of murine embryonic fibroblasts. We evaluated the effects of RepSox on expression of pathways related to pluripotency in murine embryonic fibroblast, murine embryonic stem, and bovine embryonic fibroblast cells. Each cell type was
treated with RepSox for 72 hours and subjected to standard qPCR for gene expression analysis. PCR arrays specific to stem cell pathways were used to initially evaluate the effects of RepSox on candidate genes. A subset of genes was then selected for further analysis based on these initial results. We report that RepSox inhibition of the TGF-β pathway in murine embryonic fibroblasts results in significant upregulation of components of the Wnt, Notch, and Hedgehog signaling pathways, all of which have been linked to stem cell maintenance. In addition, we observed significant upregulation of genes associated with embryonic, mesenchymal, stem cell, and neural cell lineages, indicating that RepSox may be useful in direct reprogramming of murine cells to other somatic cell types. RepSox treatment of murine embryonic stem cells did not result in consistent upregulation of Wnt, Notch, or Hedgehog pathway components, but did result in upregulation of Sox2 and Klf4 expression. Lastly, RepSox treatment of bovine embryonic fibroblasts did not result in the same effects as seen in murine fibroblasts, indicating a need for further analysis to determine the effects of RepSox on bovine cells.

(96 pages)
PUBLIC ABSTRACT

Evaluation of the Effects of the Small Molecule RepSox on the Expression of Pathways Associated with Pluripotency in Murine and Bovine Cells

Davin M. Larsen

Embryonic stem cells are cells which are isolated from early stage embryos and have the theoretical ability to become any adult cell type in the body. In the past few years much research has focused on the use of embryonic stem cells for clinical applications in the treatment of degenerative diseases. Consequently they are a promising tool in the treatment of diseases such as Diabetes and Parkinson’s disease, and could potentially be used in the repair of permanently damaged tissue. However, since these cells are isolated from early stage embryos their successful isolation often results in the destruction of the embryo from which they are derived. In light of these ethical concerns considerable research has recently focused on the production of embryonic stem-like cells from adult cells instead of embryos. Such cells are called induced pluripotent stem cells, and are usually produced using viruses as a means for delivering stem cell associated genes into the cell. Unfortunately, this production method precludes the use of these cells in clinical applications. One potential way to resolve this problem is to produce induced pluripotent stem cells using small molecules that target cellular pathways that are active in embryonic stem cells. This would eliminate the need for viruses as well as foreign genetic material for their production and make them available for clinical use.

This research focuses on one such small molecule, RepSox. We evaluated the effect of RepSox on gene expression of pathways relevant to stem cell maintenance in an effort to come closer to the aforementioned goal. This study, and future studies like it, will open up the possibility of using induced pluripotent stem cells for clinical purposes.
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Davin Larsen
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LITERATURE REVIEW

Embryonal Carcinoma Cells

Before the successful isolation and culture of embryonic stem (ES) cells, embryonal carcinoma (EC) cells were the model of choice for studies of cellular differentiation and early embryonic development. EC cells are isolated from teratocarcinomas, which are tumors composed of an array of undifferentiated malignant stem cells interspersed with a chaotic array of various types of somatic cells, usually representing all three of the primary germ layers. The somatic tissues present in the tumor arise from differentiation of the undifferentiated malignant cells (Kleinsmith and Pierce, 1964). It is possible to isolate EC cells from spontaneously formed tumors, but most often they are obtained through explantation of blastocysts, early egg cylinder stage embryos, or genital ridges to ectopic sites (Rossant and Papaioannou, 1984). However, EC cells contain properties which make them not ideal for developmental and differentiation studies.

First, EC cells are only considered to be multipotent. While a typical teratocarcinoma is comprised of somatic tissues from all three primary germ layers, the differentiation capacity of each clonal EC cell line varies, and is not able to differentiate into all cell types (Kleinsmith and Pierce, 1964). Second, while EC cells can proliferate indefinitely in vitro they also are able to proliferate in an undifferentiated state in vivo, often resulting in tumors (Rossant and Papaioannou, 1984). These characteristics made necessary the isolation of an embryonic cell type not derived from tumors.

Embryonic Stem Cells – Rodents and Primates

ES cells are most often isolated from the inner cell mass (ICM) of blastocyst stage embryos (Axelrod, 1984), although attempts have been made to isolate them at earlier stages,
such as the 8 to 16 cell and morula stages (Mitalipova et al., 2001; Stice et al., 1996). There have been many attempts to isolate ES cells in various species, with varying degrees of success (Evans and Kaufman, 1981; Hatoya et al., 2006; Martin, 1981; Polejaeva and Mitalipov, 2013). In order to determine the degree of success of a particular study it is necessary to outline the defining characteristics of an ES cell. In order to be classified as such, a cell line must meet all of the following criteria. First, a true ES cell line must be able to differentiate into tissues of all three embryonic germ layers. This is tested by embryoid body formation in vitro and teratoma formation in vivo. Embryoid bodies are formed when ES cells are cultured under conditions that do not allow the cells to attach to the culture surface. When grown in this manner the ES cells aggregate into spherical structures and differentiate randomly into various tissue types. These structures are known as embryoid bodies. Similarly, when injected into immunodeficient mice, ES cells aggregate and differentiate randomly into various tissue types, forming a tumor known as a teratoma. The tissue types present in these structures provide an indication of the differentiation potential of the cell line in question. Second, ES cells generally test positive for specific cell surface antigens. However, there are differences between ES cells from different species as to which cell surface antigens are expressed. All ES cells to date express Alkaline Phosphatase (AP). However, human cells express SSEA-3 (stage-specific embryonic antigen), SSEA-4, TRA-1-60 (Tumor Rejection Antigen) and TRA-1-81 while murine ES (mES) cells only express SSEA-1 (Ginis et al., 2004; Henderson et al., 2002; Sato et al., 2003). Third, ES cells must be able to proliferate in culture indefinitely with minimal differentiation. Lastly, ES cells must be capable of producing chimera contributing to tissues from all three embryonic germ layers including the germ line. This requirement has one notable exception in humans, as production
of human chimeras runs contrary to accepted ethical standards, and is therefore not testable (Nowak-Imialek et al., 2011).

Up until 1981, ES cells had not been successfully maintained in vitro. Evans and Kaufman proposed the following unanswered questions as possible explanations. First, what is the exact stage at which pluripotent cells capable of growth in vitro exist in the embryo? Second, what is the minimum number of cells required to successfully establish a culture of embryonic cells? Third, what culture conditions are necessary to promote embryonic cell maintenance rather than differentiation? They proposed that the cells of the early post-implantation stage epiblast are at the optimal stage for in vitro isolation and expansion. Under this premise they performed ovariectomies on female mice after successful mating. This prevented implantation of the embryo, inducing a state of diapause that allowed the epiblast to increase its cell numbers. Plating of whole blastocysts resulted in the successful establishment of ES cell lines grown on STO fibroblast feeder layers. Pluripotency of these cells was established through teratoma and embryoid body formation, as well as chimera production (Evans and Kaufman, 1981).

Later that same year, the laboratory of Gale Martin also successfully produced ES cells, albeit using a slightly different method. It was hypothesized that EC cells are able to proliferate in an undifferentiated state through endogenous production of a factor that maintains pluripotency. In this case ES cells were isolated through immunosurgery of blastocysts (Solter and Knowles, 1975) to isolate the ICM and cultured on STO fibroblast feeder cells in EC conditioned medium. In theory, this conditioned medium would contain the factor or factors produced by EC cells that maintain their pluripotency, and would also be capable of maintaining ES cells in culture. In fact, ES cells were not able to be established in the absence of this
medium. However, approximately after passage 5, conditioned medium was no longer needed.

Pluripotency was demonstrated by teratoma and embryoid body formation (Martin, 1981). A follow-up study to that of Martin’s found that the soluble factor leukemia inhibitory factor (LIF) is able to maintain ES cells in vitro, mimicking the effects of EC conditioned medium or a fibroblast feeder layer. In contrast, ES cells grown in the absence of LIF differentiated over a period of approximately three to six days. ES cells maintained in LIF were also able to contribute to chimeric mice, contributing to all somatic tissues including the germ line (Williams et al., 1988).

These studies marked the first successful establishment of ES cells in vitro and elucidated the culture conditions necessary for their maintenance. It was now possible to perform developmental and differentiation studies without the need for cell lines that were established by tumor formation.

Up until this point, the exact tissue of origin of ES cells had not been established, and would not be established for ten more years. The efficiency of ES cell isolation was also typically quite low, with 30% efficiency considered to be in the high range. Also, the efficiency of mES cell isolation varied widely by strain, with more inbred strains such as 129 mice being much more permissive. By microdissecting embryos into their component tissues, trophectoderm, epiblast, and primitive endoderm, Gardner and Brook (1998) were able to determine that the viable ES cell colonies could only be obtained from the epiblast portion of the ICM, thus establishing their origin (see Figure 1). Separation of the epiblast from the other embryonic tissues also resulted in increased efficiency of ES cell isolation. This efficiency was also improved by the use of primary embryonic fibroblast cells as feeder layers instead of STO fibroblasts, as had been customary until that time. This also resulted in successful isolation of ES cells from
strains that had never been successful prior to that point. All strains tested were able to produce viable, germ line competent chimeras (Gardner and Brook, 1998).

Figure 1: Blastocyst Stage Embryo. Note the trophoblast cells, which make up the trophectoderm, around the outside, and the ICM at the top.

All of the above studies were done in the mouse model, and ES cells had not yet been successfully isolated in any primate species. Primate ES cell lines, both human and non-human, would be valuable models of human development and differentiation since there are a number of differences between mouse and human development. For example, early embryonic structures such as the placenta, extraembryonic membranes and the egg cylinder all differ substantially between mice and human embryos. The first primate ES cell lines were isolated from the Rhesus Monkey in 1995 and demonstrated a number of characteristics consistent with those of human EC cells, but differing from those of mES and EC cells. The morphology of these cells more closely resembled that of human EC cells than that of mES cells. They also expressed cell surface antigens characteristic of human EC cells, namely SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. In contrast, mES cells express SSEA-1, but not SSEA-3 or SSEA-4. These cells also failed to
maintain pluripotency in the absence of a feeder layer when cultured in medium containing LIF, in contrast to mES cells (Byrne et al., 2007; Thomson et al., 1995).

Three years after the successful isolation of non-human primate ES cells, the same laboratory successfully isolated lines of human ES (hES) cells with characteristics similar to those of the non-human primate lines. Morphology was similar between the two species and they also expressed the same cell surface antigens. LIF also failed to maintain pluripotency of hES cell lines when cultured in the absence of a feeder layer (Thomson, 1998).

The differential role of LIF in maintenance of pluripotency in hES and mES cells was confirmed in 2004 by Daheron et al. In mES cells, LIF acts by stimulating phosphorylation of signal transducer and activator of transcription 3 (STAT3), which causes STAT3 dimerization and nuclear translocation. However, it was observed that LIF failed to maintain hES cell self-renewal in the absence of a feeder layer. Even though expression of both components of the LIF receptor (LIFR), gp-130 and LIFRβ, was confirmed in hES cells, and human LIF does result in phosphorylation of STAT3, hES cell pluripotency was not maintained by LIF. Interestingly, further experimentation revealed that, when cultured under conditions that maintain pluripotency, phosphorylated STAT3 is found in the nucleus of mES cells, but not hES cells, indicating that this pathway is not involved in maintenance of hES cells. Retroviral transduction of a constitutively active stat3 construct into hES cells failed to yield a stable cell line and indicated that the presence of LIF in hES cells may actually increase differentiation and/or apoptosis (Daheron et al., 2004). Observed differences between hES and mES cells may be explained by the differences in embryonic development between the two species. During embryonic development the ICM gives rise to epiblast and hypoblast structures, and the epiblast develops into the embryo proper. Stem cells may be isolated from the epiblast and are referred
to as EpiSC’s. These cells are slightly more specialized than truly pluripotent ES cells. During rodent blastocyst formation an egg cylinder is formed, a structure not seen in other mammalian embryos. It is thought that its absence in the human developmental process allows hES cells to progress to an EpiSC state during in vitro culture. The presence of the egg cylinder in rodent development may provide a barrier of sorts that extends the window for isolating rodent ES cells in a truly pluripotent state. It is possible that this difference can account for the variations seen between hES and mES cells (Nichols and Smith, 2009).

The similarities between human and non-human primate ES cells make the non-primate lines a valuable model for studies of human embryonic development. hES cells are also a valuable model of cellular differentiation and disease studies. This is significant since the differences between mES and primate ES cell morphology and embryonic development discussed above limit the applicability of the murine model to human studies.

Isolation of ES cells in primate and murine species has already been discussed. Along with the mouse model, true ES cells have also been isolated in the rat. Like mES cells, rat ES cells express the cell surface antigen SSEA-1. Maintenance of self-renewal is also aided by the presence of LIF during culture. While rat ES cells may be cultured on laminin coated culture dishes in the absence of feeder layers, this results in a morphological change from round colony morphology to a flattened morphology. Interestingly, the round morphology may be rescued upon return to a feeder layer. Lastly, rat ES cells must be cultured in the presence of CHIR99021, a Glycogen Synthase Kinase 3 (GSK3) inhibitor, and PD0325901, a Mitogen Activated Protein Kinase/Extracellular Signal Related Kinase (MAPK/ERK) inhibitor. Culture in the presence of these two inhibitors is termed “2i” conditions, and the absence of these two factors results in
a decrease in Octamer-binding transcription factor 4 (Oct4) and Nanog expression and rapid differentiation (Buehr et al., 2008; Li et al., 2008a; Liao et al., 2009).

ES Cells in Livestock Species

Domestic livestock species are valuable large animal models for studies of development as well as stem cell therapies. However, attempts at isolation of ES cells from these species has met with varying degrees of success, with germ line transmission and teratoma formation being the main obstacles (Nowak-Imialek et al., 2011). Species specific details will be given hereafter in the sections to follow.

Horse

The earliest attempt to isolate ES cells in the horse occurred in 2002. These cells were isolated from in vivo produced blastocysts and exhibited essential features of ES cells such as Oct4, AP, and STAT3 expression, and the presence of the SSEA-1 cell surface marker. They also demonstrated acceptable self-renewal capacity and were able to be passaged in culture for up to 56 passages. However, these cells were not able to contribute to chimera formation, and consequently were not capable of germ-line transmission (Saito et al., 2002). Other studies obtained similar results. ES-like cells that were obtained immunosurgically from in vivo derived blastocysts again showed positive expression of AP, Oct4 and SSEA-1 in addition to TRA-1-60 and TRA-1-81. These cells were able to differentiate in vitro into tissues from all 3 embryonic germ layers, but unfortunately were not able to form teratomas (Li et al., 2006).

Neither In Vitro Fertilization (IVF-) produced nor cloned embryos have been used to establish ES-like cells in the horse due to a lack of efficient protocols in the mare, limiting the availability of equine embryos for ES cell studies (Nowak-Imialek et al., 2011).
Goat

The success of isolation of caprine ES cells has been hindered greatly by the limited availability of slaughterhouse goat material (Nowak-Imialek et al., 2011). The studies that have been done demonstrate that it is possible to produce caprine ES-like cells from in vitro produced blastocysts, but these cells lose ES cell morphology after only a few passages (Pawar et al., 2009).

Sheep

The earliest attempts at isolation of ES cells from sheep occurred in 1987. Unfortunately, these cells seem to have a much reduced proliferation rate compared to ES cells from other species. Attempts at isolation from blastocysts result in the ICM being overgrown by endoderm-like cells, making ES cell isolation difficult (Handyside et al., 1987). Morphology of ovine ES-like cells is also different from that of other species, with the cells appearing to grow in monolayers as cystic structures or epithelial-like cells (Dattena et al., 2006; Piedrahita et al., 1990). Ovine ES-like cells have been successfully used in nuclear transfer procedures and are able to produce viable offspring (Wells et al., 1997), but this is not a requirement for ES cells.

Pig

Due to the similarity in size and physiology to humans many attempts have been made at isolation of ES cells from the porcine model (Evans et al., 1990). ES-like cells generally exhibited either epithelial, fibroblast or trophoblast-like characteristics. In the earliest attempts, cells would lose stability after approximately 10 passages, after which proliferation would cease (Piedrahita et al., 1990). Pluripotent ES-like cells have also been isolated from in vitro cultured blastocysts. These cells were able to be cultured successfully for over 30 passages (Miyoshi et
al., 2000). Most recently, either in vitro or in vivo produced blastocysts were used to isolate ES-like cells that showed expression of Oct4, Nanog, and SSEA-1. These colonies were even able to produce chimeric pigs, but were not capable of germ line transmission (Vassiliev et al., 2010a, 2010b).

Attempts have also been made to isolate porcine ES cells from parthenogenic blastocysts, with cell lines being maintained in culture for over 2 years without exhibiting signs of differentiation. Parthenogenic embryos also displayed the capability of producing many more ES-like colonies than their in vitro counterparts. This is thought to be due to their increased adherence to feeder cells (Brevini et al., 2005, 2007, 2010).

Interestingly, the LIF/Janus Kinase (JAK)/STAT pathway does not seem to play a prominent role in maintenance of porcine ES cells as it does in the murine model. Rather, it has been shown that Fibroblast Growth Factor (FGF) and Activin/Nodal signaling are more important in this regard (Alberio et al., 2010). ES cell culture conditions that have been established for mES and hES cells are not applicable to porcine ES cells, as these conditions fail to enhance ES cell maintenance and proliferation.

Unfortunately, successful isolation of porcine ES cells has not yet been established, the major obstacle being production of germ-line competence as well as self-renewal capacity. Porcine ES-like cells have been produced that are able to form teratomas when injected into immunodeficient mice and produce chimeras, but these chimeras are not germ-line competent (Telugu et al., 2010).

**Cattle**

The earliest attempt at isolation of bovine ES cell lines occurred in 1992, in which Saito et al used a protocol slightly modified from those used by Evans and Kaufman, and Martin in
1981. Whereas Evans and Kaufman plated whole blastocysts (Evans and Kaufman, 1981) and Martin used immunosurgical methods (Martin, 1981), Saito et al plated bisected, zona-pelucida free embryos onto mouse embryonic fibroblast (MEF) feeder layers. The resulting ES-like colonies were comparatively slow growing, as passaging took place only every 2 weeks. While spontaneous differentiation was rarely observed, the cell colonies were lost after passage 4. However, the cell lines contained normal karyotypes and were able to produce nuclear transfer derived embryos, some of which were able to reach the 8 to 16 cell stage in vitro. Pluripotency and differentiation studies such as teratoma and embryoid body formation, and chimera production were not conducted on these cell lines (Saito et al., 1992).

In 1996, bovine ES-like cells were tested for their utility in nuclear transfer procedures. Embryonic cell lines were established from both in vitro and in vivo produced blastocysts as well as morula stage embryos. In order to be picked from primary culture, colonies had to have a small cytoplasmic/nuclear volume ratio, nuclei with multiple nucleoli, and cytoplasmic vesicles. When these criteria were applied cell lines were obtained that could be cultured for over 50 passages, and formed embryoid bodies, but these embryoid bodies contained only muscle tissue and red blood cells. When these cell lines were used to produce nuclear transfer embryos, fetal development would progress to early organogenesis, but pregnancies would abort shortly thereafter. Fetal examination would reveal abnormal or absent placentation in all cases. Development to term could only be accomplished by combining eight cell stage nuclear transfer embryos with two blastomeres from an in vitro produced embryo of the same stage. Such animals were termed “aggregate chimaeras.” Unfortunately, embryonic cell line contribution was only demonstrated in placenta, skin, muscle, and heart, with no contribution to blood or other tissue types. Germ-line contribution was also not demonstrated (Stice et al., 1996).
together, these data reveal that the isolated embryonic cell lines from this study were not truly pluripotent ES cells.

Mitalipova et al. (2001) were able to establish bovine ES-like cell lines through the use of 8 to 16 cell stage precompacting embryos. Initial colonies were picked using the same criteria as Stice et al. (1996) above. These cell lines were sensitive to enzymatic treatment, making necessary the use of mechanical dissociation methods during passaging. However, when these methods were used, the cell lines were able to be maintained in culture for over three years, during which time some spontaneous differentiation into endoderm-like cells was observed. The cells stained positive for SSEA-1, SSEA-3, and SSEA-4 cell surface antigens as well as the c-kit cell surface receptor, and embryoid bodies exhibited tissue types from all three germ layers. However, these cell lines would not form teratomas, and chimera production was not reported, calling into question the in vivo differentiating capacity of these cell lines (Mitalipova et al., 2001).

The first demonstration of in vivo differentiation capacity in bovine ES-like cells was shown by Lim et al. (2011). Prior to this neither teratoma formation nor true chimera production had been observed in the bovine model. By treatment of the cells with a compound known as 5-azacytidine, ES-like cells were established that were able to form embryoid bodies in vitro and teratomas in vivo when injected into immunodeficient mice. 5-azacytidine is a DNA methyltransferase inhibitor, which acts by inhibiting DNA methylation, a marker of gene silencing. Cells isolated using this treatment expressed the cell surface antigens SSEA-1 and SSEA-4. However, chimera production was not reported with these cell lines, and the self-renewal capacity of these cells was not tested, as only early passage cells were used for differentiation studies (Lim et al., 2011).
One major problem with the bovine ES-like cell lines isolated to date is the inconsistency in the observed pluripotency markers between cell lines from different laboratories (Cao et al., 2009; Muñoz et al., 2008). For example, Mitalipova et al. (2001) reported detection of the cell surface antigens SSEA-1, SSEA-3 and SSEA-4, and the cell surface marker c-kit, while Saito (2003) reported positive expression of only SSEA-1 along with alkaline phosphatase. Wang et al. (2005) reported positive expression of only SSEA-4 along with alkaline phosphatase, while Lim et al. (2011) reported positive expression of SSEA-1 and SSEA-4, but not SSEA-3. Also, there are discrepancies in the distribution of Oct4 expression in bovine embryonic cells, as Oct4 has been reported to be isolated to the epiblast (Vejlsted et al., 2005), but has also been reported to be expressed in both the ICM and trophectoderm (Kirchhof et al., 2000). Such inconsistencies indicate a lack of standardized culture and isolation methods for bovine ES cells, and these inconsistencies need to be resolved before isolation of pluripotent bovine ES cells can be considered successful (Muñoz et al., 2008).

**The Reprogramming Transcription Factors**

To date, various laboratories have used different methods to produce induced pluripotent stem (iPS) cells. Before these methods are discussed, it is first necessary to discuss the reprogramming transcription factors used in these methods, and why they are necessary for stem cell maintenance. The following is a discussion of the roles of these transcription factors in ES cell maintenance and iPSC production.

Production of iPS cells was first accomplished in the mouse model in 2006 using the transcription factors Oct4, Sox2, Krueppel-like factor 4 (Klf4), and c-Myc (Takahashi and Yamanaka, 2006). One year later, the same laboratory used the same set of factors to reprogram human cells. However, Yu et al. (2007) used a slightly different set of factors, namely
Oct4, Sox2, Nanog, and Lin-28. By comparing the two sets of factors, it is suggested that Oct4 and Sox2 are indispensable for stem cell maintenance, while the other four factors are interchangeable or are alternative supporting factors. The factors considered here also have a high level of CpG islands compared to other genes, suggesting a higher level of epigenetic regulation in the form of methylation (Guo et al., 2009).

Oct4 & Sox2

Oct4 is a member of the POU family of transcription factors, and is also known as POU5f1 or Oct3/4. It is considered to be indispensable in iPS cell production at this point, as true iPS cells have not been produced successfully without this factor in some form. Sox2 is a member of the Sex determining region Y (SRY)-related High Mobility Group (HMG) box family of transcription factors and always acts in conjunction with Oct4 during regulation of gene expression in pluripotent cells (Guo et al., 2009). An Oct4/Sox2 heterodimer binds to and controls expression of Nanog, as well as FGF4, UTF1, Rex1 and Fbx15 (Ben-Shushan et al., 1998; Nishimoto et al., 1999; Tokuzawa et al., 2003; Yuan et al., 1995). The Oct4/Sox2 heterodimer is also self-regulating in a feed forward style loop (Okumura-Nakanishi et al., 2005).

The Wnt signaling pathway (see Figure 2) has also been shown to play a role in stem cell maintenance via expression of Oct4, Nanog, and Rex1. In the absence of Wnt binding to its receptor, the GSK3-β complex causes degradation of β-Catenin. However, upon Wnt binding to its receptor, the GSK3-β complex is disassembled, allowing β-Catenin to accumulate in the cytoplasm. Free β-Catenin then enters the nucleus and affects expression of genes including Oct4, Nanog and Rex1. 6-Bromoindirubin-3-Oxime (BIO) has been shown to be an inhibitor of GSK3-β and is sufficient to maintain expression of Oct4 in vitro (Meijer et al., 2003; Polychronopoulos et al., 2004; Sato et al., 2004).
Oct4 expression levels must be strictly maintained in specific cell types during embryonic development. Interestingly, a less than 2-fold increase in Oct4 levels in ES cells leads to cellular differentiation into ectoderm and mesoderm, while a reduction in levels of Oct4 leads to de-differentiation into trophectoderm (Niwa et al., 2000). Similarly, an increase in Sox2 levels causes down-regulation of several important genes, including Nanog (Kopp et al., 2008).

**Nanog**

Nanog is a 305-amino acid protein expressed in both embryonic and extra-embryonic tissues and is important for ES cell maintenance (Guo et al., 2009; Mitsui et al., 2003). As mentioned before, its expression is regulated by the Oct4-Sox2 heterodimer, which binds to Nanog in the promoter region. This is presumably the reason that Nanog is not considered one of the essential factors during iPS cell Reprogramming, as Oct4 and Sox2 would be able to induce endogenous Nanog expression. Nanog expression is also regulated by Phosphoinositide 3-kinase (PI3K), as inhibition of PI3K results in down-regulation of Nanog expression (Storm et
Lastly, as with Oct4, the Wnt pathway has also been shown to be able to sustain Nanog expression levels in the cell (Sato et al., 2004).

**Klf4**

Klf4 is a factor that plays a role in regulation of cell growth and differentiation (Shields et al., 1996), and is associated both with tumor suppression and oncogenesis. It has been shown that Klf4 levels rise as a result of cellular stress, such as DNA damage, and cause inhibition of cellular proliferation. In addition, Klf4 expression seems to be lost in various types of human cancer. Interestingly, Klf4 has also been shown to be overexpressed in certain types of cancer and is able to suppress p53 mediated apoptosis. For example, when Klf4 is depleted from breast cancer cells, p53 dependent apoptosis resumes. Thus, the effects of Klf4 on cell proliferation seem to be context dependent, causing cell cycle arrest in some situations, while inducing oncogenesis in others (Rowland et al., 2005).

**c-Myc**

The c-Myc gene, in its native form, is involved in a number of cellular processes, including cell growth, differentiation, proliferation, apoptosis and stem cell self-renewal. It is also yet another target of the Wnt signaling pathway, as the β-Catenin/TCF transcription complex binds directly to the c-Myc promoter and drives its transcription. c-Myc has been shown to be a crucial factor in embryonic development in mice, with targeted deletion of the gene resulting in embryonic lethality. However, mutations in the c-Myc gene have been shown to be a contributing factor in a number of different cancers, including lung, breast and colon carcinomas (Dang, 1999). Due to its oncogenicity, it is a controversial factor in iPS cell
production, but reprogramming efficiencies are significantly greater with this factor than without it (Guo et al., 2009; Nakagawa et al., 2008).

**Lin28**

Lin28 is involved in maintenance of pluripotency through the blockade of microRNA (miRNA) processing. More specifically, Lin28 blocks production of Let-7, a miRNA that plays a role in cellular differentiation (Viswanathan et al., 2008). Thus, inhibition of Let-7 also helps to inhibit cellular differentiation. However, Let-7 is also involved in inhibition of c-Myc expression, an activity that helps decrease tumorgenicity of cells (Sampson et al., 2007). It is through this activity that Lin28 has been shown to be indirectly involved in oncogenicity. Since Lin28 suppresses the activity of Let-7, c-Myc expression increases, which leads to increased tumorgenicity.

**Induced Pluripotent Stem Cells**

Embryonic Stem cells have the potential to become any cell type in the body. This potential gives them extreme promise in a number of useful applications, such as therapeutics, especially in the area of treatment of degenerative diseases. However, there are ethical concerns associated with their use. The successful isolation of ES cells requires the destruction of a living embryo, which makes their utility prohibitive, especially in the human model. Also, because ES cells must originate from an embryo, there are concerns of immune rejection by the recipient, largely due to genetic differences between the donor cell line and the recipient's native cells. These issues may potentially be overcome with the successful production of iPS cells, which are somatic cells that have been genetically reprogrammed back to a pluripotent state. Such cells could potentially be produced from any tissue type and could originate from
the recipient’s own cells, thus overcoming problems of immune rejection. The first generation of iPS cells occurred in 2006 in the mouse model using retroviruses to deliver exogenous genetic factors to murine embryonic fibroblasts (Takahashi and Yamanaka, 2006; Wernig et al., 2007). This method and many others have been used since then to produce iPS cells in a number of different species (Cao et al., 2012; Carey et al., 2009; Esteban et al., 2009; Han et al., 2011; Hotta et al., 2009; Nagy et al., 2011).

As previously mentioned, the pioneering research in the field of iPS cell production occurred in 2006. This work utilized retroviruses as a delivery method for specific exogenous stem cell factors to MEF cells. Initially, 24 genes were selected as candidate factors for induction of pluripotency. Expression of the gene Fbx15 was used as a marker for successful reprogramming. Fbx15 contains an enhancer region upstream of the transcription start site that contains an Oct4 and Sox2 binding site (Koestenbauer et al., 2006). Expression of Fbx15 therefore indirectly indicates expression of Oct4 and Sox2. Using this selection method the list of necessary reprogramming factors was reduced to only four, namely Oct4, Sox2, Klf4, and c-Myc. The iPS cells produced using these methods were able to form embryoid bodies in vitro and teratomas in vivo, but were not able to produce chimeric mice (Takahashi and Yamanaka, 2006). Germ-line competent chimeric mice were finally produced the next year by the same laboratory. It was discovered that by using Nanog as the selection marker in place of Fbx15, not only could germ-line competent chimeras be produced, but the resulting iPS cells were more similar to ES cells in methylation patterns and showed more consistent gene expression of most ES cell marker genes. However, it was also discovered that among the chimeric mice produced in this experiment, approximately 20% of them developed tumors, presumably caused by reactivation of the c-Myc transgene (Okita et al., 2007), a hypothesis that was confirmed by
removal of c-Myc from the reprogramming factors. Chimeric mice produced in this manner did not develop tumors during the experimental period. However, it should be noted that the reprogramming efficiency using this method was significantly lower without c-Myc than with it (Nakagawa et al., 2008).

The successful production of iPS cells in the human model was accomplished by two different laboratories using slightly different methods. Takahashi et al. (2007) were able to accomplish this feat through use of the same four factors as before, namely Oct4, Sox2, Klf4, and c-Myc. The resulting cells were similar to hES cells in gene expression, morphology, cell surface antigens, and proliferation and were able to differentiate into tissues of all 3 germ layers in vivo (teratoma formation) and in vitro (embryoid bodies; Takahashi et al., 2007). Yu et al., on the other hand, produced human iPS cells using a slightly different set of factors, Oct4, Sox2, Nanog, and Lin28, and also used lentiviruses instead of retroviruses as the delivery method. These cells also displayed similarities to hES cells in cell surface marker and gene expression and were able to differentiate into derivatives of all three primary germ layers (Yu et al., 2007).

Comparing the two sets of factors, it is again suggested that Oct4 and Sox2 expression is essential for successful reprogramming of somatic cells, while Klf4, c-Myc, Nanog, and Lin28 are interchangeable, and can be considered supporting factors in the reprogramming process (Guo et al., 2009).

The aforementioned studies in iPS cell production use viruses, either retroviruses or lentiviruses, to introduce exogenous genetic vectors into the target cell. These types of viruses cause a number of obstacles to the ultimate goal of the use of iPS cells for therapeutic purposes. First, reprogramming efficiency using this method is typically quite low, usually approximately 0.01 to 0.1% (Takahashi and Yamanaka, 2006). Also, the use of these viruses results in
integration of exogenous genetic material into the host genome. The integration of exogenous c-Myc into the genome, for example, could potentially result in cancer development in recipients of these cells. Ectopic expression of either Oct4 or Klf4 can also cause dysplasia (Kaji et al., 2009). If iPS cells are to be used in human therapeutics, alternative forms of reprogramming must be developed in order to avoid these issues. Some progress has been made toward this goal, as discussed hereafter, but much work still remains.

Attempts at resolving the problem of genomic integration of exogenous genetic material involved the use of adenoviruses or Sendai viruses (Ban et al., 2011; Stadtfeld et al., 2008) instead of lentiviruses or retroviruses as the gene delivery method, as adenoviruses do not integrate their genetic material into the host genome. Initial attempts with this method involved viral transduction of murine tail tip fibroblasts with Oct4, Sox2, Klf4, and c-Myc. However, these initial attempts failed completely. Reprogramming was successfully achieved through the use of fetal liver cells containing an inducible Oct4 locus with a multiplicity of infection of 20 to 50. Subsequently, reprogramming was also successfully achieved using tail tip fibroblasts with the same Oct4 inducible allele. However, multiplicities of infection of 50 to 250 were required for this cell type. Successful reprogramming was finally achieved without the Oct4 inducible allele through the use of adult hepatocytes, requiring a multiplicity of infection of only 1 to 4 (Stadtfeld et al., 2008).

While the adenoviral method was a step forward for iPS cell production, it still carries with it a number of disadvantages. First, successful reprogramming was only seen in hepatocytes, with other cell types requiring an Oct4 inducible allele. Such transgenic cells cannot be used for therapeutics. Second, the efficiency of reprogramming was extremely low at approximately 0.0001% to 0.001% indicating that genomic integration during the
reprogramming process greatly enhances efficiency. Lastly, the authors report that 3 out of 13 iPS cell lines obtained were tetraploid, a phenomenon that is generally not seen through use of retro- or lentiviral vectors (Stadtfeld et al., 2008). This indicates that while production of iPS cells without genomic integration is possible, the use of adenovirus is not an ideal method.

Another study sought to resolve the issue of genomic integration through elimination of the use of viruses altogether during the reprogramming process. Plasmids were used independently of viruses to transfect murine fibroblast cells. Two separate plasmids were used in these experiments. One plasmid contained constructs for Oct4, Klf4, and Sox2, in that order, and was transfected into cells on days 1 and 3 of the experiment. Another plasmid containing only c-Myc was transfected on days 2 and 4. While iPS cells were obtained using this protocol, plasmid incorporation into the host genome was detected. Interestingly, plasmid incorporation could largely, though not completely, be avoided if both plasmids were transfected together on days 1, 3, 5, and 7. Using this protocol iPS cells were obtained without evidence of genomic integration in 6 out of 10 experiments (Okita et al., 2008). This study demonstrates that viruses are dispensable in the production of iPS cells, which is a necessary step forward. Interestingly, it also demonstrates the importance of timing in introducing exogenous factors during iPS cell production if genomic integration is to be avoided. However, it should be noted that the reprogramming efficiency of this method was much lower than that of viral reprogramming, presumably due to the eventual loss of the plasmids during cell division. Also, genomic integration was not completely avoided in all cases, indicating a necessity to screen the cells for plasmid integration before their use in subsequent experiments (Chou et al., 2011; Okita et al., 2008).
One way to avoid the side effects of viral vectors in iPS production would be to avoid the use of exogenous genes altogether. This has been done through the use of recombinant proteins for Oct4, Sox2, Klf4, and c-Myc instead of genetic vectors. This prevents genomic insertion and avoids reliance on the necessary transcription of delivered genes. The proteins were produced in *E. coli* and a poly-arginine (11R) protein transduction domain was added to the C-terminus of each protein to allow penetration of the cell membrane (Nagahara et al., 1998). MEF cells were treated with the recombinant proteins and Valproic Acid (VPA) overnight in 4 cycles and colonies appeared around days 30-35. However, the efficiency of this method was decidedly lower than with previous methods at approximately 0.00006% or 3 colonies per 5 × 10⁴ cells. The elimination of c-Myc protein, although desirable, lowers this efficiency by approximately 3-fold (Zhou et al., 2009). This lower efficiency makes this method impractical for therapeutic purposes unless efficiency can be improved.

miRNA has been used both to increase the efficiency of viral reprogramming methods, as well as to replace the reprogramming factors typically used during reprogramming (Oct4, Sox2, Klf4, and c-Myc; Pfaff et al., 2011). The miR-290 cluster of miRNA typically constitutes approximately 70% of the miRNA population in ES cells and was consequently used to enhance reprogramming of iPS cells. More specifically, the study’s authors used miR-291-3p, miR-294 and miR-295. These miRNA’s were transfected individually into Oct4-GFP (green fluorescent protein) MEF cells on days 0 and 6 post Oct4, Sox2, Klf4 (OSK) infection. Each was able to enhance reprogramming efficiency, but miR-294 had the greatest effect, enhancing reprogramming efficiency from 0.01-0.05% to 0.1-0.3%. miRNAs with similar seed sequences also were able to enhance reprogramming efficiency. It was also found that these miRNAs did not enhance reprogramming efficiency when transfected together, but that miR-294 was able to
enhance efficiencies in a dose dependent manner, up to a maximum of approximately 0.4-0.7% of starting MEFs. miR-294 was also able to substitute for c-Myc during the reprogramming process, producing a much more uniform population of GFP positive colonies than with c-Myc, which would typically produce colonies, 80% of which were GFP negative (Judson et al., 2009).

miRNAs have also been used to completely replace Oct4, Sox2, Klf4, c-Myc (OSKM) during reprogramming, albeit still using lentiviral vectors. The miR302/367 cluster of miRNAs has been shown to be a direct target of Oct4 and Sox2. When the 5 miRNAs in this cluster were combined into a single lentiviral vector they were able to reprogram MEFs into iPS cells 2 to 4 days faster than with traditional OSKM vectors, but only when used in conjunction with VPA. Reprogramming efficiency is also reported to be almost 2-fold more efficient using the miRNA vectors, with approximately 79% of colonies expressing GFP vs. approximately 50% of colonies expressing GFP using OSKM vectors (Anokye-Danso et al., 2011).

**Cellular Reprogramming with Small Molecules**

The movement to improve reprogramming efficiency and eliminate the use of viruses and exogenous vectors in the reprogramming process has led to much research on the use of small molecule compounds that target cellular pathways related to pluripotency (Feng et al., 2009). In order to understand how these small molecules work it is first necessary to review the specific pathways targeted by them. Specific small molecules and their effects on reprogramming will then be discussed.

**Histone Deacetylase inhibition**

Histone deacetylation has been hypothesized to be one reason why both animal cloning and iPS production procedures are so inefficient. Evidence has accumulated that indicates that
the reprogramming process is hindered by faulty epigenetic modifications during the reprogramming process, leading to low survival rates among clones (Zhao et al., 2010) and inefficiency of iPS reprogramming. Acetylation of histones is equated with a more permissive state of gene transcription in the cell, and consequently, histone deacetylation is an undesirable event in nuclear reprogramming assays.

Small molecules, such as Trichostatin A (TSA) and VPA have been used to prevent histone deacetylation during reprogramming and have improved efficiencies somewhat. Specifically, TSA has been used to improve cloning efficiency by as much as 75% in various species (Kishigami et al., 2006; Zhao et al., 2010) and to increase cell numbers and expression of Sox2 and c-Myc in cloned mouse blastocysts (Li et al., 2008b). VPA has been used extensively in iPS studies to improve reprogramming efficiency and has been shown to have a dramatic effect on reprogramming efficiencies in 4-factor reprogrammed cells (Oct4, Sox2, Klf4 and c-Myc), increasing colony numbers as much as 40 fold after only 8 days of treatment compared to control 4-factor treated cells (Huangfu et al., 2008a). Efficiencies have improved as much as 1000 fold in 3-factor reprogrammed cells (Oct4, Sox2, Klf4). When treated with VPA in conjunction with only Oct4 and Sox2 reprogramming efficiencies reach approximately 0.0001%, equal to that of 3-factor reprogramming in the absence of VPA (Huangfu et al., 2008b). When used in conjunction with other small molecules such as CHIR99021 and 616452 (RepSox), both discussed in detail later, successful reprogramming has been achieved using only Oct4 with an efficiency of 1 iPSC-like colony per $2 \times 10^5$ cells 30 days after infection (Li et al., 2010).

**Methyltransferase Inhibition**

Another epigenetic modification that affects reprogramming efficiency is DNA methylation, a marker associated with gene silencing. It has been shown that different cell
types have different methylation patterns. These cell types may be distinguished based on these patterns and even iPSC cells can be distinguished from ES cells based on DNA methylation alone (Doi et al., 2009). Inhibition of DNA methylation has been demonstrated, in various cases, to improve reprogramming efficiencies of iPSC cells (Huangfu et al., 2008b; Park et al., 2011; Shi et al., 2008a, 2008b) as well as cloning efficiencies and embryonic developmental potential in both mice and cattle (Ding et al., 2008; Lim et al., 2011). The two main small molecules used to inhibit DNA methylation are 5-Azacytidine and 5-Aza-2'-deoxycytidine, which differ from each other in structure only by the presence or absence of a hydroxyl group on the 2' carbon of the ribose sugar (see Figure 3). 5-Azacytidine was synthesized in 1964 for use as an antimetabolite for the treatment of acute myelogenous leukemia and inhibits methylation of both DNA and RNA by incorporating itself into the nucleotide strands themselves. It is also able to inhibit protein synthesis through incorporation into transfer RNA (tRNA). 5-Aza-2'-deoxycytidine, on the other hand, is only able to inhibit methylation of DNA, but is approximately 10 times more cytotoxic than 5-Azacytidine. Inhibition of methylation occurs passively by causing covalent linkage of DNA methyltransferase 1 (Dnmt1) during the maintenance methylation step of DNA replication (Christman, 2002).

![Figure 3: Methyltransferase Inhibitors. 5-Azacytidine (A) and 5-Aza-2'-deoxycytidine (B), showing a presence and absence, respectively, of a 2' hydroxyl group on the ribose sugar.](image-url)
5-Azacytidine has recently been shown to be able to improve isolation efficiency of both bovine ES-like and mES cells. Specifically, when treated with 5 μM 5-Azacytidine, the success rate of isolation of ES-like cells from pressed bovine blastocysts more than doubled that of controls and demonstrated the ability to differentiate into derivatives of all three embryonic germ layers both in vitro and in vivo. Unfortunately, chimera competence was not demonstrated due to logistical difficulties in chimera production in cattle, leaving the true potential of these cell lines in question (Lim et al., 2011). 5-Azacytidine has also been used as part of a chemical cocktail with 15 other small molecule compounds in the reprogramming of murine fibroblasts to an intermediate state of differentiation. While reprogramming was not complete, it nonetheless demonstrates the potential utility of this method in cellular reprogramming (Park et al., 2011).

5-Aza-2’-deoxycytidine has been shown to improve the cloning efficiency of bovine embryos. Treatment of oocytes both before and after nuclear transfer resulted in significant improvement in development over controls, and blastocyst stage embryos were shown to have normal DNA methylation patterns, indicating the potential for normal development (Ding et al., 2008).

A slightly lesser known compound, BIX-01294 (BIX), is a G9a histone methyltransferase inhibitor that has also been useful in improving reprogramming efficiencies. Specifically, it has been shown to be capable of replacing Sox2 and c-Myc during reprogramming of neural progenitor cells, improving efficiency approximately 10 fold over controls and approximately 50% over typical 4-factor reprogrammed cells. BIX was also able to reprogram fetal neural progenitor cells in combination with Sox2, Klf4, and c-Myc, demonstrating the ability to replace Oct4 during reprogramming (Shi et al., 2008b). BIX has also been shown to be capable of
reprogramming MEF cells in combination with Oct4 and Klf4, as well as the small molecule BayK8644, an L-Calcium channel agonist, with approximately half the efficiency of typical 4-factor reprogramming (Shi et al., 2008a).

Chaetocin is another histone methyltransferase inhibitor that has been implicated to help with cellular reprogramming. It is a fungal mycotoxin that preferentially targets and inhibits the histone methyltransferase SU(VAR)3-9, which specifically di-methylates Lysine 9 of histone 3 to induce a state of condensed heterochromatin (Greiner et al., 2005; Schotta et al., 2003). It has also been demonstrated to upregulate Sox2 when used as part of a small molecule cocktail in the absence of exogenous transgenes (Park et al., 2011).

The MEK/ERK Pathway

It has been previously mentioned that there are cellular pathways with conflicting roles between hES and mES cells, such as Bone Morphogenetic Protein (BMP) signaling (Chen et al., 2011a), the effects of LIF (Daheron et al., 2004), and Transforming Growth Factor Beta (TGF-β) signaling (Ichida et al., 2009; James et al., 2005). Another such pathway with seemingly conflicting roles between these two species is the MEK/ERK pathway (Burdon et al., 1999; Li et al., 2007). In mES cells, this pathway has a negative effect on pluripotency. This has been demonstrated through mutation of the gp130 receptor, which plays a role along with LIF receptors in stem cell maintenance through LIF and subsequent activation of the JAK/STAT pathway. It also activates MEK/ERK signaling upon binding the ligand Sarcoma (Src) Homology Phosphatase 2 (SHP-2). Ligand binding causes phosphorylation of Tyrosine 118 on gp130. When this residue is mutated, there is no effect on pluripotency of mES cells, indicating that SHP-2 and MEK/ERK signaling are not required for murine stem cell maintenance. This was confirmed through introduction of a mutant inactive form of SHP-2, which resulted in an increase in stem
cell self-renewal. Indeed, phosphorylated gp130 also seems to attenuate STAT3 signaling, reducing the effects of LIF in murine stem cell maintenance (Burdon et al., 1999).

As mentioned previously, the maintenance of rat ES cells is accomplished only through inhibition of the MEK/ERK pathway using the small molecule PD0325901 (Bain et al., 2007), along with GSK3 inhibition. In the absence of these molecules, rat ES cells lose Oct4 and Nanog expression almost immediately (Buehr et al., 2008; Li et al., 2008a).

In contrast to rodent species, hES cells are dependent, at least in part, to MEK/ERK signaling for stem cell maintenance. Inhibition of this pathway using either PD98059 or U0126 results in significant down-regulation of the hES cell markers Tra-1-60, Tra-1-81, SSEA-4, Oct4, and Nanog, along with a differentiated cellular morphology. Treated hES cells also display a reduced capability to form embryoid bodies in vitro, instead giving rise to cellular aggregates with an irregular and opaque morphology and a lack of markers of the 3 germ layers. They instead exhibit strong expression of trophectodermal and primitive endodermal markers. In contrast, treatment of mES cells with these same small molecules results in no significant change in Oct4, Nanog, or SSEA-1 expression and no change in morphology (Li et al., 2007).

The above studies illustrate yet another difference between hES and mES cells in the way that pluripotency is maintained. Potential differences such as this will need to be taken into account during iPS studies in livestock species if small molecules are to be used successfully in cellular reprogramming.

**p53 inhibition**

There are many indications that p53 plays a negative role in iPS cell production, as it works to prevent proliferation of cells with DNA damage by inducing apoptosis (Marión et al., 2009). Also, keratinocytes, which typically have a higher reprogramming efficiency than other
cell types, express a significantly lower level of p53 (Kawamura et al., 2009). Indeed, p53 null MEF cells have been shown to exhibit an efficiency approximately 4-fold higher than wild type MEF cells, while human foreskin fibroblasts transfected with a p53 small interfering RNA (siRNA) showed a reprogramming efficiency approximately 10-fold higher than wild type cells. In addition, p53 null iPS cell colonies appeared on average 3 days sooner than wild type cells. Also, as one might expect, p53 null iPS cells displayed an increase in chromosomal aberrations, but were still able to produce germ-line competent chimeras (Marión et al., 2009; Utikal et al., 2009).

Another study also finds an increase in reprogramming efficiency through p53 inhibition, demonstrating increases in efficiency from 0.002% in wild type MEF cells to 0.01% in heterozygous p53 mutants and 0.05% in p53 null mutant cells when reprogrammed with only three factors (no c-Myc). Clonal expansion was also increased in p53 null cells, with approximately 10% of wells successfully expanding vs. almost none in wild type iPS cells. Addition of c-Myc further increased efficiency, with up to 20% of wells successfully expanding. However, this also caused a lack of transgene silencing in the resulting iPS cells. Transfection using non-viral plasmid constructs was also quite successful, with about 100 iPS colonies in p53 null cells vs. no GFP positive control colonies (Hong et al., 2009).

Knockdown of p53 has also been demonstrated to facilitate cellular reprogramming. using only Oct4 and Sox2, with colonies appearing approximately 4 weeks after infection. Reprogramming of human fibroblasts using four factors also increased in speed, with colonies appearing only 2 weeks after infection versus four weeks in wild type cells (Kawamura et al., 2009; Zhao et al., 2008).
Interestingly, p53 has been shown to play a role in ES cell self-renewal. Inhibition of p53 activity in unstressed ES cells using the small molecule Pifithrin-α resulted in a significant decrease in the size and number of ES cell colonies. Rate of DNA synthesis, as indicated through incorporation of Bromodeoxyuridine (BrdU) into DNA, was also decreased significantly. Cyclin D1 expression was also decreased, and cells were arrested in the G1 phase. Pluripotency was not affected by p53 inhibition in unstressed ES cells. Based on this data, the authors propose that, during unstressful conditions, p53 localizes to the cytoplasm and promotes self-renewal, while during times of stress, p53 localizes to the nucleus to downregulate expression of Nanog, causing differentiation (Abdelalim and Tooyama, 2012).

Overall, caution must be exercised if p53 inhibition is to be used in iPS cell production, as p53 seems to provide a quality control step during the reprogramming process. While efficiency is lower when p53 is active, it seems to prevent production of iPS cells with DNA damage (Marión et al., 2009). Consequently, iPS cells produced in such a way may not be suitable for clinical use.

Wnt Signaling

The Wnt signaling pathway has been shown to be involved in direction of cell polarity, proliferation, and fate determination during embryonic development (Logan and Nusse, 2004; MacDonald et al., 2009). In addition, it has been shown to be capable of maintaining pluripotency in both hES and mES cells (Lyashenko et al., 2011; Reya and Clevers, 2005; Sato et al., 2004; Wray et al., 2011). We have previously discussed this pathway briefly, and here we discuss its mechanism of action in more detail (see Figure 2 for illustration).

In the absence of Wnt ligand, cytoplasmic β-Catenin forms a complex with Axin, Adenomatous Polyposis Coli (APC), GSK3 and Casein Kinase 1 (CK1). Axin acts as a scaffolding
protein and coordinates phosphorylation of β-Catenin at Serine 45 via CK1, followed by phosphorylation at threonine 41 and Serines 33 and 37 via GSK3 (Kimelman and Xu, 2006). The latter two phosphorylation events create a binding site for the E3 ubiquitin ligase Beta Transducin Repeat containing Protein (β-Trcp), which causes ubiquitination and subsequent degradation of β-Catenin in the proteosome.

Activation of the Wnt pathway occurs through binding of Wnt ligands to Frizzled (Fzd) receptors and Low density lipoprotein related receptor 5/6 (LRP5/6) co-receptors. There are 19 known Wnt ligands and 10 known Fzd receptors, and the specific effect of the Wnt pathway depends on the ligand-receptor pair (Binnerts et al., 2007; Van Amerongen et al., 2008). Wnt binding leads to phosphorylation of LRP5/6 at a PPPSPxS motif (P=Proline, S=Serine, x=variable). Interestingly, evidence suggests that this phosphorylation event is caused by the coordinated actions of GSK3, which causes phosphorylation of PPPSP (Binnerts et al., 2007; Khan et al., 2007), and CK1, which causes subsequent phosphorylation at xS (Zeng et al., 2005). These phosphorylated sites then act as a docking site for Axin, resulting in disruption of the β-Catenin phosphorylation complex (Davidson et al., 2005; Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005). This results in accumulation of β-Catenin in the cytoplasm and its subsequent translocation to the nucleus, the mechanism for which is poorly understood. β-Catenin then forms a complex with TCF/LEF and affects expression of target genes (MacDonald et al., 2009).

As mentioned previously, the Wnt pathway has been shown to play a role in maintenance of both hES and mES cells. It has also been shown that TCF co-occupies multiple DNA binding sites with Oct4, Sox2 and Nanog (Marson et al., 2008; Yi et al., 2011). Sato et al., found that treatment of ES cells with the small molecule BIO, a GSK3 inhibitor, is able to maintain pluripotency in ES cells in the absence of feeder layers, and sustains expression of
Oct4, Rex-1, and Nanog, and that withdrawal of BIO results in differentiation (Sato et al., 2004).

Another GSK3 inhibitor, CHIR99021, has also been used in various pluripotency studies. In particular, in conjunction with the MEK inhibitor PD0325901, it has been shown to maintain pluripotency and germline competence of rat ES cells, which had been an elusive goal up until 2008 (Li et al., 2008a). CHIR99021 has also been shown to be highly supportive of iPS cell production, increasing the efficiency of reprogramming as much as 30 fold in some experiments (Chen et al., 2011b; Li et al., 2009, 2010; Lyssiotis et al., 2009). Lastly, Lithium, an anti-psychotic drug and GSK3 inhibitor, has also been shown to greatly enhance reprogramming efficiency of MEF cells. Wang et al found that after treatment of MEF cells with Lithium as many as 15% of cells expressed Oct4 2 weeks after viral treatment, and that this effect is partially dependent on inhibition of GSK3. Interestingly, other potent inhibitors of GSK3, such as CHIR99021 and BIO, had marginal effects on reprogramming compared to Lithium, indicating only a partial dependence on GSK3 inhibition for reprogramming efficiency in this case (Wang et al., 2011).

Not all compounds that activate Wnt signaling do so through GSK3 inhibition. Two known compounds, Cytochalasin D and Phorbol 12-myristate 13-acetate (PMA) do so through upregulation of Wnt genes, specifically Wnt5a (Jönsson et al., 1998). The upregulation of Wnt also leads to an increase in cytoplasmic β-Catenin levels (Tobimatsu et al., 2006). Cytochalasin D has also been shown to upregulate expression of Sox2, Klf4 and c-Myc, while PMA upregulates Sox2 expression during partial reprogramming of MEF cells using a small molecule cocktail without use of exogenous transgenes (Park et al., 2011).

**Notch Signaling**

Notch signaling is another example of the apparent differences between hES and mES cells. It has been shown to be necessary for hES cell proliferation (Fox et al., 2008), but has been
shown to play a role in regulating differentiation and determining cell fate of mES cells (Androutsellis-Theotokis et al., 2006; Chiba, 2006).

The Notch pathway itself is a pathway of cellular communication that acts through single pass transmembrane receptors that bind corresponding single pass transmembrane ligands on neighboring cells. There are several Notch receptors that bind to one of five Notch ligands, Delta-like1 (Dll1), Delta-like3 (Dll3), Delta-like4 (Dll4), Jagged1 (Jag1), and Jagged2 (Jag2), although Dll3 may not actually function as a Notch ligand. Upon binding of receptor to ligand, the Notch intracellular domain (NICD) is cleaved, translocates to the nucleus and binds to the CBF1, Suppressor of hairless, Lag-1 (CSL) complex, turning it from a transcriptional repressor to a transcriptional activator (Chiba, 2006).

Notch signaling has been demonstrated to have seemingly differing roles in hES and mES cells. In hES cells, knockdown of Notch receptors results in a corresponding decrease in growth rate and increase in apoptosis, indicating that it plays a role in hES cell self-renewal. Notch activation in hES cells also leads to increased colony formation, but does not affect colony size (Androutsellis-Theotokis et al., 2006; Fox et al., 2008). In contrast, Notch signaling in mES cells plays a role in directing differentiation to specific lineages rather than influencing self-renewal. High Notch expression corresponds to a preference for the neural cell lineage, while low Notch expression corresponds to mesodermal lineages. Thereafter Notch signaling acts to maintain tissue specific adult stem cell populations and loss of Notch expression leads to terminal differentiation (Chiba, 2006; Kobayashi and Kageyama, 2010).

Support for the role of the Notch pathway in self-renewal is provided by a study in which upregulation of p53 expression in human osteosarcoma cells also led to an increase in Notch1 expression. Exposure of human prostate cancer cells to the small molecule Etoposide, a
drug that induces genotoxic stress by causing DNA damage, led to a corresponding increase in Notch1 expression. Conversely, inhibition of Notch signaling led to an increase in susceptibility to apoptosis, indicating that Notch signaling is upregulated in response to p53 expression and acts to inhibit apoptosis in cells undergoing genotoxic stress (Alimirah et al., 2007). Etoposide has also been shown to upregulate gene expression of Sox2 and Klf4 when used to treat MEF cells, indicating its possible usefulness in Cellular reprogramming (Park et al., 2011). Its effects on human cancer cells indicate that it may also be useful in reprogramming human cells, as it may act to promote self-renewal and inhibit apoptosis through upregulation of Notch expression (Alimirah et al., 2007; Fox et al., 2008).

**TGF-β/BMP Signaling**

The TGF-β pathway is another such pathway with seemingly differing roles between hES and mES cells, as will be discussed further below. There are two branches of this pathway, TGF-β signaling and BMP signaling, and they have differing effects on the cell, especially in the context of pluripotency, and seem to be antagonistic toward one another (Xu et al., 2008). Components of the pathway include the receptors, of which there are two types. The type 1 receptors include Activin receptor like kinases (Alk)1 – Alk7, and these receptors form a complex with type 2 receptors, of which there are five. BMP binding generally occurs through ALK1, 2, 3 and 6 while TGF-β/Activin/Nodal signaling generally occurs through ALK4, 5, and 7. Upon ligand binding type 1 and type 2 receptors form a complex, resulting in phosphorylation of type 1 receptors by type 2 receptors. Mothers against decapentaplegic (SMAD) proteins are then phosphorylated by the intracellular domain of the type 1 receptor (Lee et al., 2011). SMAD2 and 3 are associated with TGF-β signaling while SMAD1, 5 and 8 are associated with BMP signaling. Phosphorylated SMADs then form a complex with SMAD4, and this complex enters the nucleus
to affect transcription of target genes (Ichida et al., 2009; James et al., 2005; Wu and Hill, 2009; Xu et al., 2008).

In hES cells the TGF-β pathway has been shown to be important for stem cell maintenance as inhibition of this pathway by the small molecule SB431542 (TGF-β receptor inhibitor) leads to a decrease in both Oct4 and Nanog expression, with the decline in Nanog expression being the most pronounced (James et al., 2005; Xu et al., 2008). In support of this phenomenon, SMAD2/3 has been shown to bind directly to the proximal region of the Nanog promoter to upregulate its transcription in hES cells (Xu et al., 2008).

Conversely, mES cells do not seem to depend on the TGF-β pathway for their maintenance and have in fact, been shown to benefit from BMP treatment (Li et al., 2010; Ogawa et al., 2007). In contrast to hES cells, treatment of mES cells with SB431542 does not lead to any significant decrease in expression of Oct4 and pluripotency is maintained (James et al., 2005). In support of this, the small molecule known as RepSox (aka E-616452), an ALK5 inhibitor named for its ability to replace Sox2 during reprogramming, was used in the production of murine iPS cells. Treatment of Oct4-GFP MEF cells with RepSox in conjunction with viral transduction of Oct4 and Klf4 leads to successful production of GFP-positive iPS cells. It is noteworthy that both Sox2 and c-Myc are able to be omitted when RepSox is used. Interestingly, RepSox treatment of GFP-negative iPS cells did not result in a significant increase in Sox2 expression, but did result in upregulation of Nanog, indicating that RepSox is able to replace Sox2 indirectly through increasing Nanog expression (Ichida et al., 2009). It is interesting to note that TGF-β inhibition leads to a decrease in Nanog expression in human pluripotent cells and an increase in Nanog expression in murine pluripotent cells, presumably through a similar mechanism in each species (Ichida et al., 2009; Xu et al., 2008).
Based on the number of small molecules available currently for use in manipulation of pathways related to pluripotency (see Table 1), it should be possible, in theory, to create a “cocktail” of small molecule compounds that is capable of completely reprogramming somatic cells to iPS cells without the use of exogenous factors or viruses. This has been done already to some extent (Park et al., 2011), but the cells were only partially reprogrammed. More research is needed in order to create a combination of molecules that is capable of targeting specific pathways related to pluripotency so that cells may be fully reprogrammed.

Table 1: A list of small molecules used in pluripotency experiments with their respective modes of action and references

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Mode of Action</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Bromoindirubin-3′-oxime</td>
<td>GSK3 Inhibition (Wnt+)</td>
<td>Sato et al., 2004; Park et al., 2011</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 Inhibition (Wnt+)</td>
<td>Buehr et al., 2008; Li et al., 2008</td>
</tr>
<tr>
<td>Lithium</td>
<td>GSK3 Inhibition (Wnt+)</td>
<td>Wang et al., 2011</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>Wnt5a upregulation</td>
<td>Jönsson et al., 1998; Park et al., 2011</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-Acetate</td>
<td>PKC, causes increase in β-Catenin</td>
<td>Tobimatsu et al., 2006; Park et al., 2011</td>
</tr>
<tr>
<td>Valproic Acid (VPA)</td>
<td>Histone Deacetylase Inhibition</td>
<td>Huangfu et al., 2008a; Zhou et al., 2009</td>
</tr>
<tr>
<td>Trichostatin A</td>
<td>Histone Deacetylase Inhibition</td>
<td>Kishigami et al., 2006; Zhao et al., 2010</td>
</tr>
<tr>
<td>5-azacytidine; 5-aza-2′-deoxycytidine</td>
<td>DNA methyltransferase Inhibition</td>
<td>Christman 2002; Lim et al., 2011; Park et al., 2011</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>G9a Histone Methyltransferase Inhibition</td>
<td>Shi et al., 2008a, 2008b</td>
</tr>
<tr>
<td>Chaetocin</td>
<td>Histone Methyltransferase Inhibition</td>
<td>Greiner et al., 2005; Park et al., 2011</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Notch1 upregulation</td>
<td>Alimirah et al., 2007; Park et al., 2011</td>
</tr>
<tr>
<td>Pifithrin-α</td>
<td>PS3 Inhibition</td>
<td>Abdelalim &amp; Tooyama, 2012</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK/ERK Inhibition</td>
<td>Buehr et al., 2008; Li et al., 2008</td>
</tr>
<tr>
<td>BayK8644</td>
<td>L-Calcium Channel Agonist</td>
<td>Shi et al., 2008b</td>
</tr>
<tr>
<td>SB431542</td>
<td>TGF-β Receptor Inhibition</td>
<td>Xu et al., 2008; James et al., 2005; Lin et al., 2009</td>
</tr>
<tr>
<td>RepSox</td>
<td>TGF-β Receptor Inhibitor</td>
<td>Ichida et al., 2009</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Inhibition of Senescence</td>
<td>Esteban et al., 2010</td>
</tr>
</tbody>
</table>
**Pathway Interactions**

Many of the previously discussed pathways have been shown to interact with one another, either directly or indirectly in various cellular contexts. The following section will discuss the known interactions between some of these pathways and will focus specifically on interactions between the TGF-β, Wnt, Notch, and p53 pathways and their implications for iPS cell production.

**TGF-β & Wnt Pathway Interactions**

Components of the TGF-β and Wnt pathways have been shown to positively interact with each other in various cellular contexts. For example, Wnt3a has been shown to cooperate with TGF-β signaling to induce mesoderm formation during mouse development, and both signals are required for this to occur (Kemp and Hendrickx, 2007). Also, treatment of mouse osteoblastic cells with TGF-β results in an increase in levels of β-Catenin in a dose and time dependent fashion. In addition, overexpression of Smad3 leads to an overall decrease in levels of phosphorylated β-Catenin, a post-translational modification associated with its degradation. Treatment of mouse osteoblasts with Etoposide, which causes genotoxic stress, increases levels of apoptosis, and subsequent treatment with Lithium Chloride, a previously discussed inhibitor of GSK3, antagonizes Etoposide induced apoptosis (Tobimatsu et al., 2006). This is an indicator that the TGF-β and Wnt pathways may interact in a positive manner to promote rescue of cells from apoptosis in response to genotoxic stress. Lastly, co-precipitation studies have revealed that β-Catenin physically interacts with Smad4 during Xenopus development to affect expression of target genes, specifically Xwnt8, and that this association also occurs in human kidney and mouse fibroblast cell lines (Nishita et al., 2000).
TGF-β & Notch Pathway interactions

TGF-β signaling has been shown to interact with the Notch pathway in a negative fashion during the cellular repair process in mouse muscle stem cells. Increased TGF-β signaling is associated with inhibition of cell cycle progression, in part through inactivation of c-Myc. Excessive TGF-β signaling takes place in the muscle of older mice after injury as the signaling balance is shifted from active Notch to active TGF-β signaling as muscles age. Experimental evidence indicates that the two pathways are antagonistic toward one another, as activation of endogenous Notch signaling attenuated the negative effects of TGF-β treatment after muscle injury and increased muscle regeneration (Carlson et al., 2008).

Wnt & Notch Pathway Interactions

The Notch and Wnt signaling pathways have been shown to have both positive and negative interactions with each other, depending on cellular context (Hayward et al., 2008; Kwon et al., 2011). First, the two pathways act in a cooperative manner during development and patterning of the Drosophila wing. Initially Notch signaling promotes Wnt expression at the future wing margin, after which Wnt promotes Notch expression, creating a positive feedback loop that maintains signaling of both pathways. These two pathways have also been shown to interact in a positive manner during early germ layer formation in the sea urchin, as well as during development of skin precursors in vertebrates (Hayward et al., 2008).

Notch signaling is also important for the maintenance of adult stem cell populations. For example, overexpression of Notch1 in hematopoietic stem cells (HSCs) results in increased HSC generation. Notch signaling is greatly reduced in differentiated cell types and is downregulated as HSCs differentiate. Inhibition of Notch signaling in HSCs also accelerates the differentiation process. In addition, Wnt signaling is also important for HSC maintenance as
inhibition of Wnt signaling leads to a decrease in proliferation and viability. On the other hand, stimulation of Wnt signaling upregulates expression of Notch reporter constructs as well as Hairy and Enhancer of Split (Hes)1 and Deltex (Dtx)1, both of which are Notch target genes (Duncan et al., 2005).

All of these studies indicate that these two pathways are capable of positively interacting with each other, as expression of one pathway is able to maintain expression of the other in various cellular contexts.

**Notch, Wnt & TGF-β pathways interact with p53**

The p53 pathway is largely responsible for programmed cell death, or apoptosis, and acts mainly in response to cellular stress. More specifically, p53 activates expression of p21 in response to DNA damage (Datto et al., 1995). The p53 pathway has been shown to interact with the Notch, Wnt and TGF-β pathways in many different situations (Alimirah et al., 2007; Cordenonsi et al., 2003; Datto et al., 1995; Lee et al., 2010; Zhang et al., 2011).

First, as previously discussed, upregulation of p53 in human osteosarcoma and prostate cancer cell lines also leads to an expected increase in p21 expression, but also to an increase in Notch1 expression. The same treatment in p53 null prostate cancer (PC-3) cells led to no such effect. This provides an indication that p53 upregulates Notch signaling in response to genotoxic stress in an effort to attenuate p53 mediated proapoptotic functions (Alimirah et al., 2007).

TGF-β signaling also interacts with p53 signaling in different ways depending on cellular context. First, TGF-β treatment of human keratinocytes results in a 6 to 7 fold increase in p21 expression levels as little as 1 hour after treatment and also results in increased association of p21 with its cellular targets, specifically cyclin D1 and cyclin dependent kinase (Cdk)2. This
phenomenon occurs through a p53 independent mechanism as TGF-β is able to increase p21 expression as shown through the use of a p21 luciferase reporter in which the p53 response element has been removed from the promoter (Datto et al., 1995). Interestingly, p53 is also required for various TGF-β gene responses through a direct interaction with Smad2. Indeed, p53 knockdown in Xenopus embryos leads to decreased Activin mediated induction of endodermal and mesodermal markers. Use of an anti-p53 siRNA also leads to a decrease in TGF-β mediated gene responses in mammalian hepatoma cells. Finally, TGF-β mediated growth arrest in MEF cells also requires p53, as p53 negative MEF cells are insensitive to the anti-proliferative effects of TGF-β1 treatment (Cordenonsi et al., 2003).

As discussed previously, the Wnt pathway has been linked to stem cell maintenance, however, it does not seem to be sufficient by itself to sustain long term self-renewal of ES cells (Lee et al., 2010; Li et al., 2009; MacDonald et al., 2009). The p53 pathway also affects ES cell pluripotency, as expression of Nanog is reduced in hES cells in response to p53 activation. Because of this it is thought that differentiation in response to p53 is one mechanism used to ensure the genomic stability of ES cell populations. Wnt signaling is affected by p53 in a positive manner as DNA damaged ES cells show an enrichment of 5 Wnt ligands, 5 receptors and 1 component of the TCF/LEF transcription complex in addition to 9 regulators and downstream targets of Wnt signaling. This also occurs in MEF cells, although the response is greatly diminished. This seems to occur as part of an anti-differentiation response as evidenced by an increase in the percentage of Nanog and Oct4 positive cells in mES cell populations treated with UV light, and inhibition of Wnt signaling after UV treatment greatly attenuated this response (Lee et al., 2010).
Wnt signaling has also been shown to have an effect on expression of p53 expression in mesenchymal stem cells (MSCs) and induces the aging of these cells through this interaction. Old rat serum was used to treat MSCs in order to determine its effect on these cells. In response to old rat serum treatment, MSC senescence was greatly increased, while proliferation rate was greatly reduced. β-Catenin expression and nuclear accumulation were also upregulated and GSK3 expression was downregulated in response to this treatment. Inhibition of Wnt signaling through an anti-β-Catenin siRNA attenuated these affects. An increase in p53 expression was also seen in response to old rat serum treatment, and inhibition of Wnt signaling also led to decreased expression of p53, indicating that Wnt signaling upregulates p53 expression in aging MSCs (Zhang et al., 2011). Interestingly, this coupled with previous research indicates that the Wnt and p53 pathways are able to mutually affect expression of the other depending on cellular context (Lee et al., 2010; Zhang et al., 2011).

**Research Objectives**

Previous research has shown interactions between the TGF-β, Wnt and Notch pathways in various cellular contexts, and these pathways have all been linked to pluripotency in some fashion (Alimirah et al., 2007; Carlson et al., 2008; Cordenonsi et al., 2003; Datto et al., 1995; Duncan et al., 2005; Hayward et al., 2008; Kemp and Hendrickx, 2007; Lee et al., 2010; Nishita et al., 2000; Zhang et al., 2011). The TGF-β pathway differentially regulates pluripotency based on species. It maintains pluripotency in humans through upregulation of Nanog (James et al., 2005), while it inhibits pluripotency in mice through inhibition of Nanog expression (Ichida et al., 2009). The Notch pathway is necessary for hES cell proliferation (Fox et al., 2008), while in mice it regulates differentiation of ES cells and maintains adult stem cell populations (Chiba, 2006). The Wnt pathway is important for ES cell maintenance in both species (Sato et al., 2004).
The small molecule RepSox, a TGF-β pathway inhibitor, has been used as a supplement during viral reprogramming of murine MEF cells to improve reprogramming efficiency and is able to replace Sox2 by upregulating Nanog expression (Ichida et al., 2009). However, its effect on other pathways has not been determined. We treated MEF cells with RepSox with the goal of determining its effect on gene expression at a more in depth level, specifically in relation to pathways associated with pluripotency. Based on the differential roles of TGF-β in stem cell maintenance of different species, we also desired to compare the effects of RepSox treatment on bovine embryonic fibroblasts (BEF) to that of MEF cells in order to see if RepSox affects cells of these two species is the same manner. We also have the long term goal of developing a cocktail of small molecules that may be used to produce iPS cells and discuss the viability of using RepSox as a part of this cocktail in both species. We report our results here and hypothesize possible mechanisms for the observed pathway interactions.
MATERIALS AND METHODS

Culture of Murine and Bovine Embryonic Fibroblasts

SCRC-1008™ MEF cells were obtained from the American Type Culture Collection (ATCC®), and BEF cells were graciously provided by Dr. Clay Isom’s laboratory (Utah State University). Both cell types were cultured in DMEM/F12 medium (HyClone™, Catalog #SH30271.01) supplemented with 10% fetal bovine serum (HyClone™, Catalog #SH30070.03) and 1% Penicillin and Streptomycin Solution (MP Biomedical, Catalog #1670049) and incubated at 37°C in a 5% CO₂ in air environment. MEF cells were seeded at or below passage 6 while BEF cells were seeded at or below passage 10. Both cell types were seeded at a concentration of 3 x 10⁵ total cells in 20 ml of media in a T75 flask (Corning®, Catalog #430720). Cells were allowed to attach to the culture surface overnight and grow for 24 additional hours before addition of RepSox (Biovision, Catalog #1894). After 24 hours fresh medium was added to the cultures and supplemented with either 25μM RepSox or 2.35 x 10⁻⁵ μM TGF-β1 (Sigma-Aldrich®, Catalog #T7039). Medium was changed every 24 hours and fresh supplement added for a total of 72 hours in preparation for RNA extraction.

Culture of Murine ES Cells

mES cells were obtained from ATCC® (SCRC-1033™). In preparation for ES cell culture, MEF cells at or below passage 6 were seeded into 60 mm cell culture dishes and grown in the same culture medium and environmental conditions as experimental MEF cells and were allowed to grow to approximately 90% confluency. MEF cells were then treated with 10 μg/ml of Mitomycin C solution (Sigma-Aldrich®, Catalog #M0503) in serum free Dulbecco’s Modified Eagles Medium/Hams F12 (DMEM/F12) for 2 hours. After 2 hours of treatment, the serum free
medium was replaced with serum containing fibroblast medium and the cells were allowed to rest for at least 2 hours prior to addition of ES cells. mES cells were then seeded onto feeder layers at a concentration of approximately $2 \times 10^5$ total cells and allowed to attach overnight. ES cell medium consisted of Knockout™ DMEM (Life Technologies™, Catalog #10829-018) supplemented with 15% Knockout™ serum replacer (Life Technologies™, Catalog #10828), 2 mM L-Glutamine (HyClone™, Catalog #SH30034.01), 1X Non-essential amino acid solution (EMD Millipore, Catalog #TMS-001-C), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich®, Catalog #M7522), 1% Penicillin and Streptomycin Solution (MP Biomedical, Catalog #1670049), and 1000 U/ml murine LIF (Life Technologies™, Catalog #PMC4054). After overnight attachment the cells were allowed to grow for 24 hours before addition of RepSox. After 24 hours the cells were supplemented with 25 μM RepSox or $2.35 \times 10^{-5}$ mM of TGF-β1. Medium was changed and fresh supplement added every day for 3 days in preparation for RNA extraction.

**RNA Extraction**

After the 3 day treatment period, the cell culture medium was removed by pipet and the cells were washed with Phosphate Buffered Saline (PBS; Hyclone™) solution to remove all remaining traces of serum. The cells were then detached using 0.25% trypsin solution (Thermo Scientific, Catalog #SH30042.01). The cells were then washed from the culture surface using fresh medium and pelleted by centrifugation at approximately 300 x g for 5 minutes. Medium was carefully decanted from the cell pellet. In the case of ES cells, the cell pellet was resuspended in 5 ml culture medium and incubated at 37°C in a 5% CO₂ in air environment for 45 minutes to allow the fibroblast cells from the feeder layer to attach and separate from the ES cells. The unattached cells were considered to be ES cells and were again pelleted by centrifugation at 300 x g for 5 minutes. At this point, all cell pellets (MEF, BEF and ES) were
resuspended in PBS solution in order to remove all traces of medium from the cell samples and centrifuged once again at 300 x g for 5 minutes. RNA extraction was then performed on the cells using the RNeasy® Mini Kit (Qiagen®, Catalog #74104) following the manufacturer’s protocol. The cell lysis step was performed by passing the cell sample in lysis buffer through a 20 gauge needle at least 5 times. RNA concentration was determined by dispensing 2 μl of RNA sample onto a Nanodrop® 2000 spectrophotometer (Thermo Scientific). RNA was then stored at -80°C as per manufacturer instructions until reverse transcription (RT) to complimentary DNA (cDNA).

**Reverse Transcription to cDNA**

After RNA extraction, RNA samples were converted to cDNA using the SABiosciences™ RT² First Strand Kit (Qiagen®, catalog #330401) following manufacturer instructions. Briefly, a total of 3μg RNA was added to 5x genomic DNA elimination buffer in a sterile Polymerase Chain reaction (PCR) tube and incubated at 42°C for 5 minutes. Samples were then chilled on ice for at least 1 minute. The 2x RT Cocktail was then prepared according to the supplied protocol and added to the genomic DNA elimination mixture to a final 1x concentration. The samples were then incubated at 42°C for 15 minutes followed immediately by a 5 minute 95°C enzyme inactivation step. Ninety-one μl of RNase free water was then added to each reaction in preparation for quantitative PCR (qPCR).

**PCR Arrays**

Murine cDNA samples were first used for qPCR on 2 separate SABiosciences™ RT² Profiler™ stem cell PCR arrays (Qiagen®, Catalog #PAMM-405A and PAMM047A) for initial analysis following the recommended protocol. Bovine samples were not run on PCR arrays due
to the expense of performing a species conversion, but were stored for later analysis. Briefly, qPCR reaction master mixes were prepared for each cDNA sample using RT² SYBR® Green ROX™ qPCR Master Mix (Qiagen®, Catalog #330520) following the manufacturer’s protocol, and 25µl of each master mix was dispensed into each well of the 96-well PCR arrays. The arrays were then briefly centrifuged and incubated on a Mastercycler® ep Realplex4 qPCR machine (Eppendorf) using the following cycling conditions: 1 cycle at 95°C for 10 minutes for Hot Start activation and initial denaturation followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. This was followed immediately by a melt curve analysis to determine primer specificity using the following parameters: 95°C for 15 seconds, then 60°C for 15 seconds (no optics), a gradual temperature increase from 60°C to 95°C over a 20 minute period (optics on), and a final 95°C incubation for 15 seconds. Following qPCR the Ct baseline level was set manually at 170 and the melt curve peak threshold level was left at the default value of 33.

Primer Design

After initial PCR arrays were run on murine samples, genes that showed an initial fold change of at least 4 were chosen for further analysis. Individual primers were designed in house for each of these genes (see Tables 2 & 3 in the Appendix). cDNA sequences were obtained from GenBank® and used as templates for primer design. Primers were designed using the Primer3Plus website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and checked for specificity using the National Center for Biotechnology Information’s (NCBI) BLAST® software. Murine primers were aligned against the mouse genomic plus transcript database and bovine samples were blasted against Bos Taurus specific sequences. Primer sequences that significantly aligned to more than one gene with similar E values were not accepted. Primer sets whose amplicons were greater than 200 base pairs were also not accepted. Individual primer
sets were then checked for complementarity using Beacon Designer™ free edition (Premier Biosoft). Any primer sets that had a complementarity score below -3.5 were not accepted. Qualifying primer sets were then used for standard qPCR analysis of the chosen genes.

**Standard qPCR Analysis**

Individual genes were chosen for further analysis based on results from PCR Arrays and individual primer sets designed as described above. The following five genes were used as housekeeping genes for normalization of murine samples: ß-Actin, Glyceraldehyde phosphate dehydrogenase (Gapdh), Glucuronidase-ß (Gusb), Hypoxanthine guanine phosphoribosyl transferase (Hprt), and Heat shock protein 90 alpha class B member 1 (Hsp90ab1). The same housekeeping genes were used to normalize bovine samples with the exception of Hsp90ab1, for which sufficiently specific primers were not obtained. Reactions were prepared using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Catalog #172-5120) according to the manufacturer’s protocol. cDNA was obtained in the same manner as described above and approximately 30 ng was applied to each individual qPCR reaction. Individual reactions were loaded onto a 96 well qPCR plate (Bioexpress, Catalog #T-3085-1), briefly centrifuged and loaded onto a Mastercycler® ep Realplex4 qPCR machine (Eppendorf) and amplified using cycling conditions identical to those used for PCR Arrays. Melt curve analysis was also performed using conditions identical to those of PCR Arrays. Following qPCR analysis, a random subset of reactions was chosen and run on a 1% agarose gel in order to double check primer specificity.

**Data Analysis**

Following qPCR, data analysis was performed using the SABiosciences™ RT² Profiler™ PCR Array Data Analysis Template v4.0 (Qiagen®). The software consists of an Excel spreadsheet
that automatically normalizes sample data based on housekeeping gene information and calculates fold changes upon insertion of raw cT values as well as statistical significance using the standard t-test. P-values at or below a value of 0.05 were accepted as statistically significant. Fibroblast control and treatment samples were compared using this method. Murine fibroblast controls and treatment samples were also compared to mES cell control samples using this method. Graphs and tables were prepared from this data using Microsoft Excel and Word 2010.
RESULTS

Recent efforts in iPS cell production have focused on eliminating the need for exogenous genetic material as well as viruses for successful reprogramming. Successful reprogramming has been achieved using non-viral plasmids (Okita et al., 2008) and miRNA (Anokye-Danso et al., 2011; Judson et al., 2009), thus avoiding the use of viruses, but these methods still require the use of exogenous genetic material to accomplish reprogramming. Recombinant proteins have also been developed and used successfully to reprogram cells (Zhou et al., 2009), but recombinant protein production is a complex and time consuming process. None of these processes sufficiently increases efficiency of reprogramming, which is necessary for practical applications of iPS cells.

The efficiency of reprogramming has been modestly improved through the use of various small molecules as a supplement to or in place of, viral reprogramming factors (see table 1), but the use of viruses and exogenous genetic material has not been fully eliminated using small molecules as of yet. In order for this to occur, the discoveries of small molecules that directly target specific pathways related to pluripotency, and are therefore more likely to be able to replace reprogramming factors, are necessary. One such factor, RepSox (aka E-616542), has recently been used to successfully reprogram MEF cells using only Oct4 and Klf4 reprogramming vectors (Ichida et al., 2009). RepSox is an Alk5 (TGF-β receptor) inhibitor that prevents phosphorylation of Smad2/3 proteins and has the capability to replace Sox2 during reprogramming through upregulation of Nanog expression.

Since the TGF-β pathway is a prominent pathway in the cell, we hypothesized that RepSox treatment would most likely affect expression of other genes and pathways in addition to Nanog expression. In order to determine these effects we performed genetic analysis on
RepSox treated fibroblasts using qPCR and assessed the implications of these changes with regards to pluripotency and iPSC production. We report our results here and hypothesize possible mechanisms of action which would possibly explain our results.

We performed our initial analysis on MEF cells treated with RepSox using the PCR arrays PAMM-405a and PAMM-047a (Qiagen®) because they contain gene markers specific to stem cells and stem cell regulation. We chose to use PCR arrays due to their cost effectiveness compared to other methods of multi-gene analysis. Each array is able to test expression levels of 84 specific genes and has built-in controls for genomic DNA contamination and RT as well as 5 housekeeping genes and 3 positive PCR controls. The PCR arrays yielded a total of 27 genes out of the 168 total genes tested on the arrays that showed an initial fold change in expression of at least 4. These genes were chosen for further analysis. We designed custom primers for each gene and tested their expression using standard qPCR methods in order to obtain statistical significance calculations. We also tested expression of Oct4, Sox2, Klf4, c-Myc, and Nanog using standard qPCR.

**RepSox Upregulates c-Myc Expression in MEF Cells**

We tested expression of the major pluripotency factors in response to RepSox treatment in MEF cells. We found that RepSox treatment alone does not significantly affect expression of any of the pluripotency factors except c-Myc (+3.86, p=0.018; see Figure 4). This is consistent with the results reported by Ichida et al. (2009) who also reported a lack of effect on pluripotency factor expression in MEF cells when treated with RepSox in the absence of any other factors. They also reported the ability to exclude c-Myc during the reprogramming process while maintaining the ability to produce true iPSC cells. Our result would seem to explain
why this is possible. It is important to note the lack of upregulation of Nanog expression, even though RepSox has been reported to directly upregulate its expression through inhibition of TGF-β signaling. Ichida et al reported similar results and were only able to achieve Nanog upregulation when RepSox was used in conjunction with viral reprogramming vectors. The reason for this is not conclusively known, but could presumably be due to epigenetic factors.

![MEF Pluripotency Factors](image)

**Figure 4:** MEF Pluripotency Factors. Fold Changes in gene expression of the five major pluripotency factors in response to RepSox treatment. * = statistically significant.

**RepSox Upregulates Wnt Signaling in MEF Cells**

Wnt signaling is a prominent process in cellular signaling and is characterized by degradation of β-Catenin in the absence of Wnt ligand. Upon Wnt binding to Fzd receptors, the degradation complex is destabilized and β-Catenin is allowed to accumulate in the nucleus. β-Catenin then enters the nucleus where it forms a complex with TCF/LEF and acts as a
transcription factor, affecting expression of target genes (see Figure 2). We found significant upregulation of a number of genes associated with Wnt signaling (see Figure 5). Specifically, Fzd1, Fzd4, and Fzd9, all Wnt pathway receptors, were upregulated 2.99 (p = 0.00098), 11.42 (p = 0.0003) and 8.12 (p = 0.001) fold, respectively. The transcription factor Lef1 was also upregulated in response to RepSox treatment (3.13 fold, p = 0.0019). Interestingly, the receptor Fzd6 was significantly downregulated by a factor of 5.62 fold (p = 0.04) in response to RepSox treatment, and this result was highly reproducible. This is consistent with upregulation of Wnt signaling as Fzd6 is known to be a negative regulator of this pathway in human cells (Golan et al., 2004). Also, upregulation of Wnt signaling would seem to explain the observed change in c-Myc expression in MEF cells, as c-Myc is a known target of the Wnt pathway (Zhang et al., 2012).

Taken together, these data indicate that the Wnt pathway is positively affected by treatment of MEF cells with RepSox.

Figure 5: MEF Wnt Signaling. Fold changes in gene expression of components of the Wnt pathway in response to RepSox treatment of MEF cells. * = Statistically significant.
RepSox Treatment Upregulates Notch Signalin in MEF Cells

The Notch signaling pathway is a cellular communication pathway that is involved in various developmental processes. In humans this pathway is necessary for proliferation of ES cells (Fox et al., 2008). In mice it is involved in regulation of differentiation, and positive and negative changes in Notch activity are correlated with ES cell differentiation to specific cellular lineages. It is also involved in maintenance of adult stem cell populations, and downregulation of Notch signaling leads to terminal differentiation (Chiba, 2006). The pathway itself is activated when transmembrane Notch receptors bind and activate transmembrane ligands (Dll1, 3, 4, Jag1, 2) on a neighboring cell. This leads to cleavage of the intracellular Notch domain followed by translocation to the nucleus where it complexes with the CSL complex and activates transcription of target genes.

We found that components of the Notch signaling pathway are significantly upregulated in response to RepSox treatment (see Figure 6). Specifically, the Notch receptors Jag1 and Notch3 were upregulated 3.55 (p = 0.0009) fold and 2.82 (p = 0.02), respectively, compared to controls. The Notch pathway target genes Dtx1 was highly upregulated compared to controls as well (16.4 fold, p = 0.0057). Interestingly, Dtx1 is also a known positive regulator of Notch signaling. This, coupled with its high level of upregulation, suggests a possible positive feedback loop in response to RepSox treatment, although additional research is needed to determine if this is actually the case. Hairy/Enhancer of split (Hey1), another Notch target gene, was also upregulted by a factor of 4.2 (p = 0.000097). Expression of the Notch ligands Dll1 and Dll3 was slightly upregulated as well, although these changes were not statistically significant. Overall, these data indicate that Notch signaling is elevated in MEF cells in response to RepSox.
treatment, providing another possible mechanism by which RepSox is able to promote pluripotency and contribute to Cellular reprogramming in conjunction with other factors.

Figure 6: MEF Notch Signaling. Fold changes in gene expression of Notch pathway components in response to RepSox treatment. Note the significant increase in Dtx1 expression, a known regulator of Notch signaling. * = Statistically significant.

RepSox Affects Expression of TGF-β Pathway Components

The TGF-β pathway is the immediate target of the small molecule RepSox. The pathway itself is activated upon ligand binding to a type 2 receptor, which then forms a complex with a type 1 receptor. Smad2/3 is then phosphorylated by the type 1 receptor. Smad2/3 then forms a complex with Smad4 and enters the nucleus to affect transcription of target genes. RepSox functions by inhibiting phosphorylation of Smad2/3 by Alk5, a type 1 receptor. Interestingly, RepSox treatment of MEF cells led to an unexpected increase in expression of components of the TGF-β signaling family, namely Activin A receptor type 1C (Acvr1c, aka Alk7) and zinc finger
E-box binding homeobox 2 (Zeb2; see Figure 7). These two genes encode a type 1 Activin receptor and a transcriptional inhibitor, respectively. However, their upregulation was modest at 4.99 (p = 0.0017) fold and 2.98 (p = 0.02) fold, respectively. The reason for their upregulation is unknown. However, it could possibly be a compensation mechanism by the cell in response to Alk5 inhibition, since ligand binding to Acvr1c also results in phosphorylation of Smad2/3 proteins. More research is needed to answer this question, but it was not pursued further as it was not the main focus of this project.

BMP signaling is an alternative pathway within the TGF-ß superfamily of signaling and is often associated with phosphorylation of Smad1/5/8 instead of Smad2/3 (Massagué, 1998). BMPs have also been associated with stem cell maintenance and improved iPS production in mice (Chen et al., 2011a). We saw a substantial increase in BMP3 expression (14.14 fold, p = 0.016) in response to RepSox treatment, providing another possible mechanism by which RepSox may increase reprogramming efficiencies in the mouse model.

Figure 7: MEF TGF-ß Signaling. Fold changes in gene expression of components of the TGF-ß superfamily. Note the substantial increase in BMP3 expression, a signaling pathway associated with pluripotency in the murine model. * = Statistically significant.
Hedgehog Signaling in MEF Cells Treated with RepSox

Hedgehog signaling is involved in many aspects of embryonic development in mammals, and its disregulation has been associated with tumor formation. The pathway itself involves a number of interactions between membrane-bound receptors and affects expression of downstream target genes. In the absence of ligand the membrane-bound Patched (Ptch) receptor inhibits the actions of smoothened (SMO), also membrane bound, which normally activates GLI-Kruppel family member (Gli) transcription factors. Upon ligand binding to Ptch receptors, SMO is left free to activate Gli, which then enters the nucleus and acts as a transcription factor. Secretion of and subsequent signaling by hedgehog ligands requires the participation of Ptchd proteins, also membrane bound, and otherwise known as dispatched.

We show here that two important components of hedgehog signaling are upregulated in response to TGF-β inhibition by RepSox (see Figure 8). The transcription factor Gli2 was significantly upregulated by a factor of 3.76 \( (p = 0.000068) \). Expression of the membrane bound Ptchd2 was upregulated by an even greater factor, 10.34 fold \( (p = 0.000002) \). Hedgehog signaling has been linked to stem cell maintenance in various adult stem cell types, including neural and hematopoietic stem cells (Ahn and Joyner, 2005; Liu et al., 2006). BMPs have also been reported to cooperate downstream with Hedgehog signaling in the maintenance of both normal and cancerous mammary stem/progenitor cell populations (Bhardwaj et al., 2001), providing a possible link between this pathway and the observed upregulation in BMP signaling in the present study. In addition, the transcription factor Gli2 has been shown to positively regulate expression of Sox2 by binding to a Sox2 enhancer region which is vital to Sox2 expression (Takanaga et al., 2009). Taken together, these results provide another mechanism
by which TGF-ß inhibition through RepSox treatment positively affects reprogramming efficiency of MEF cells.

Figure 8: MEF Hedgehog Signaling. Fold changes in gene expression of components of the Hedgehog signaling pathway in RepSox treated MEF cells vs. control samples. * = Statistically significant.

RepSox Upregulates Markers of Other Cell Lineages in MEF Cells

In addition to stem cell specific genes, the PCR arrays we used for initial analysis also contain markers of various cellular lineages, including embryonic, hematopoietic, mesenchymal and neural cell lineages. Interestingly, we found that RepSox treatment resulted in significant upregulation of a number of genes associated with these lineages (see Figure 9). Expression of S100b, a neural cell lineage marker, was modestly increased by approximately 5.02 fold (p = 0.000002). We also saw an increase in expression of Actin, alpha, cardiac muscle (Actc1) and Keratin 15 (Krt15), both embryonic cell lineage markers, by factors of 4.54 (p = 0.0006) and
10.07 (p = 0.028) fold, respectively. RepSox treatment also resulted in a very substantial increase in expression of the mesenchymal cell lineage marker bone gamma carboxyglutamate protein (Bglap) (43.83 fold, p = 0.05). In light of this result, it is surprising that expression of Collagen type 2 alpha 1 (Col2a1) and Col9a1, also both markers of a mesenchymal cell lineage, only saw modest increases that were not statistically significant. Lastly, we observed a very modest increase in expression of the hematopoietic lineage marker Cd3d (2.23 fold, p = 0.004), a slight, but statistically significant increase. Taken together, these data indicate that RepSox treatment results in upregulation of various cell lineage markers that may be useful in assays involving direct reprogramming of somatic cells to other cell types (Caiazzo et al., 2011).

Figure 9: Lineage Markers in MEF Cells. Fold changes in gene expression of various lineage markers in MEF cells in response to RepSox treatment. N = Neural cell lineage; E = Embryonic cell lineage; M = Mesenchymal cell lineage; H = Hematopoietic cell lineage; SC = Stem Cell specific biomarker. * = Statistically significant.
RepSox treatment also resulted in upregulation of a few cytokines and growth factors that have previously been associated with stem cell maintenance (see Figure 10). Expression of the stem cell associated marker chemokine (C-X-C Motif) ligand 12 (Cxcl12) was increased by a factor of 5.36 ($p = 0.00084$). Notably, expression of insulin-like growth factor 1 (Igf1) was increased approximately 9.47 fold ($p = 0.004$), and expression of fibroblast growth factor receptor 3 (Fgfr3) was increased 9.97 fold ($p = 0.000063$). Igf1 secretion from Leydig cells in the testes has been associated with maintenance of spermatogonial stem cell populations, and blocking of Igf1 activity in this context resulted in significant downregulation of Oct4 and Nanog (Huang et al., 2009). FGF signaling is associated with maintenance of pluripotency in hES cells, and FGF has long been a standard supplement in hES cell medium. Specifically, FGF cooperates with Activin/Nodal signaling in hES cell populations to maintain pluripotency (Vallier et al., 2005). Since TGF-β/Activin/Nodal downregulation is associated with maintenance of mES cell populations, it is possible that the increase in Fgfr3, a component of FGF signaling, is a direct result of TGF-β inhibition by RepSox treatment.

![MEF Cytokines & Growth Factors](image)

**Figure 10:** MEF Cytokines and Growth Factors. Fold changes in gene expression of cytokines and growth factors associated with stem cell maintenance. * = Statistically significant.
Differentially Expressed Genes in Murine ES Cells

In addition to MEF cells, we decided to test the effects of TGF-β inhibition on SCRC-1033™ mES Cells (ATCC®) in order to test whether or not RepSox has the same or similar affect as on MEF cells. We grew mES cells on MEF feeder layers and treated them with 25μM RepSox for 3 days. Following treatment we trypsinized the ES cell cultures and separated them from the MEF cells by incubation at 37°C for 45 minutes. This allowed the MEF cells to attach to the culture surface while the ES cells remained in suspension. We then tested the effects of RepSox treatment on expression of the five main pluripotency factors as well as the same set of 27 genes taken from PCR Arrays. We found that mES cells respond differently to RepSox treatment than do MEF cells, only showing upregulation of a subset of the genes that are affected in MEF cells (see Figure 11). ES cells showed modest but significant upregulation of Fzd1 (1.57 fold, p = 0.017) and Fzd9 (2.13 fold, p = 0.0295), indicating that there is a small increase in Wnt signaling in ES cells in response to RepSox treatment. The growth factor receptor Fgfr3 was upregulated 4.05 fold (p = 0.004), and the growth factor Cxcl12 was upregulated by a factor of 2.82 (p = 0.007). The pluripotency factors Sox2 and Klf4 also saw small but significant increases in expression of 2.08 fold each (p = 0.048 and 0.017, respectively; see Figure 12). Taken together, this data would seem to indicate an increase in genes associated with maintenance of pluripotency. However, we also saw upregulation of markers of other cell lineages. In particular, Krt15, which was upregulated approximately 10 fold (p = 0.028) is expressed in the developing fetus, but is also highly expressed in skin and epithelial cells, indicating that RepSox treatment in mES cells results in increased expression of some genes that could be associated with differentiation in an ES cell context. Even so, the increase in expression of pluripotency
factors and other pathways associated with stem cell maintenance indicate that RepSox treatment of mES cells has an overall positive effect on pluripotency.

Figure 11: mES Differentially Expressed Genes. The same set of genes tested in MEF cells was tested here. Non-significant changes in expression are not pictured here.

Figure 12: mES Pluripotency Factors. Fold changes in gene expression of pluripotency genes in mES cells. * = Statistically significant.
RepSox Treated MEF Cells Compared To ES Cell Gene Expression

Because our long-term goal is to produce iPS cells using only small molecules we desired to compare gene expression of control and RepSox treated MEF cells to that of mES cells in order to assess how RepSox treatment might affect reprogramming. We found that, for many genes, expression levels in RepSox treated MEF cells rose to a level closer to that of ES cells (see Figure 13A). Such genes included members of the Wnt pathway (Frat1, Lef1, Nfatc2), Notch pathway (Dll1, Dll3, Dtx1) and the Hedgehog signaling pathway (Gli2, Ptchd2). It is important to note, however, that for most genes there is still a significant difference in RepSox treated MEF expression levels and mES expression levels, confirming the obvious point that more treatment factors are needed in order to complete the reprogramming process. It is also noteworthy that expression levels of members of the Notch signaling pathway are included in this group as the level of Notch signaling is important in ES cell maintenance, as increases and decreases in Notch activity in ES cells are both associated with differentiation.

We also found that many genes were upregulated to expression levels well beyond that of ES cell expression levels in response to RepSox treatment (see Figure 13b). Such genes included Cxcl12 and Fgfr3, both genes that have been associated with stem cell maintenance. Cxcl12, in particular, saw a dramatic increase in expression after treatment when compared to ES cell expression (425 fold). Other genes in this group included those that were markers of other cell lineages, providing more evidence that RepSox treatment could possibly be a valuable component of direct transformation of one somatic cell type to another when combined with other factors. It will be necessary to test expression levels of these factors in combination with other small molecule treatments in addition to RepSox in order to truly assess possible effects on reprogramming efficiency.
Figure 13: mES vs. MEF Gene Expression. Comparison of gene expression between mES cells and MEF control and RepSox treated samples. A) RepSox treatment resulted in expression levels closer to those of mES cells for these genes. This group notably includes members of the Wnt, Notch and Hedgehog signaling pathways, whose expression levels are highly regulated in mES cells. B) RepSox treatment led to an increase in expression greater than that of ES cells in this group. N = Neural lineage; E = Embryonic lineage; M = Mesenchymal lineage; SC = Stem Cell lineage. * = Statistically significant difference between mES and MEF gene expression.
The Effect of RepSox Treatment on Bovine Embryonic Fibroblasts

Since the TGF-β pathway has differing effects on pluripotency between species, we decided to test the effects of RepSox treatment on gene expression of BEF cells and compared them to that of MEF cells. BEF cells were subjected to identical treatment conditions as MEF cells, and the same set of 27 genes plus pluripotency factors were analyzed. Our analysis found that RepSox treatment did not significantly affect gene expression of the pluripotency factors (data not shown). We also found that, out of the 27 additional genes tested, only three saw a significant upregulation in BEF cells (see Figure 14), indicating that RepSox treatment does not affect BEF cells in the same way that it affects MEF cells. This also provides an indication that RepSox treatment may not be effective in contributing to reprogramming efficiency during bovine iPS cell production, but more testing is needed to come to this conclusion definitively.

![Figure 14: BEF Differentially Expressed Genes. Fold changes in gene expression in BEF cells treated with RepSox and TGF-β1. * = statistically significant.](image-url)
CONCLUSION AND DISCUSSION

The small molecule known as RepSox has previously been used to increase the efficiency of reprogramming in MEF cells (Ichida et al., 2009). Specifically, it was able to eliminate the need for both Sox2 and c-Myc during the reprogramming process while still allowing for the successful production of mouse pluripotent iPS cells. As RepSox inhibits the TGF-β pathway, which is involved in many cellular processes, we desired to test the effects of RepSox on gene expression of MEF cells on a more extensive level in order to determine how these changes might also affect pluripotency during reprogramming. We started by treating MEF cells with RepSox for a period of 3 days and tested the effects.

We found first, that RepSox treatment resulted in upregulation of the Wnt pathway in MEF cells, a pathway associated with stem cell maintenance (Sato et al., 2004; Yi et al., 2011). Upon treatment we found increases in expression of Wnt receptors and transcription factors, indicating that the Wnt pathway is significantly more active in RepSox treated MEF cells than in control samples. Consistent with this, we saw a significant decrease in expression levels of the Fzd6 receptor. Since Fzd6 is associated with negative regulation of the Wnt pathway its downregulation is consistent with increased Wnt activity. Further evidence is provided toward this conclusion by the fact that RepSox treatment also resulted in an upregulation of c-Myc expression, a known target of the Wnt pathway (Zhang et al., 2012). This would also provide a possible mechanism by which Ichida et al were able to omit c-Myc from the reprogramming process. We also observed that expression of Wnt pathway components was closer to expression levels seen in mES cells, indicating that RepSox treatment is beneficial to the reprogramming process with regards to this pathway.
In addition to the Wnt pathway, Notch signaling was also increased. We observed significant increases in Jag1 expression, a Notch receptor, as well as Dtx1, a known regulator of Notch signaling. In hES cells, Notch signaling is necessary for stem cell maintenance, and its downregulation results in decreased proliferation (Fox et al., 2008). The Notch pathway is active in mES cells as well, but its role is slightly different. A decrease in Notch signaling results in differentiation to the mesodermal lineage, while an increase results in a preference for the neural lineage (Kobayashi and Kageyama, 2010). In light of this information it is difficult to assess how RepSox treatment affects pluripotency with regards to Notch signaling. However, we also observed that expression levels of Notch pathway components were closer to levels seen in mES cells upon RepSox treatment. Notch signaling is also heavily involved in maintenance of adult stem cell populations, providing an indication that RepSox treatment is beneficial to reprogramming efficiency in this aspect.

Interestingly, we observed an increase in genes associated with TGF-β/Activin/Nodal signaling upon RepSox treatment. RepSox acts by preventing Smad2/3 phosphorylation via inhibition of the Alk5 receptor. Expression levels of Acvr1c were significantly increased upon treatment with RepSox. Since this gene encodes an Alk7 receptor, which also phosphorylates Smad2/3 proteins, it is possible that its upregulation is a type of compensation mechanism by the cell in response to RepSox treatment, but more research is needed to support this conclusion. RepSox treatment also resulted in a substantial increase in expression levels of BMP3. BMP’s have previously been shown to be important in mES cell pluripotency, and their use during reprogramming functionally replaces Klf4 and supports reprogramming using only Oct4 (Chen et al., 2011a).
In addition to Notch and Wnt signaling pathways, we also observed an increase in expression of Gli2 and Ptchd2, both components of the Hedgehog signaling pathway. Hedgehog signaling is highly active during embryonic development and has also been shown to be important during maintenance of adult stem cell types including mammary, hematopoietic, and neural stem cells (Ahn and Joyner, 2005; Bhardwaj et al., 2001; Liu et al., 2006). Expression levels of Gli2 and Ptchd2 in RepSox treated MEF cells were upregulated significantly to a level that is highly similar to levels observed in mES cells. Hedgehog signaling also cooperates with BMP’s to induce proliferation of hematopoietic stem cells, providing a possible mechanism by which both BMP and hedgehog signaling are upregulated.

RepSox treatment of MEF cells also resulted in an increase in expression levels of genes associated with stem cell maintenance, including Igf1 and Fgfr3. In particular, FGF signaling is associated with maintenance of human stem cell populations. In addition to this, we observed an increase in expression of genes associated with various cell lineages, including neural, embryonic, mesenchymal and hematopoietic lineages. Levels of all of these genes were increased to levels well beyond levels seen in mES cells, making it unclear how this would affect reprogramming efficiencies upon RepSox treatment. However, since many of these genes are associated with cell lineages other than MEF cells, this provides an indication that RepSox may be a valuable component during assays involving direct reprogramming of one somatic cell type to another (Caiazzo et al., 2011; Pfisterer et al., 2011; Vierbuchen et al., 2010).

In addition to MEF cells, we treated mES cells with RepSox in order to observe any effect on gene expression it may have in this cell type. We found that RepSox treatment does not have the same effect in this cellular context, as only nine total genes that we tested were significantly upregulated. These genes included Fzd1 and Fzd9, both members of the Wnt
pathway, as well as Sox2 and Klf4, both pluripotency factors. Ichida et al. (2009) reported that RepSox replaces Sox2 during reprogramming through TGF-β inhibition and that this inhibition resulted in a direct increase in Nanog expression. Interestingly, we observed no significant increase in Nanog expression upon treatment of ES cells with RepSox. This, together with the observed increase in Sox2 expression, leads us to conclude that RepSox affects Sox2 expression via some alternative mechanism in addition to that observed previously by Ichida et al.

Lastly, we tested the effects of RepSox treatment on BEF cells. Since the TGF-β pathway has divergent roles with regards to mES and hES cell maintenance, we reasoned that the same difference may exist between the murine and bovine species. On the other hand, if the role of the TGF-β pathway is the same between the two species with regard to stem cell maintenance, perhaps RepSox would be a valuable tool for use during isolation/creation of pluripotent bovine cell lines, a goal which has not yet fully been realized. We observed that RepSox treatment only resulted in significant upregulation of three genes in BEF cells (Actc1, Bglap, Fzd4). In addition, we did not observe an increase in expression of any pluripotency genes in response to treatment. We therefore conclude that RepSox treatment does not have the same effect on bovine cells as on murine cells. However, since there is a lack of standardized culture conditions regarding pluripotent bovine cells it is possible that inadequate culture conditions is a contributing factor in the lack of results seen in this study. It is possible that other factors are needed in bovine cells in order for significant changes in pluripotency pathways to be observed in response to RepSox treatment. Also, in light of the fairly limited set of genes tested here, more research is needed to conclusively determine if RepSox is effective in the creation of pluripotent bovine cells. However, our data provides an indication that RepSox may not be a valuable tool in this species.
Overall, we conclude that RepSox treatment of MEF cells results in significant upregulation of the Wnt, Notch and Hedgehog pathways, as well as c-Myc, and that based on previous research, these changes are beneficial to pluripotency and are contributing factors in the increase in reprogramming efficiency seen during Cellular reprogramming. RepSox treatment is also beneficial to mES cell maintenance, as it results in significant increases in expression levels of Sox2 and Klf4, as well as Fzd1 and Fzd9, both components of the Wnt pathway. RepSox treatment has differential effects on mES cells and MEF cells based on different cellular contexts. Lastly, we conclude that RepSox treatment does not have the same effect on bovine cells as it does on murine cells, only resulting in significant changes in expression levels of 3 of the genes we tested. More research is needed in order to elucidate the effects of RepSox on bovine cells, and to find small molecules that will be beneficial to the creation of pluripotent cells in the bovine model.
REFERENCES


### Table 2: Murine primer sequences

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