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# Self-Renewal Requirements of Human Embryonic Stem Cells and Their Engraftment Potential in Mouse Blastocysts

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# Self-renewal requirements of human embryonic stem cells and their engraftment potential in mouse blastocysts

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

## Daylon Jefferson James

June 2006

# Acknowledgements

A central principle of graduate education is that it goes beyond the curriculum and demands a novel contribution to our understanding of the world around us. What I have come to realize in my graduate years is that that novel contribution is synthesized, in large part, from the collective influence of numerous individuals. I would like to thank all of those who have helped me up to this point, in particular:

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# ABSTRACT

**Self-renewal requirements of human embryonic stem cells and their engraftment**

**potential in mouse blastocysts**

Daylon Jefferson James, Ph.D.

The Rockefeller University 2006

Human embryonic stem cells (hESCs) are a unique population of cells derived from a 6 day old human embryo that can be maintained indefinitely *in vitro* and have the ability to differentiate to all adult cell types. In addition to their potential for cell based therapies in the treatment of disease and injury, the broad developmental capacity of hESCs offers potential for studying the origins of all human cell types. Embryonic stem cells were first derived from mouse embryos (mESCs), and years of work have demonstrated their utility to developmental research, but relatively little is known about human ESCs. The experiments described below address two fundamental questions in hESC biology: First, what are the molecular signaling pathways that are relevant to hESC "stemness"? And second, can hESCs contribute to specialized human cell types in the context of mouse embryogenesis?

Because it plays a prominent role in the early cell fate decisions of embryonic development, we examined the role of TGFβ superfamily signaling in hESCs. We found that, in undifferentiated cells, the TGFβ/activin/nodal branch is activated (through the signal transducer Smad2/3) while the BMP/GDF branch (Smad1/5) is only active in isolated mitotic cells. Upon early differentiation, Smad2/3 signaling is decreased while Smad1/5 signaling is activated. We next tested the functional role of TGFβ/activin/nodal signaling in hESCs and found that it is required for the maintenance of markers of the undifferentiated state. We extended these findings to show that Smad2/3 activation is required downstream of Wnt signaling, which we have previously shown to be sufficient to maintain the undifferentiated state of hESCs. Strikingly, we show that in *ex vivo* mouse blastocyst cultures, Smad2/3 signaling is also required to maintain the inner cell mass

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(from which stem cells are derived). These data reveal a critical role for TGFβ signaling in the earliest stages of cell fate determination and demonstrate an interconnection between TGFβ and Wnt signaling in these contexts.

To date, the emergence of specialized cells from hESCs has commonly been studied in tissue culture or upon injection into adult mice, yet these methods have stopped short of demonstrating the potential exhibited by mESCs, which can give rise to every cell type when combined with embryos at the blastocyst stage. Due to obvious barriers precluding the use of human embryos in similar cell mixing experiments with hESCs, human/non-human chimeras may need to be generated for this purpose. In order to define the developmental potential of hESCs in the context of embryogenesis, we explored the ability of hESCs to engraft into mouse blastocysts. In advance of these cell mixing experiments, we derived a new hESC line, RUES1, and characterized its marker expression, functional characteristics and gene expression profiles. Using this new line, we showed that hESCs engrafted into mouse blastocysts, where they proliferated and differentiated *in vitro* and persisted in mouse/human embryonic chimeras that implanted and developed in the uterus of pseudopregnant foster mice. Embryonic chimeras generated in this way offer the opportunity to study the behavior of specialized human cell types in a non-human animal model. Our data demonstrate the feasibility of this approach, using mouse embryos as a surrogate for hESC differentiation.

# GENERAL INTRODUCTION

Contained within each cell of the human body there is a genetic blueprint for all its many parts, from calloused toe to thinking brain. Indeed, all vertebrates come into being as a single cell, the fertilized egg, proliferates and differentiates to billions of specialized cells that are arranged to make up the adult. This process is called embryogenesis and it has been scrutinized for millennia. In 350 BC, Aristotle cracked open a chicken egg every day during its three week incubation period and noted the formation of major organs over time from a tiny streak. In 1924, Hans Spemann and Hilde Mangold ushered in the age of experimental embryology with pioneering work defining the "organizer" in amphibian embryos (Spemann, 1918; Spemann, 1921; Spemann and Mangold, 1923), findings that were not only revolutionary in their own time, but also seminal to our modern understanding that it is the molecular interactions between cells that dictate form and fate in vertebrate embryogenesis. And in recent decades, scientists have begun to reveal the means by which a conserved genetic blueprint can give rise to the complex array of cell types generated during vertebrate embryogenesis.

Between vertebrate species, the dynamics of embryogenesis vary in many respects; for example, in mammals, eggs are fertilized within the female and embryos can gestate for as many as 22 months (Elephant), while amphibian eggs are laid and fertilized in the external environment and can develop to free swimming tadpoles within 36 hours (*Xenopus laevis*). Developmental differences such as these account for considerable variety between species, but all vertebrates share similarities in the morphological process that establishes the early embryonic body plan (Scialli, 2003). In humans, this takes place following implantation into the uterus, when a trilaminar organization of the embryo emerges as cells migrate through the primitive streak to form mesoderm (Figure 1). This process is called gastrulation, and it describes the way in which an unstructured early embryo undergoes morphogenetic movements to become organized into three primary germ layers – endoderm, mesoderm, and ectoderm. Endoderm is the innermost germ layer and gives rise to organs including liver, lungs and pancreas; mesoderm, the

# Figure 1
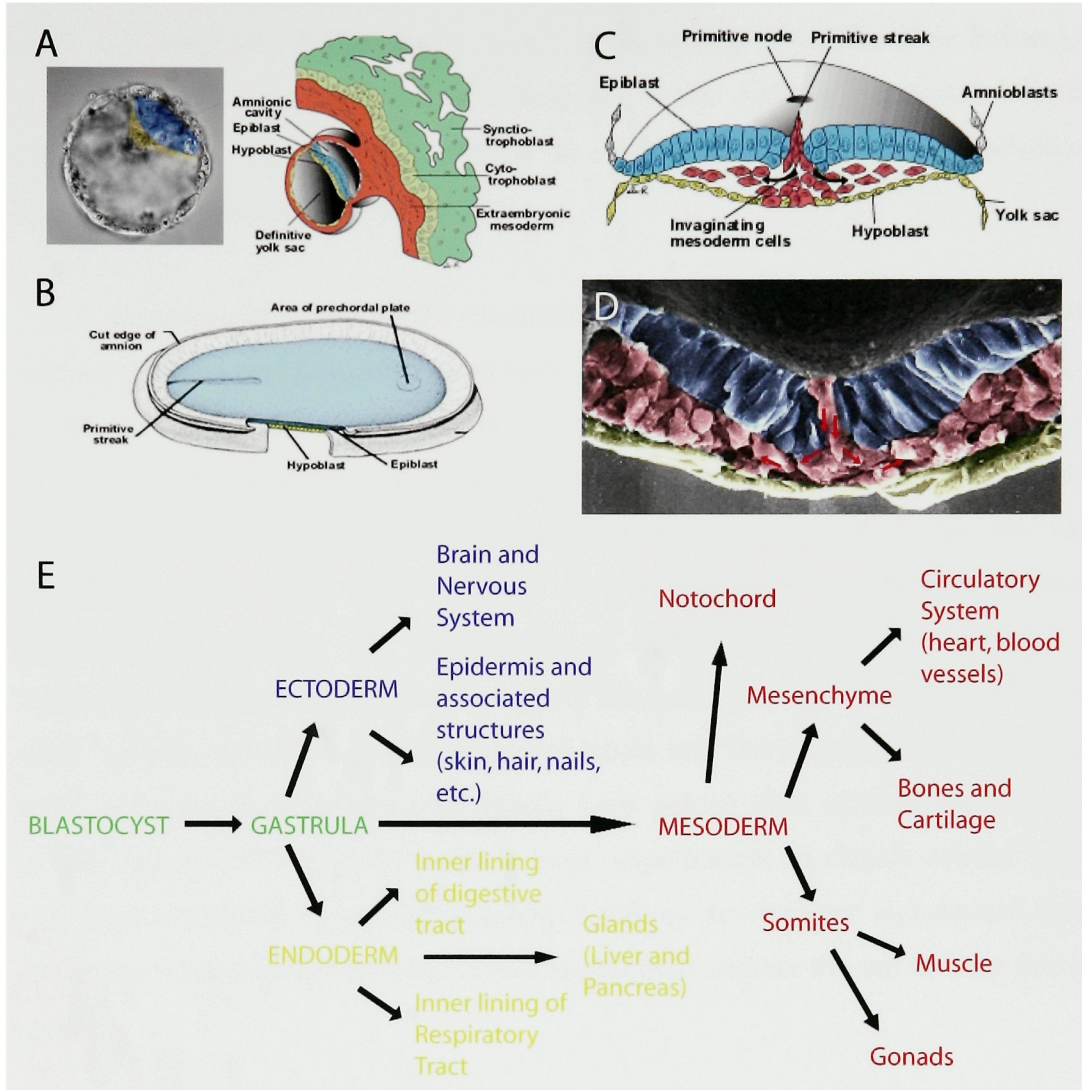


**Figure 1. Schematic representation of primary germ layers and their derivatives before, during and after mammalian gastrulation.** (A) Representation of mammalian blastocyst before and during implantation into uterine epithelium. (B) View of prospective human embryo before the onset of gastrulation. (C) Cross-section of human embryo during gastrulation showing migration of mesodermal cells through the primitive streak. (D) Electron micrograph of mouse embryo during gastrulation showing three primary germ layers. (E) Chart describing the emergence of tissue and organs from primary germ layers and their derivatives. In all panels, ectoderm is represented in blue, mesoderm is shown in red and endoderm in yellow.
(Adapted from UNC Medical School Embryo Images Online)

derivatives of which include the heart, blood and bone, is the middle germ layer; and ectoderm, which gives rise to skin and the central nervous system, is the outermost germ layer. All vertebrates begin development by organizing into these three primary germ layers, and each layer gives rise to homologous organ systems in evolutionarily related species. Hence, by studying animals as distant as frogs, we can advance our understanding of human development.

During embryogenesis, the emergence of a complete repertoire of tissues from the primary germ layers demands a symphony of signaling and gene expression in order to coordinate millions of cells. In the same way that morphogenetic movements of the early embryo are similar between vertebrate species, the major signal transduction pathways that mediate gastrulation are also conserved among vertebrates. In the last half century, molecular studies using animal models have revealed many conserved transduction pathways that mediate the specification of specialized cell fates/identities during vertebrate embryogenesis (Figure 2). Indeed, from *situs inversus* (reversal of siddedness of organ system) to acheiropody (absence of hands and feet), insights into the molecular bases of human birth defects have mostly been gained from experiments using animal models (Leroi, 2003, p.114). In these ways, experiments on closely related organisms have been invaluable to an understanding of human development and disease, but some of the most challenging questions cannot be resolved without the use of bona fide human tissue.

With the emergence of human embryonic stem cells (hESCs) as a resource, scientists today are uniquely situated to capitalize on the last century of developmental research. hESCs are a population of cells derived from a 6 day old human embryo (Figure 3) (Thomson et al., 1998). At this stage, the embryo is at the blastocyst stage, a hollow sphere with an eccentrically placed clump of cells called the inner cell mass (ICM). Embryonic stem cells that are derived from mouse ICM (mESCs) have been shown to give rise to any and all cells of the adult mouse (Reviewed in Smith, 2001), and it is presumed that hESCs have the same capacity. In fact, the stage from which hESCs are derived comes before the specification of primary germ layers via gastrulation, so hESCs

# Figure 2
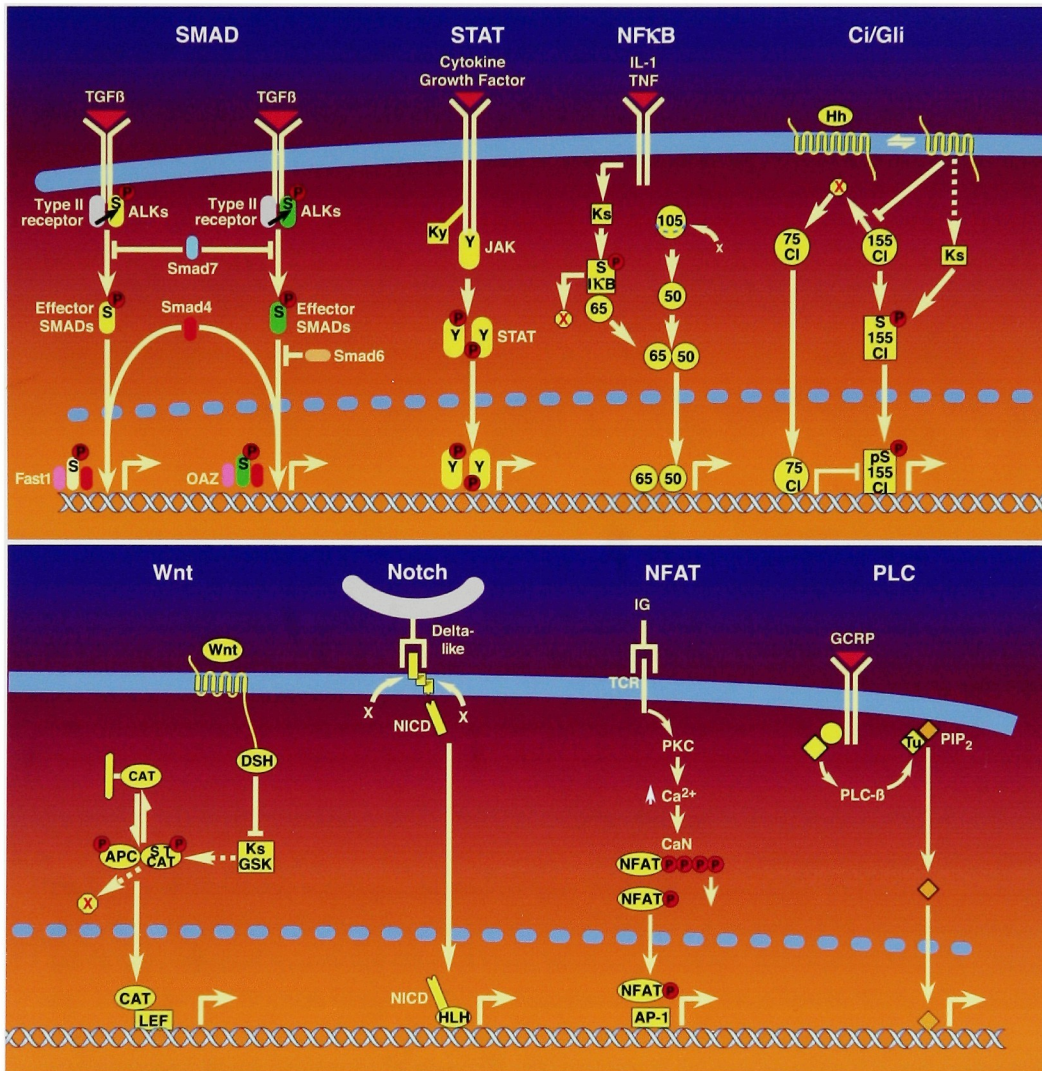


**Figure 2. Eight developmentally relevant signal transduction pathways.** Ligands, intracellular effectors and transcriptional cofactors that mediate signaling by eight canonical signal transduction pathways. These signaling pathways are instrumental to much of the intercellular communication during vertebrate embryogenesis. (Adapted from Brivanlou and Darnell, *Science*, 2002)

# Figure 3
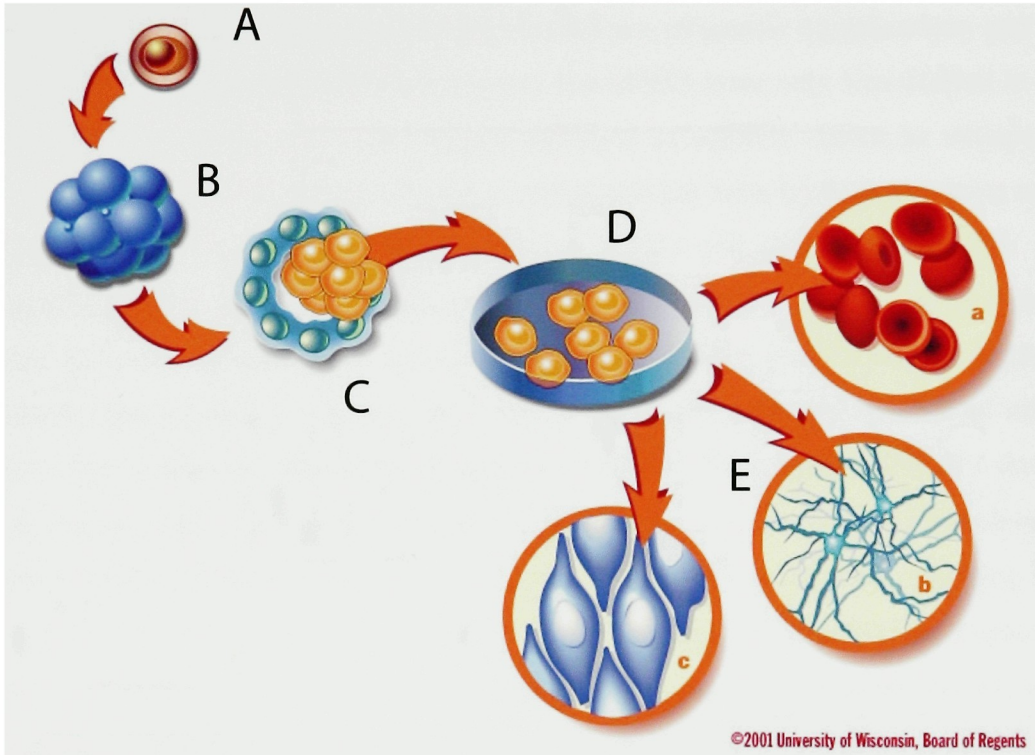


**Figure 3. Schematic representation of the derivation process of human embryonic stem cells from IVF embryos.** (A) Human zygotes generated by in vitro fertilization are cultured to morula (B) and then blastocyst stages (C) in vitro. (D) The inner cell mass of the human blastocyst is isolated and plated on a feeder layer of mouse embryonic fibroblasts. (E) Stable cultures of human embyonic stem cells can give rise to specialized cell types in vitro, including blood (a), nerve cells (b) and muscle (c). (Adapted from University of Wisconsin, Board of Regents, 2001)

are able to assume endodermal, mesodermal, or ectodermal fates, as well as their derivatives. They also have the ability to self-renew and can be maintained for indefinite passages *in vitro*. For these reasons, hESCs offer great potential for the study of human development, as well as the treatment of disease and/or injury using cell-based therapies.

Embryonic stem cells were first discovered in the mouse (mESCs), and for years, mESCs have commonly been used for the introduction of genetic modifications into mice and the study of tissue differentiation *in vitro*. But hESCs were only first derived in 1998 (Thomson et al, 1998), and many known attributes of mESCs cannot be extrapolated directly to apply to hESCs. Hence the pace of research has been brisk, as scientists race to bridge the gap between our understanding of hESCs and their potential. The work comprising this doctoral thesis focuses on two related aspects of hESC biology; the first section addresses the molecular nature of the undifferentiated state of hESCs: Leukemia inhibitory factor (LIF), a cytokine that was shown to maintain self renewal of mESCs (Smith, 2001), does not maintain self renewal of hESCs (Sato et al., 2004). We describe the activation state of another developmentally relevent pathway, the TGFβ pathway, in undifferentiated hESCs and demonstrate a requirement for Activin/Nodal signaling in the maintenance of hESC self-renewal. In the second section, we examine the capacity of hESCs to engraft into blastocyst stage mouse embryos. A significant barrier to the exploration of hESC potential is the absence of human embryos in which to examine the behavior of engrafted hESCs. Engraftment of hESCs into the mouse blastocyst is a potential way of observing their differentiation in an embryonic milieu that approximates the environment from which they were derived, the ICM. In the experiments presented and discussed below, we set out to define some molecular aspects of "stemness" and explore the potential for non-human embryos to serve as a surrogate for the differentiation of hESCs.

# CHAPTER 1

## Maintaining the Undifferentiated State of Human Embryonic Stem Cells

## Summary

Human embryonic stem cells self-renew indefinitely and give rise to derivatives of all three primary germ layers, yet little is known about the signaling cascades that govern their pluripotent character. Because it plays a prominent role in the early cell fate decisions of embryonic development, we have examined the role of TGFβ superfamily signaling in hESCs. We found that, in undifferentiated cells, the TGFβ/activin/nodal branch is activated (through the signal transducer Smad2/3) while the BMP/GDF branch (Smad1/5) is only active in isolated mitotic cells. Upon early differentiation, Smad2/3 signaling is decreased while Smad1/5 signaling is activated. We next tested the functional role of TGFβ/activin/nodal signaling in hESCs and found that it is required for the maintenance of markers of the undifferentiated state. We extend these findings to show that Smad2/3 activation is required downstream of Wnt signaling, which we have previously shown to be sufficient to maintain the undifferentiated state of hESCs. Strikingly, we show that in *ex vivo* mouse blastocyst cultures, Smad2/3 signaling is also required to maintain the inner cell mass (from which stem cells are derived). These data reveal a critical role for TGFβ signaling in the earliest stages of cell fate determination and demonstrate an interconnection between TGFβ and Wnt signaling in these contexts.

# Introduction

Embryonic stem cells (ESCs) are a population of multipotent, self-renewing cells that are derived from the epiblast of mammalian blastocyst embryos and that retain this developmental identity even after prolonged culture *in vitro* (Rossant, 2001). ESCs can be induced to differentiate to functional cell types of all three primary germ layers *in vitro*, and upon integration into host animals, they have the capacity to contribute to all cell types of the embryo, including the germ line (Smith, 2001). These qualities have made ESCs valuable resources for the introduction of complex genetic modifications into mice; and the isolation and culture of human embryonic stem cells (Thomson et al., 1998) has drawn a great deal of attention to their biology and the potential they offer for regenerative medicine, as well as the study of early human development.

Little is known about the signaling pathways that govern the unique properties of hESCs. Maintenance of mESC identity was initially found to be dependent on extrinsic factors that were produced by a feeder layer of mouse embryonic fibroblasts (MEFs). Subsequently, it was found that leukemia inhibitory factor (LIF) was produced by MEFs and was sufficient to maintain ES cell identity in the absence of a feeder layer. LIF, however, is not the lone factor responsible for the maintenance of stem cell identity in the mouse, as null mutants in which LIF/Stat3 signaling is eliminated show no defect in the establishment of the stem cell compartment (reviewed in Smith, 2001). Furthermore, LIF is incapable of maintaining stem cell identity in hESCs (Thomson et al, 1998, Reubinoff et al, 2000, Sato et al, 2004), suggesting a contribution from other signaling pathways to the establishment and/or maintenance of stem cell identity in mammalian development. Reports have linked at least two other transduction pathways to the maintenance of the undifferentiated state. Recent work has shown that the undifferentiated state of hESCs can be maintained in the absence of MEFs by two methods: activation of the Wnt pathway by BIO, a small bioactive molecule that inhibits GSK3β (Sato et al., 2004); or culture in high concentrations of FGF (Xu et al., 2005). But at present, hESCs can only be reliably and consistently maintained undifferentiated through culture in the presence of unknown factors in MEF-conditioned medium.

Assuming hESCs have a cognate population in a developing human embryo, the identity of stem cells may be rooted in the molecular environment found in early embryogenesis. That is, the transduction pathways that dictate cell identity in the embryo may also be involved in stem cell maintenance *in vitro*. Models of early vertebrate development have described a role for multiple signaling cascades in the emergence of pattern and cell identity (Harland and Gerhart, 1997; Scialli, 2003). These models have suggested a prominent role for TGFβ signaling in the earliest cell fate decisions of embryogenesis (Figure 4). For example, in the amphibian *Xenopus leaevis*, the TGFβ pathway is implicated in neural induction and mesendoderm specification, two of the earliest developmental events (reviewed in Munoz-Sanjuan and Brivanlou, 2002); and it is also involved in primitive streak and mesoderm formation in the mouse (reviewed in Goumans and Mummery, 2000).

The TGFβ superfamily, which contains approximately 40 potential ligands in the human genome, signals through two main branches (Figure 5): The bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) ligands account for one branch; and transforming growth factor beta (TGFβ)/Activin/Nodal ligands comprise the second branch. Upon receptor activation, TGFβ signals are conveyed to the nucleus by Smad proteins. Smad1 and Smad5 transduce on behalf of BMP and GDF ligands via the type I receptors ALK 1, 2, 3, and 6; and the TGFβ/Activin/Nodal branch is transmitted by Smad2 and Smad3 via ALK 4, 5 and 7 (reviewed in Shi and Massague, 2003). There are also two inhibitory Smads that provide a repressive input on the pathway; Smad6, which selectively inhibits Smad1/5; and Smad7, which inhibits both branches of TGFβ signaling. And finally there is the common Smad, Smad4, which is an accessory Smad that binds receptor activated Smad1/5 or Smad2/3. Upon activation by phosphorylation and association with a common Smad4, the receptor activated Smads translocate to the nucleus and, in concert with other transcription factors, regulate gene expression (Shi and Massague, 2003).

# Figure 4
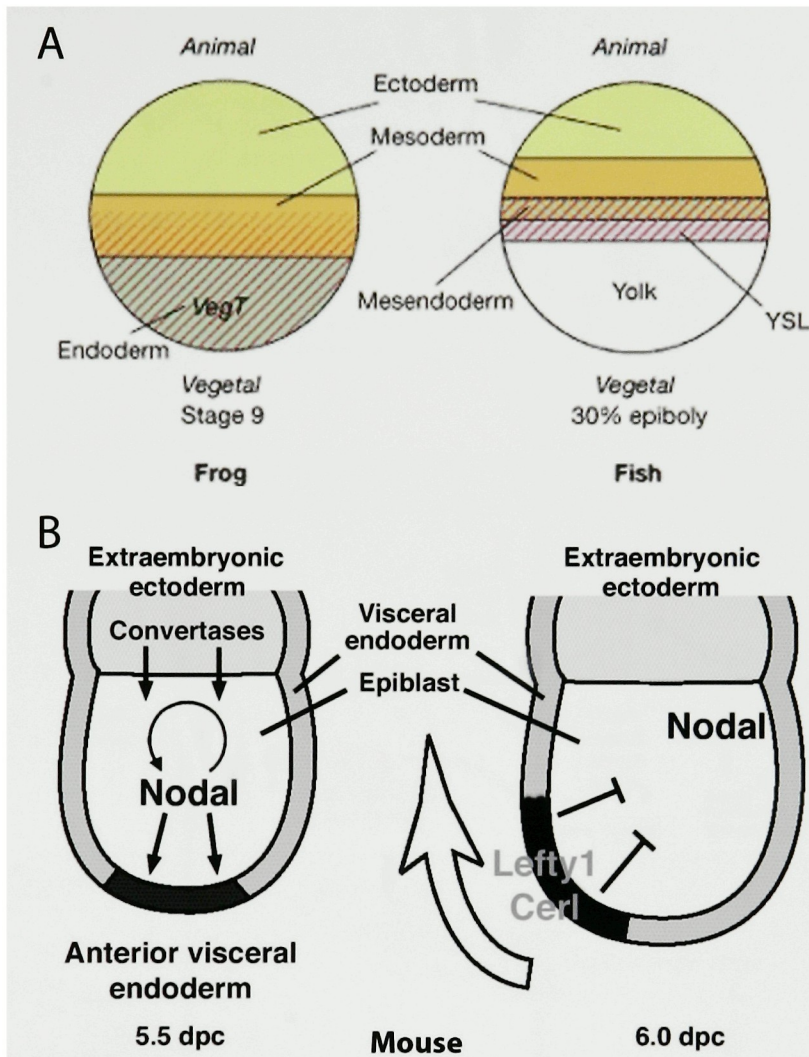


**Figure 4. TGFβ signals figure prominently in early cell fate decisions during vertebrate development.** (A) Expression patterns of Nodal-related genes within the TGFβ superfamily (red hatching) are superimposed on simplified fate maps of early frog and fish embryos. Classical embryological studies have shown that the vegetal region in frog and fish embryos is the source of mesoderm-inducing factors. Nodal-related genes are experessed in these regions. (B) A schematic representation of an early mouse embryo at 5.5 and 6 days post-coitus shows a model of Nodal action. At 5.5 dpc Nodal is expressed in the mouse epiblast and is maintained and enhanced by auto-regulation. Nodal signaling induces lefty1 and cerl in the distal visceral endoderm. Nodal signaling is required for the displacement of anterior visceral endoderm, determining the position of the anterior-posterior axis by 6 dpc. (Adapted from Schier and Shen, Nature 2000; and Schier, AnnuRevCellBio, 2003)

# Figure 5
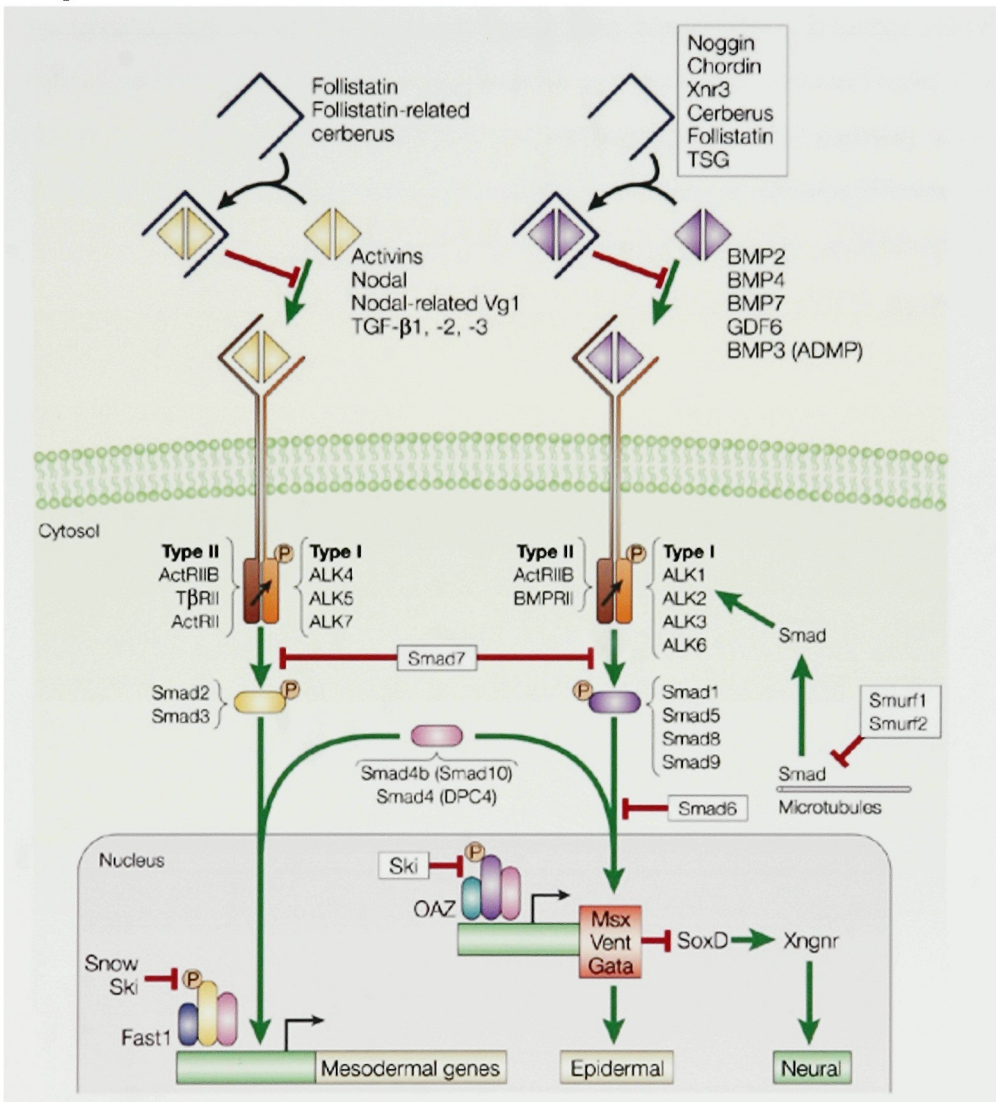


**Figure 5. Extra and Intracellular components of the TGFβ signaling pathway.**
TGFβ ligands signal to the nucleus via two distinct branches. The Activin/Nodal
branch (left) signals via Activin, Nodal and TGFβ ligands which bind receptors at
the cell membrane, resulting in phosphorylation of the effector Smads 2 and/or 3.
The BMP/GDF branch (right) signals via BMP and GDF ligands which bind recep-
tors, resulting in phosphorylation of Smads 1/5 and/or 8. Upon phosphorylation,
effector Smads from both branches bind Smad4 and accumulate in the nucleus,
where they collaborate with transcriptional co-factors to drive gene expression.
There are also inhibitory Smads 6 and 7, which apply a repressive input on the
accumulation of effector Smads in the nucleus.
(Adapted from Munoz-Sanjuan and Brivanlou, *Nature Neuro Reviews*, 2002)

Here we show that activation of the Activin/Nodal branch through Smad2/3 is associated with pluripotency and is required for the maintenance of the undifferentiated state in hESCs and in *ex vivo* mouse blastocyst outgrowths. Whether hESCs were maintained in MEF-conditioned medium or BIO, we show that Activin/Nodal signaling is required downstream of both conditions, in that inhibition of this pathway with a small molecule inhibitor or soluble receptors results in a loss of the undifferentiated state. Accordingly, we find that exogenous ActivinA is supportive of the undifferentiated state. In our study of hESCs, we have revealed a critical role for TGFβ signaling in the regulation of ES cell identity.

## Results

**The undifferentiated state of hESCs is characterized by activation of Smad2/3 signaling and inhibition of Smad1/5 signaling**

While TGFβ signaling has been shown to play a role in primary cell fate decisions in many developmental models, its role in ESC fate determination remains uncertain. To study the involvement of the TGFβ superfamily pathway in ESC fate decisions, we first analyzed the activation status of both arms of this family (TGFβ/activin/nodal signaling and BMP/GDF signaling) in human embryonic stem cells, both in the undifferentiated state and during the early phase of differentiation in response to certain modulators of TGFβ superfamily activation (Figure 6).

In the undifferentiated state that is maintained by growth in MEF conditioned medium (CM), we found that Smad2/3 was phosphorylated and localized to the nucleus of hESCs, indicating activation of the TGFβ/activin/nodal pathways. Furthermore, the phosphorylation and nuclear localization were both reduced in cells that were allowed to differentiate by growth in non-conditioned medium (nCM) (Figure6A and B). We have previously shown that the GSK3β inhibitor BIO is also capable of maintaining hESCs in the undifferentiated state, even in the absence of CM, through activation of canonical Wnt signaling (Sato et al, 2004). Accordingly, BIO maintained phosphorylation of Smad2/3 above levels seen in hESCs grown in nCM alone, and this effect was accompanied by maintenance of Oct3/4, a marker of pluripotency. To test the ability of hESCs cultured in nCM to activate Smad2/3 signaling, we cultured the cells in nCM supplemented with ActivinA. Under these conditions, hESCs maintained high levels of Smad2/3 phosphorylation as well as increased Oct3/4 relative to cells cultured in nCM alone, though morphologically, cells were not identical to undifferentiated hESCs. These results suggest that Smad2/3 activation in the absence of extrinsic factors present in CM may be supportive of the undifferentiated state, though not entirely sufficient to maintain typical hESC morphology nor markers of pluripotency through extended culture (Figure 6A-C).

# Figure 6
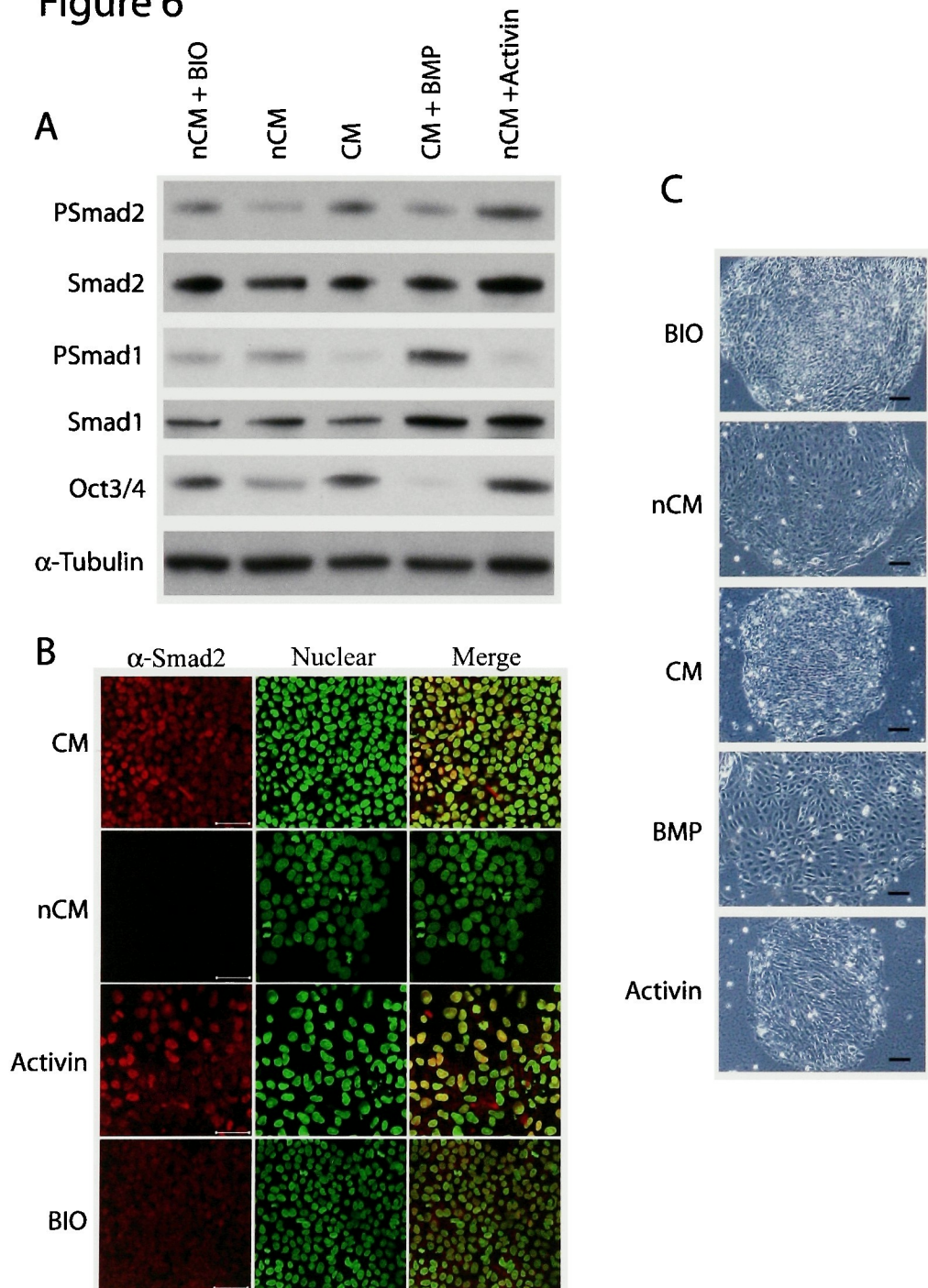


**Figure 6. The undifferentiated state of hESCs is characterized by activation of Smad2/3 mediated signal transduction and inhibition of Smad1/5 mediated signal transduction.** (A)Western blot analysis of H1 hESCs cultured in various conditions. Cells were cultured for four days in the presence of nCM supplemented with 2 µM BIO, non-conditioned medium (nCM), MEF conditioned medium (CM), CM supplemented with 25 ng/ml BMP4 and nCM supplemented with 25 ng/ml ActivinA. Membranes were probed with antibodies specific for phosphorylated Smad2, Smad2/3, phosphorylated Smad1/5, Smad1/5, Oct3/4 and α-tubulin (as a control for protein loading). (B) Immunofluorescence microscopy of BGN2 hESCs in the undifferentiated and differentiated state. BGN2 cells were cultured for 5 days in the presence and absence of CM, in nCM supplemented with 25 ng/ml ActivinA and with nCM supplemented with 2µM BIO. Cells were decorated with an antibody specific for Smad2/3, as well as SytoxGreen nuclear counterstain. (C) Brightfield images of H1 hESCs cultured in conditions described for panel (A). All scale bars represent 50 µm.

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We also examined the state of Smad1/5 phosphorylation in hESCs grown in the above conditions. Smad1/5 activation in these cells showed the opposite character of Smad2/3 activation; in the undifferentiated state, Smad1/5 phosphorylation was barely evident and upon differentiation, phosphorylation was globally increased and localized to the nucleus (Figure 6A and 7A). Addition of ActivinA to nCM reduced phosphorylation of Smad1/5 to levels comparable to those of hESCs grown in CM, suggesting an input of Smad2/3 mediated signaling on the suppression of Smad1/5 activation. To study the effect of induced Smad1/5 activation in undifferentiated hESC, we cultured cell in CM supplemented with BMP4. As previously reported (Xu et al, 2002b), these conditions resulted in a decrease in Oct3/4 levels and a concomitant change in morphology (Figure 6A,C). Thus, we have shown that Smad2/3 activation correlates with stemness in hESCs descriptively and functionally, while Smad1/5 activation correlates with differentiation.

Minimal global levels of Smad1/5 phosphorylation in undifferentiated hESCs may be accounted for by the observation that phosphorylation was only evident in the cytoplasm of cells undergoing mitosis (arrows in Figure 7A). In order to assess the relationship between Smad1/5 phosphorylation and mitotic index, hESCs were synchronized by incubation with colcemide, which arrests cells at the onset of metaphase. Smad1/5 phosphorylation was compared between hESCs that had been released from the colcemide block for different lengths of time and asynchronous hESCs (Figure 7B). hESCs that were released from colcemide block for 15 minutes showed higher levels of Smad1/5 phosphorylation than hESCs that had been released for 4 hours. Asynchronous hESCs show slightly reduced levels relative to cells that had been blocked and released for 15 minutes. To measure the efficacy of colcemide in hESCs synchronization, we used an antibody which is specific for phospho-serines on CDKs that are phosphorylated only in the context of mitosis. BMP stimulated hESCs were used as a control for Smad1/5 phosphorylation. Interestingly, a correlation between Smad1/5 phosphorylation and mitotis was also apparent in dividing cells of preimplantation mouse embryos (Figure 7C).

# Figure 7
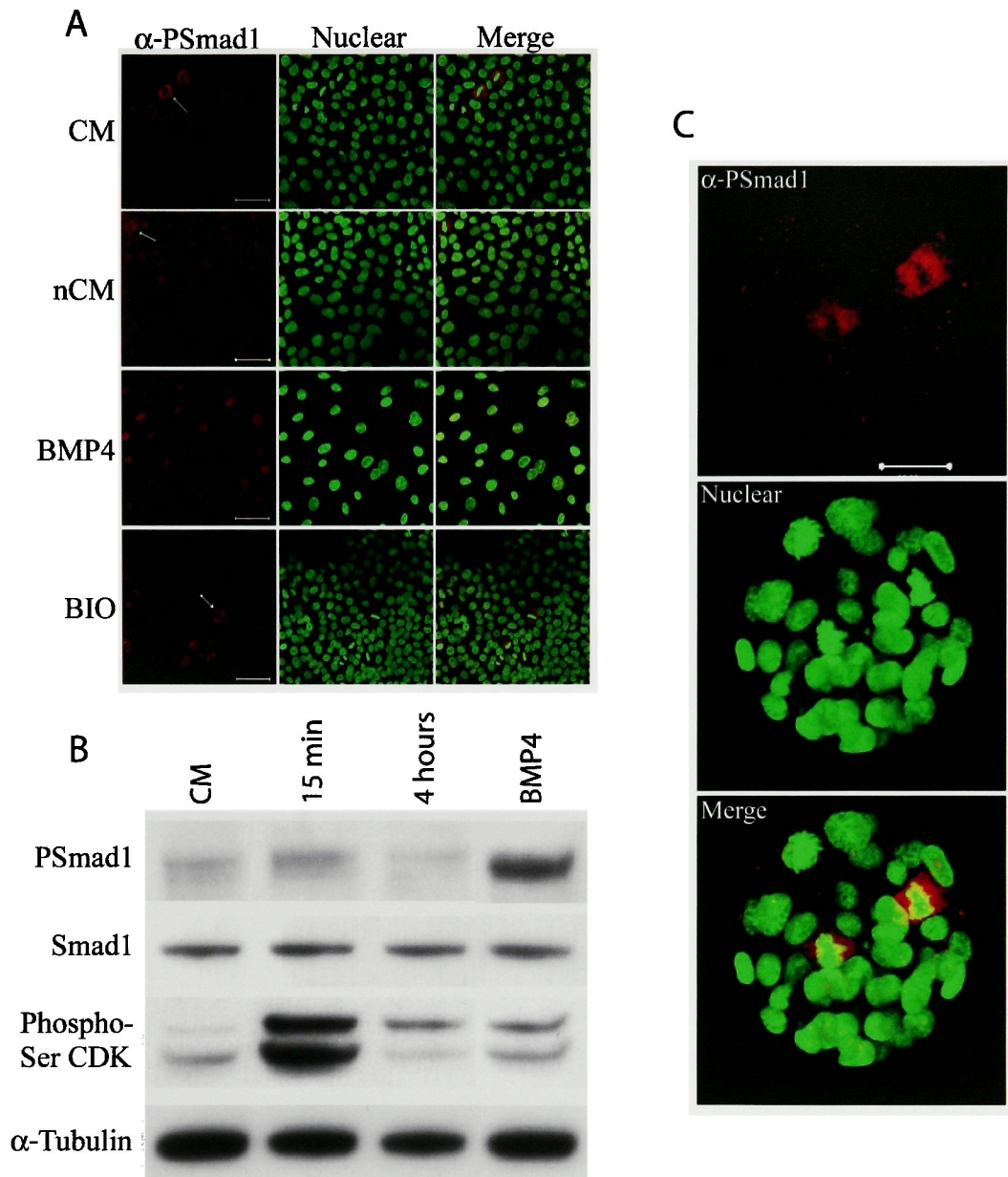


**Figure 7. Global Smad1/5 phosphorylation is increased under differentiation conditions and is evident in mitotic hESC in the undifferentiated state.** (A) Immunofluorescence microscopy of BGN2 hESCs in the undifferentiated and differentiated state. BGN2 cells were cultured for 5 days in the presence and absence of CM, in CM supplemented with 25 ng/ml BMP4 as a control for Smad1/5 activation and nCM supplemented with 2 μM BIO. Cells were decorated with an antibody specific for phosphorylated Smad1/5, as well as SytoxGreen nuclear counterstain. Arrows indicate mitotic cells. Scale bars represent 50 μm. (B) Western blot analysis of colcemide synchronized BGN1 hESCs. Cells were blocked at metaphase by incubation with 100 ng/ml demecolcine solution and harvested at 15 minutes and 4 hours post-release. Cells grown in CM alone and CM supplemented with 25 ng/ml BMP4 were used as controls for asynchronous and Smad1/5 activated cells, respectively. Membranes were probed with antibodies specific for phosphorylated Smad1/5, Smad1/5, phosphorylated Ser CDKs substrate, and α-tubulin (as a control for protein loading). (C) Immunofluorescence microscopy of blastocyst stage embryo containing mitotic cells. Mouse blastocyst embryos were fixed and decorated with anti-phospho Smad1/5 antibody and SytoxGreen nuclear counterstain and then imaged by confocal microscopy. Scale bars represent 20 μm.

**Smad2/3 activation is necessary for maintenance of the undifferentiated state in hESCs**

Having defined the nature of Smad1/5 and Smad2/3 activation in undifferentiated and differentiating hESC, and having discovered a correlation between increased Smad2/3 activation and the undifferentiated state, we set out to determine whether active Smad2/3 signaling is necessary for the maintenance of the pluripotent state (Figure 8). The TGFβ/Activin/Nodal branch of TGFβ signaling can be efficiently inhibited by SB-431542, a synthetic compound that precludes Smad2/3 phosphorylation by type 1 TGFβ receptors (Laping et al, 2002). We challenged hESCs cultured in the presence of CM or BIO with SB-431542. As expected, phosphorylation of Smad2/3 is reduced in hESCs cultured in the presence of SB-431542 and the ability of CM or BIO to maintain protein levels of the pluripotency marker, Oct3/4, was lost upon challenge with SB-431542. In addition to reducing Smad2/3 phosphorylation and Oct3/4, SB-431542 also has the effect of increasing Smad1/5 phosphorylation to the levels seen in differentiating hESCs (Figure8A).

Prompted by the result that intact Smad2/3 signaling was necessary for the maintenance of high Oct3/4 protein levels in hESCs, we examined the expression of known markers of pluripotency in hESCs with the variable levels of Smad2/3 activation described above (Figure 8B). Upon withdrawal of CM and the subsequent reduction of Smad2/3 phosphorylation, hESCs showed reduced expression of Oct3/4 as well as another established marker of pluripotency, Nanog. Inhibition of Smad2/3 activation by SB-431542 in the presence of CM or BIO resulted in significantly reduced expression of both markers. Consistently, SB-431542 promoted differentiation by morphological criteria, inducing cells to assume a flat, spread out morphology (Figure 8C). Stimulation of TGFβ/Activin/Nodal signaling by ActivinA in the presence of nCM restored the expression of both markers to levels above those exhibited by hESCs cultured in nCM alone. Hence, by measure of two independent markers of pluripotency, Smad2/3 activation is not only necessary to, but also supportive of the undifferentiated state of hESCs.

# Figure 8
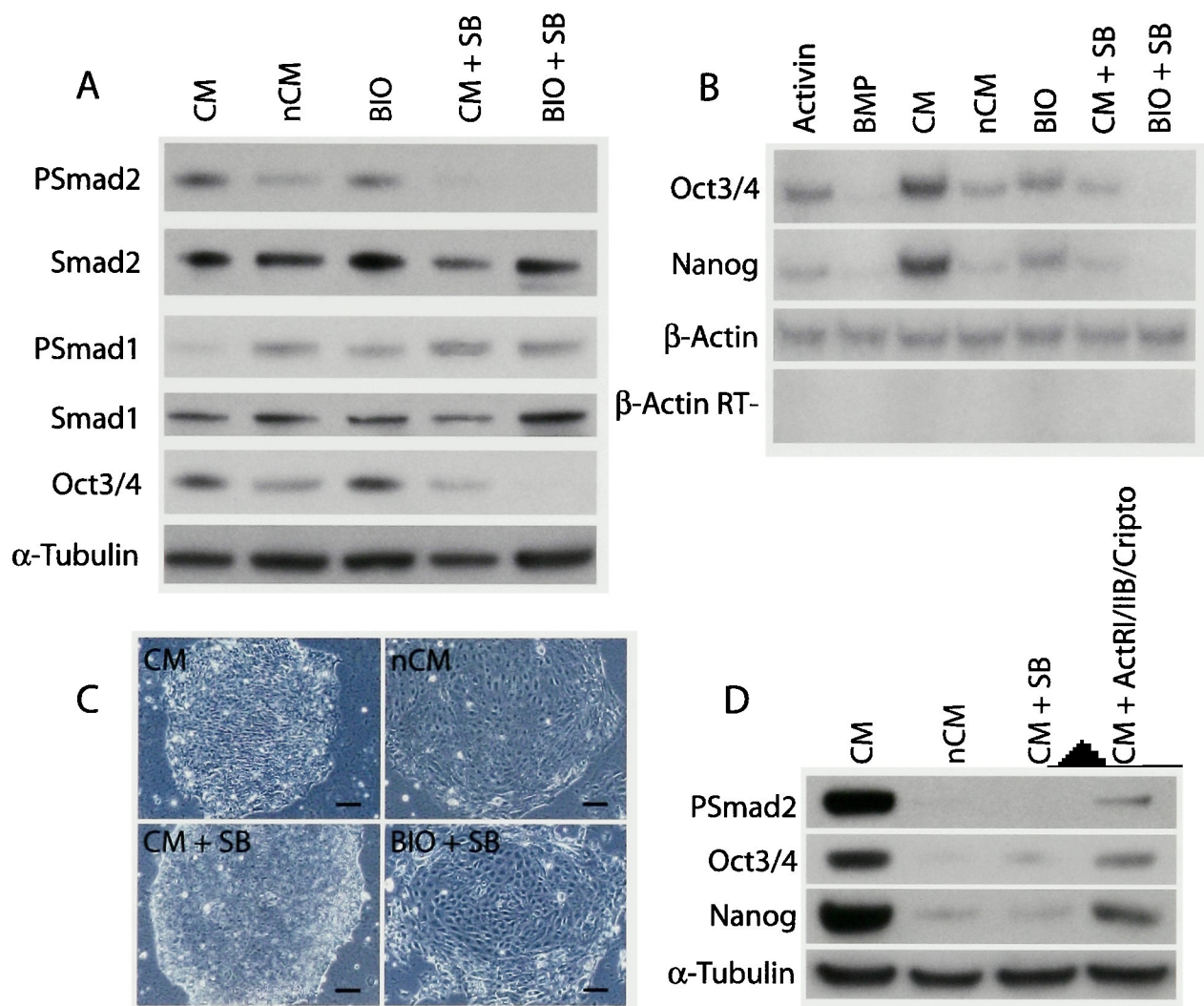


**Figure 8. Intact Smad2/3 signaling is required for the maintenance of the undifferentiated state in hESCs.**
(A) Western blot analysis of H1 hESCs cultured in conditions in which Smad2/3 signaling is intact or inhibited. Cells were cultured for 4 days in the presence of CM, CM supplemented with 10μM SB-431542, nCM supplemented with 2 μM BIO, and nCM supplemented with 2 μM BIO and 10 μM SB-431542. Membranes were probed with antibodies specific for phosphorylated Smad2, Smad2/3, phosphorylated Smad1/5, Smad1/5, Oct3/4 and α-tubulin (as a control for protein loading). (B) Expression analysis of H1 hESCs. Cells were cultured for 4 days in CM, nCM, nCM supplemented with 25 ng/ml ActivinA, CM supplemented with 25 ng/ml BMP4, CM supplemented with 10 μM SB-431542, nCM supplemented with 2 μM BIO, and nCM supplemented with 2 μM BIO and 10 μM SB-431542. RT-PCR was performed on these cells using primers for human Oct3/4, Nanog, β-actin (as a loading control) and β-actin RT minus (as a control for contamination with genomic DNA). (C) Brightfield views of hESCs cultured in conditions described in panels A and B. Scale bars - 50μm (D) Western blot analysis of H1 hESCs cultured in the presence of a cocktail of soluble receptors specific to the Activin/Nodal pathway. Cells were cultured for 5 days in the presence of CM, nCM, CM supplemented with 10 μM SB-431542 and CM supplemented with hrActRIB (5 μg/ml), hrActRIIB (5 μg/ml) and hrCripto (250 ng/ml). Membranes were probed with antibodies specific for phosphorylated Smad2, Oct3/4, Nanog and α-tubulin (as a control for protein loading).

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We next set out to confirm that our observations concerning the effect of SB-431542 were specific to its inhibition of Activin/Nodal signaling through Smad2/3. Small molecule inhibitors have tremendous experimental value because they allow strict temporal and dose-dependent control of their effect and can traverse most, if not all, compartments of a cell, but they also have the potential to affect unrelated signal transduction pathways. In order to rule out the possibility that SB-431542 was mediating its effect by disrupting signal transduction unrelated to the TGFβ superfamily, we used an independent means of inhibiting Activin/Nodal signaling, culturing hESCs in CM supplemented with a combination of human recombinant ActRIB, hrActRIIB, and hrCripto (Figure 8D). These three proteins in their native state form a complex and bind Activin/Nodal ligands in canonical TGFβ signaling, so soluble extracellular domains should provide an alternative to SB-431542 by competing for Activin/Nodal in CM. Both Oct3/4 and Nanog were significantly reduced in hESCs cultured in the presence of soluble receptors (Figure 8D), thus providing corroborative data demonstrating the requirement for this pathway in the maintenance of pluripotent hESCs.

Our results indicated that the negative effect of SB-431542 on maintenance of hESC pluripotency is specific to canonical TGFβ signaling, but this pathway is involved in many of the more fundamental aspects of cell biology, including cell viability, adhesion, migration and proliferation (reviewed in Massague et al, 2000). Thus, the global inhibition of Alk4/5/7 activation by SB-431542 could be affecting the fundamental cellular biology of hESCs in a manner independent of differentiation pathways. In order to address whether SB-431542 was affecting cell viability or proliferation of hESCs, we performed Brdu incorporation and TUNEL assays (Figure 9A) and found no significant effect of SB-431542 on these parameters.

Apart from its role in activating the TGFβ/activin/nodal branch of TGFβ signaling, Alk4/5/7 has also been implicated in activation of MAP kinases, including p38, Jnk, and Erk. It has previously been shown that, at the doses we have used, SB does not significantly affect activation of other pathways in cell culture (Inman et al, 2002). To confirm this in hESCs, we assessed the phosphorylation of MAP kinases p38, JNK, and

# Figure 9
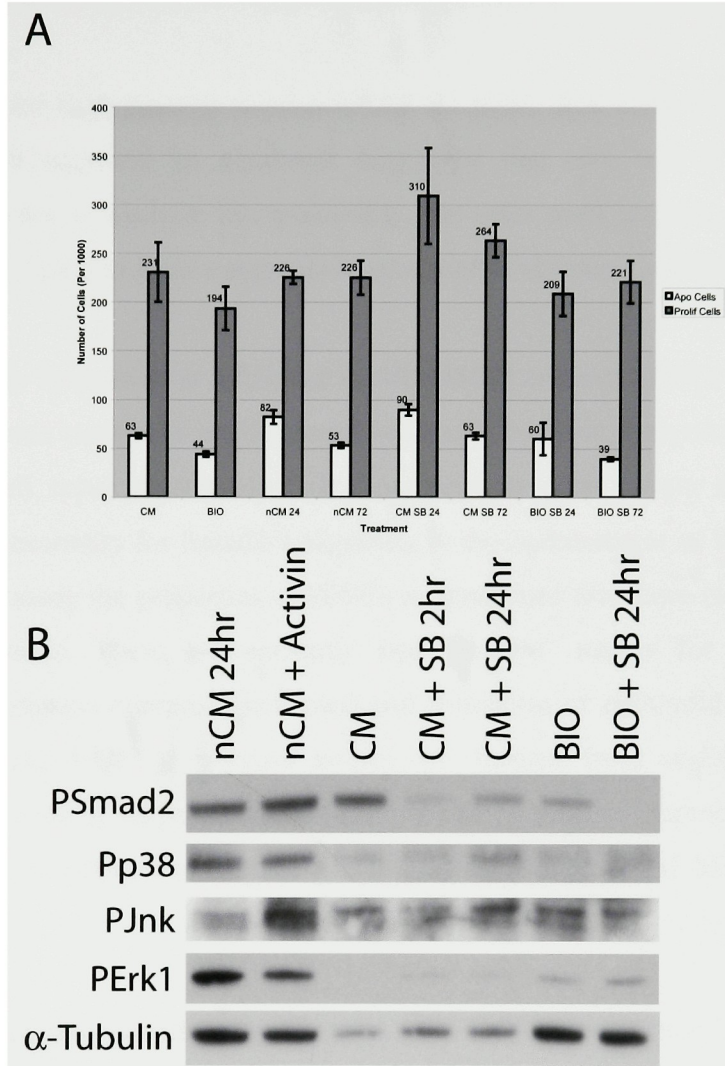


**Figure 9. Effect of SB-431542 is not due to alterations in hESC viability or related Alk4/5/7 receptor mediated signal transduction** (A) Histogram describing Brdu incorporation and TUNEL assays performed on H1 hESCs. Cells were cultured for 3 days (except when indicated otherwise) in the presence of CM, nCM, CM with 10 µM SB-431542, nCM with 2 µM BIO and nCM with 2 µM BIO and 10 µM SB-431542. Brdu incorporation and TUNEL assays were performed on three successive passages of H1 hESCs. (B) Western blot analysis of H1 hESCs cultured in conditions shown. Cells were cultured for 2 or 24 hours in the presence of nCM supplemented with 25ng/ml ActivinA, CM, CM supplemented with 10µM SB-431542 and nCM supplemented with 2µM BIO with or without 10µM SB-431542. Membranes were probed with antibodies specific for phosphorylated forms of Smad2, p38, Jnk and Erk1, as well as α-tubulin (loading control).

Erk in the presence and absence of SB-431542 and found that the phosphorylation of these effector molecules was not regulated upon addition of SB-431542 (Figure 9B).

Due to the high passage number of the H1 hESC line used, it is possible that the cells may have acquired an abnormal karyotype that endowed it with an atypical character. In order to exclude this possibility, the cells used in these experiments were karyotyped and found to have a normal complement of chromosomes (data not shown).

**Embryoid body formation is inhibited in SB-431542 treated hESCs**

Embryonic stem cells are defined functionally by their pluripotency - the ability to give rise to cell types representing all three primary germ layers of the embryo. In examining the necessity for Smad2/3 signaling in the maintenance of the pluripotency, it is important to assay the properties of hESCs in conditions that more closely approximate *in vivo* conditions. There are currently two "*in-vivo*" assays for hESCs: teratoma formation in immuno-compromised mice and formation of embryoid bodies in culture (Thomson et al., 1998). Embryoid bodies are formed from undifferentiated hESC aggregates cultured in suspension and they typically contain differentiated cell types of ectodermal, mesodermal and endodermal lineages. Differentiated hESCs do not form embryoid bodies.

We examined the efficiency of embryoid body formation from hESCs cultured for four days under condtions in which Smad2/3 signaling was activated or inhibited (Figure 10A). Addition of the inhibitor SB-431542 to cells grown in CM significantly reduced the number of embryoid bodies that formed. Cells grown in the presence of BIO were able to form more embryoid bodies than cells grown in nCM alone, and these embryoid bodies were similar morphologically to those formed from hESCs grown in CM (Figure 10B). This effect was drastically reduced in the context of Smad2/3 inhibition and the few embryoid bodies that did form were of an atypical, multi-cystic morphology (Figure 10B). Activation of Smad2/3 alone by ActivinA conferred upon the cells a marginally increased ability to generate embryoid bodies, which is consistent with the ability of ActivinA to restore Oct3/4 levels in cultured hESCs. These data support the notion that

# Figure 10
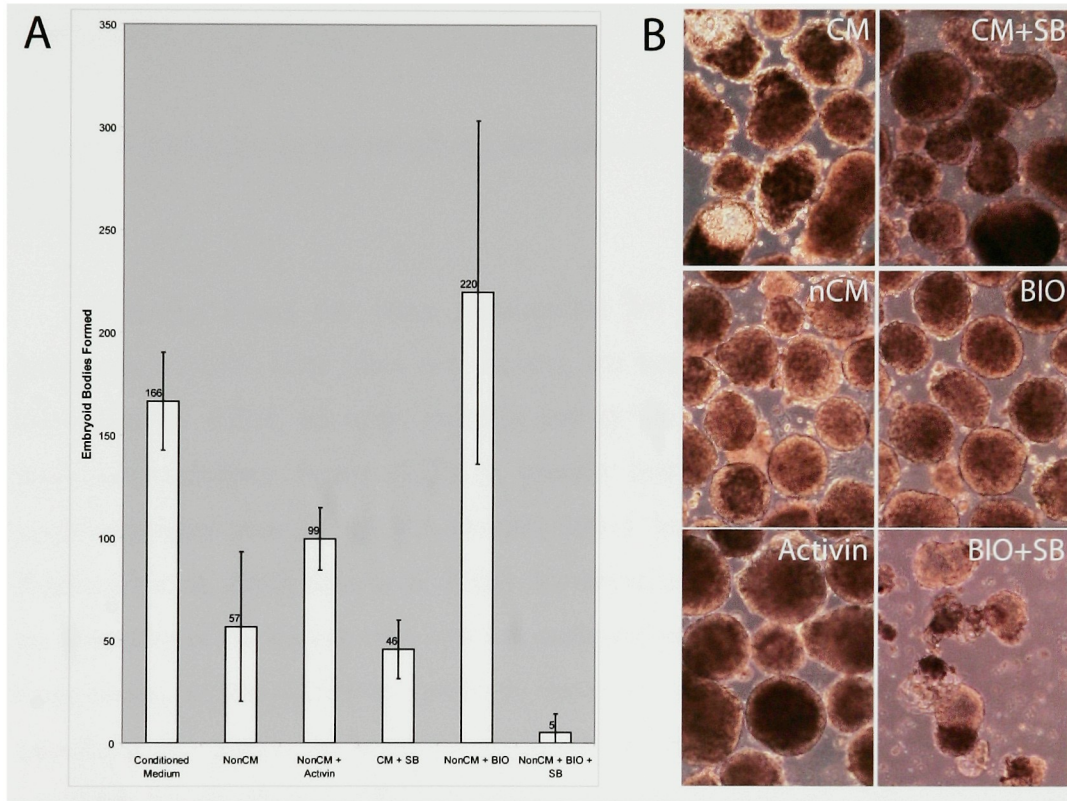


**Figure 10. Inhibition of Smad2/3 signaling adversely affects the ability of hESCs to form embryoid bodies.** (A) Histogram describing number of embryoid bodies formed from BGN2 hESCs. Cells were cultured for 7 days in CM, nCM, nCM with 25 ng/ml ActivinA, CM with 10 µM SB-431542, nCM with 2 µM BIO and nCM with 2 µM BIO and 10 µM SB-431542. Cells were then detached from substrate and cultured in a suspension of nCM for 7 days further. Histograms and error bars represent the experiment performed in triplicate and on two separate passages of BGN2 cells. (B) Brightfield images of embryoid bodies formed under the conditions represented in the histogram in (A).

Smad2/3 activation is necessary but only partially sufficient for maintenance of pluripotency.

In order to assess the character of embryoid bodies formed from hESCs cultured in the above conditions, we assayed the differentiated cell types contained within them by RT-PCR and found that markers of all three primary germ layers were expressed (data not shown).

**Smad2/3 activation is not necessary for maintenance of the undifferentiated state in mESCs**

Having found the same requirement for active Smad2/3 signaling in three independent hESC lines (data not shown), we next assayed the relevance of Smad2/3 activation to mESC identity. mESCs are typically cultured in medium containing leukemia inhibitory factor (LIF), a protein that has been shown to maintain the undifferentiated state of mESCs (Smith, 2001), but not hESCs (Thomson et al, 1998, Reubinoff et al, 2000, Sato et al, 2004). However, a role for LIF in the establishment and maintenance of the mouse stem cell compartment *in vivo* is uncertain, as null mutants for components of the LIF/Stat3 pathway have no stem cell defect (Smith, 2001). We examined the nature of Smad2/3 signaling in mESCs with the expectation that the necessity for Smad2/3 signaling in mESCs cultured in medium containing LIF would be similar to that observed for hESCs cultured in MEF conditioned medium (Figure 11A).

mESCs were cultured in defined medium without a contribution of growth factors from serum. Upon withdrawal of LIF, levels of Smad2/3 phosphorylation in mESCs were reduced, but the SB-431542-mediated inhibition of Smad2/3 signaling in the context of LIF resulted in no significant change in Oct3/4 levels. We have previously shown that BIO is able to maintain mESCs as well as hESCs in the undifferentiated state (Sato et al, 2004). In support of this result, mESCs grown in the presence of BIO were able to maintain levels of Oct3/4 relative to mESCs cultured in the presence of LIF, but unexpectedly, inhibition of Smad2/3 signaling in the context of BIO had no effect on

# Figure 11

**A**



Lanes: -LIF, +LIF, +LIF + SB, Activin, BIO, BIO + SB, CM, CM + SB

Rows: PSmad2, Smad2, Oct3/4, α-Tubulin

**B**



Columns: α-PSmad2, Nuclear, Merge

Rows: 2CS, 4CS, 8CS, Morula, Blastocyst

**C**

4 Days DMSO | 4 Days 20µM SB

Rows: α-Oct3/4, Nuclear, Merge

|  | Control | 20µM SB | Soluble Receptors |
|---|---|---|---|
| Blastocysts Extracted | 31 | 30 | 30 |
| Blastocysts Attached | 19 | 21 | 24 |
| Oct3/4 Positive Outgrowths | 14 (74%) | 6 (29%) | 8 (33%) |
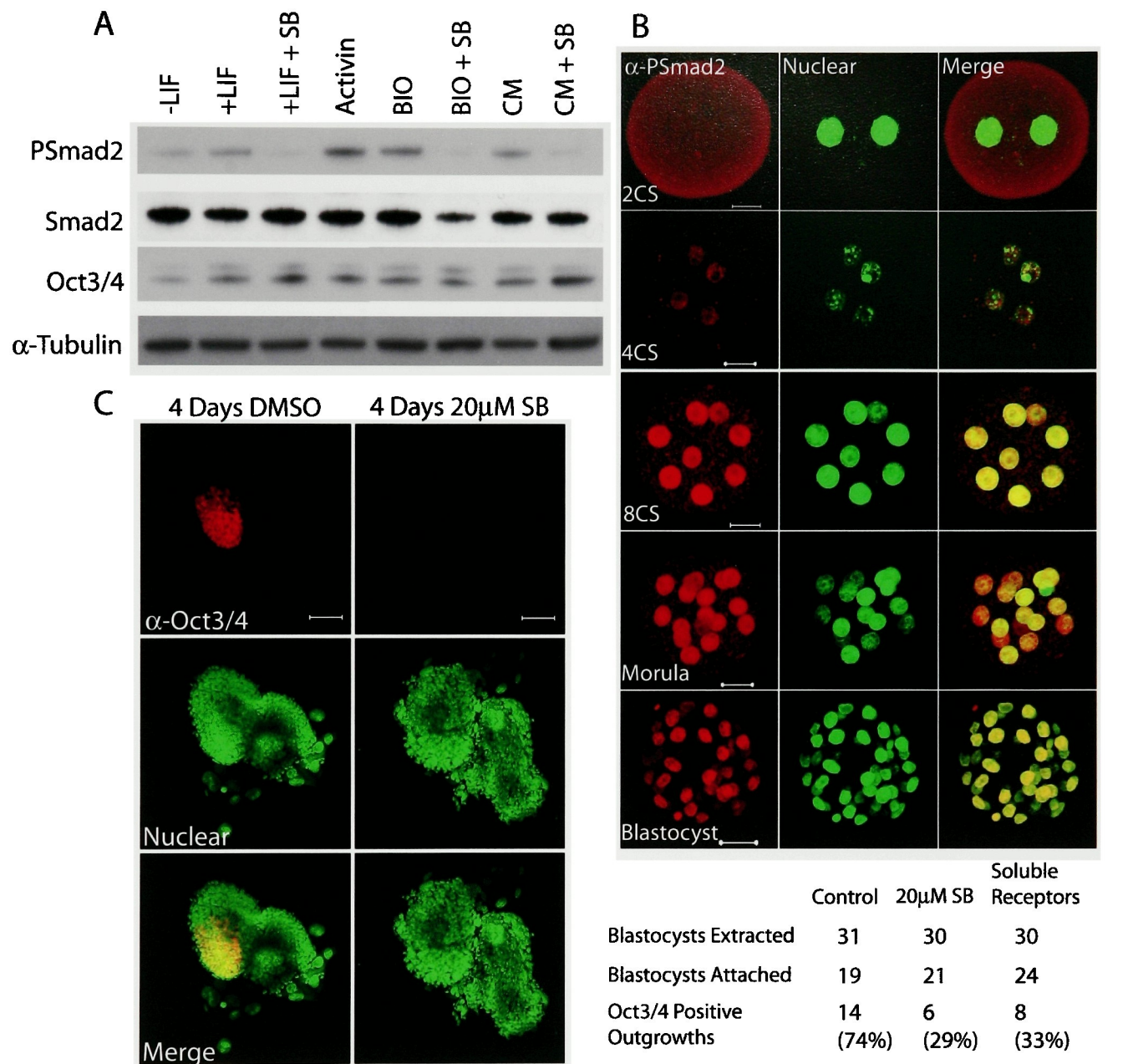
**Figure 11. Intact Smad2/3 signaling is not required for the maintenance of the undifferentiated state of mESCs, but is required for the maintenance of the stem cell compartment of blastocyst outgrowths.**
(A) Western blot analysis of 129/SVJ mESCs. mESCs were cultured for 3 days in mESC medium with and without LIF, mESC medium with LIF plus 10 µM SB-431542, mESC medium with 25 ng/ml ActivinA, mESC medium with 2 µM BIO, mESC medium with 2 µM BIO plus 10 µM SB-431542, CM, CM with 10 µM SB-431542. Membranes were probed with antibodies specific for phosphorylated Smad2, Smad2/3, Oct3/4 and α-tubulin (as a control for protein loading). (B) Whole mount immunofluorescent confocal microscopy of pre-implantation stage mouse embryos. Mouse embryos were extracted and fixed at 2 cell, 4 cell, 8 cell, compacted morula and blastocyst stages. Embryos were decorated with an antibody specific for phosphorylated Smad2 and SytoxGreen nuclear counterstain. Scale bars represent 20 µm. (C) Confocal immunofluorescent microscopy of blastocyst outgrowths. Mouse blastocyst stage embryos were extracted and cultured in the presence of mESC medium supplemented with DMSO or 20 µM SB-431542 for 4 days. Outgrowths were decorated with an antibody specific for Oct3/4 and SytoxGreen nuclear counterstain. The table indicates the percentage of embryos exhibiting Oct3/4 positive cells. Scale bars represent 100 µm.

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Oct3/4 levels (Figure 11A). Taken together, these results suggest that mESCs are dissimilar to hESCs in that they have no requirement for active Smad2/3 signaling in the maintenance of pluripotency. Furthermore, the ability of BIO to maintain the undifferentiated phenotype of these cells is dependent on active Smad2/3 signaling in hESCs but not in mESCs.

**Activation of Smad2/3 is required for maintenance of pluripotency in the ICM of mouse blastocyst outgrowths**

The behavior of embryonic stem cells *in vitro* is obviously different from that of a developing embryo *in vivo*, and the character of mESCs does not necessarily reflect that of their founder population in the ICM of the mouse blastocyst. So the fact that TGFβ signaling seems to be negligible for the maintenance of pluripotency in mESCs is no indication as to the requirement for TGFβ signaling in peri-implantation mouse embryos. TGFβ ligands and receptors are expressed very early in mouse preimplantation embryos (Albano et al, 1993; Mummery et al, 1993; Paria and Dey, 1990; Roelen et al, 1994; Slager et al, 1991), and a recent report showed that genetic deletion of both Smad2 and Smad3 results in reduced epiblast and Oct3/4 levels in mouse embryos at embryonic day 7.5 (Dunn, et al, 2004). This prompted us to investigate the requirement for Smad2/3 activation in the maintenance of the stem cell compartment of mouse blastocysts. Mouse blastocyst outgrowths are used to assess the character and potency of two cell types present in blastocyst stage embryos, the trophectoderm and inner cell mass (ICM). When cultured *ex vivo*, the trophectoderm cells of the blastocyst adhere to the matrix substrates and migrate across them, while the inner (ICM) maintains a compact morphology. It is the inner cell mass from which pluripotent embryonic stem cells are derived, and only these cells maintain Oct3/4 expression upon outgrowth.

In order to assess the state of Smad2/3 activation in early mouse development, we performed confocal immunofluorescence microscopy on mouse embryos from two-cell to late blastocyst stages (Figure 11B). Smad2/3 was phosphorylated and localized to the nucleus beginning at the four cell stage and remained phosphorylated in both the trophectoderm and ICM up to the blastocyst stage. To assess the requirement for

Smad2/3 activation in peri-implantation mouse embryos, we cultured blastocyst stage embryos for 4 days in the presence or absence of SB-431542. We assayed the presence of Oct3/4 in outgrowths cultured from the blastocyst stage for 4 days, both in the presence and absence of SB-431542 and found that 74% of control outgrowths (14 of 19) were positive for Oct3/4 staining while only 29% of SB-431542 treated outgrowths (6 of 21) showed Oct3/4 staining; typical control and SB-431542 treated embryos in which the Oct3/4 compartment is maintained and lost, respectively, are represented in Figure 11C. Strikingly, while Oct3/4 protein is present in a majority of outgrowths cultured for 4 days in the presence of DMSO, most of those cultured in SB-431542 display a complete loss of Oct3/4 staining. Hence, Smad2/3 activation is required not only for maintenance of pluripotency of hESC cultured *in vitro*, but it is also required for the maintenance of the Oct3/4 positive compartment of the ICM upon blastocyst outgrowth. Mouse blastocyst outgrowths cultured in the presence of the soluble receptors hrActRIB, hrActRIIB and hrCripto showed a similar trend to those cultured in the presence of SB-431542, with 33% (8 of 24) containing an Oct3/4 positive compartment.

**Discussion**

For embryonic stem cells to realize their potential in clinical applications, it is first necessary to address fundamental questions regarding their biology and the molecular nature of "stemness." We demonstrate here a requirement for the TGFβ/activin/nodal branch of the TGFβ signaling pathway in the maintenance of human embryonic stem cell identity. Either in the context of unknown extrinsic factors secreted by MEFs (CM) or the GSK3 inhibitor BIO, inhibition of Smad2/3 activation results in significantly reduced expression of markers of pluripotency. While it may contribute to the maintenance of the undifferentiated state, Smad2/3 activation alone does not confer upon hESCs the stem cell identity. Indeed, Wnt and TGFβ/activin/nodal signaling collaborate in the maintenance of pluripotency, and the Wnt signaling pathway impinges, directly or indirectly, on Smad2/3 activation. We also found a similar requirement for Smad2/3 signaling in the maintenance of the stem cell compartment of cultured mouse blastocyst outgrowths, though activated Smad2/3 does not seem to be necessary for the ability of LIF to maintain the undifferentiated state of mESCs.

The primary cell fate decision of mammalian development occurs in morula stage embryos when the outer cells of the embryo form trophectoderm, which mediates attachment and implantation into uterine tissue, and the inner cells form the inner cell mass, which contributes to all the tissues of the embryo and from which embryonic stem cells are derived (reviewed in Rossant, 2001). Our results indicate a role for TGFβ signaling in the maintenance of pluripotency of the cellular derivatives of the inner cell mass. While TGFβ superfamily ligands have been shown to contribute to primary cell fate determination in other vertebrate models, such as the establishment of the dorsal organizer in Xenopus (Harland and Gerhart, 1997), an input for TGFβs in mammalian embryogenesis has not been well described at pre-gastrula stages. Studies of ligand and receptor expression suggest that the TGFβ cascade is activated in pre-implantation mouse embryos (Albano et al, 1993; Paria et al, 1992; de Sousa Lopes et al, 2003) and our analysis of Smad2 phosphorylation at these stages showed Smad2/3 activation as early as

the four-cell stage. Yet the role of the TGFβ superfamily in early mammalian embryogenesis is not well understood.

Previous analyses of the role of TGFβ signal transduction in early mammalian embryogenesis have mostly arisen from the study of knockout mice. An array of null mutations of TGFβ signaling components have been made in the mouse, but among these, very few have an effect before gastrulation and none affect the establishment and/or maintenance of the stem cell compartment at peri-implantation stages (reviewed in Goumans and Mummery, 2000). For example, null mutants of Smad2 or the Activin/Nodal receptor ActRIIB result in failure of mesoderm formation and malformed primitive streak (Weinstein et al, 1998; Song et al, 1999). Recently, mice were created with null mutations for both Smad2 and Smad3 (Dunn et al, 2004). These mice displayed a similar, though more severe developmental phenotype to Smad2 null mutants, with complete failure to form mesoderm or gastrulate. A striking character of these embryos was the loss of pluripotent epiblast by e7.5, as measured by Oct3/4 expression, while the formation of extraembryonic ectoderm was retained. In that extraembryonic ectoderm arises from trophectoderm (Nagy et al, 2003), this double mutant phenotype supports the notion that proper maintenance of the ICM/epiblast requires an intact Smad2/3 signaling pathway, as derivatives of this compartment are lost while trophectodermal derivatives are able to form. The double mutant phenotype also agrees with our finding that the Oct3/4 positive compartment of blastocyst outgrowths is lost, at the equivalent of embryonic day 7.5, when Smad2/3 signaling is globally inhibited (Figure 11C). From these data, in combination with our results describing the necessity for Smad2/3 signaling in maintenance of the undifferentiated state of hESCs, a paradigm emerges in which Smad2/3 signaling plays a role in the maintenance of pluripotent cell types *in vivo* as well as *in vitro*.

Mice deficient for Smad4, the common Smad that mediates translocation of effector Smads to the nucleus, display a similar phenotype to the Smad2/3 double knockout, failing to gastrulate or form mesoderm, though Oct3/4 expression in the epiblast of these embryos has not been examined (Sirard et al, 1998). It will be interesting

to see whether markers of pluripotency are affected for this and related phenotypes, both *in vivo* and upon blastocyst outgrowth. Indeed, many of the null mutations of TGFβ superfamily ligands and receptors should be reconsidered with respect to their effect on the epiblast.

TGFβ signaling has recently been shown by many studies to figure prominently in the maintenance of the undifferentiated state of hESCs. Among the factors found to be specifically enriched in undifferentiated hESCs are *nodal* (Rosler et al, 2004), *cripto*, *lefty-A* and *lefty-B* (Sato et al, 2003), all components of TGFβ signal transduction; *cripto* encodes an EGF-CFC co-receptor that is essential for responsivity to Nodal, and LeftyA and LeftyB are both inhibitors of Nodal signaling. Expression of Nodal and both Lefties has been shown to be high in undifferentiated hESCs and reduced upon differentiation (Besser, 2004), and hESCs cultured in recombinant Nodal exhibit prolonged expression of pluripotency markers (Vallier et al, 2004). Furthermore, TGFβ has recently been shown to contribute to a cocktail of growth factors that maintain the undifferentiated state of hESCs in feeder-free culture (Amit et al, 2004) and Smad1/5 activation by BMP4 is known to induce trophectoderm in the context of CM (Xu et al, 2002b). Our results extend the role of the TGFβ pathway in the maintenance of the undifferentiated state of hESCs, demonstrating a requirement for the Activin/Nodal branch downstream of canonical Wnt activation or extrinsic factors present in CM. And following from our work, Vallier et al. have recently demonstrated a requirement for active Smad2/3 signaling in the maintenance of hESC pluripotency mediated by high concentrations of FGF (Vallier et al, 2005).

The correlation between Smad1/5 phosphorylation and mitosis we have described suggests a compelling role for ligands of the BMP/GDF branch of the TGFβ superfamily in cell proliferation. This pathway has been linked to cell proliferation in other developmental contexts. For example, in *Drosophila*, overexpression of the BMP4 homologue *dpp* promotes primordial germ cell (PGC) proliferation and causes accumulation of more PGCs in the *Drosophila* gonad (Xhu and Zie, 2003). In mice, null mutations of Alk3, the type I TGFβ receptor that mediates Smad1/5 activation, exhibit

reduced cell proliferation in the epiblast (Mishina et al, 2002). In light of these phenotypes, it is not surprising to see that mitosis in two further developmental contexts (pre-implantation mouse embryos and undifferentiated hESCs) is coincident with Smad1/5 phosphorylation, yet there is no evidence to support any causal link between the two.

That Smad2/3 signaling is not required for the LIF or BIO-mediated maintenance of the undifferentiated state of mESCs underscores the dissimilarity between hESCs and mESCs. LIF is sufficient to maintain pluripotency and self-renewal of mESCs, but these cells differentiate when cultured with LIF in the absence of serum (Ying et al, 2003), suggesting a necessary input from other extrinsic factors. It has recently been shown that mESC identity can be maintained in cells cultured in the absence of serum, as long as LIF is supplemented with either BMP4 or the forced expression of the downstream BMP4 target, *Id* (Ying et al, 2003). This suggests an essential contribution from the TGFβ superfamily to the maintenance of mESCs, though this contribution activates the Smad1/5 signaling cascade that induces differentiation to trophectoderm when it is activated in hESCs (Xu et al., 2002b). Considering the disparity between our results describing the necessity for Smad2/3 activation in the maintenance of pluipotency in cultured blastocyst outgrowths versus mESCs, it is possible that one or both of these paradigms of "stemness" may not recapitulate the behavior of ICM derivatives *in vivo*. Indeed, this contradiction raises questions as to whether ES cells in culture are an adequate tool for the study of embryonic development.

We have previously demonstrated the ability of BIO to maintain pluripotency of mESCs and hESCs (Sato et al, 2004) and our results describing the character of Smad1/5 and Smad2/3 activation in hESCs extend these findings to implicate a combinatorial role for the TGFβ and Wnt signal transduction pathways in the molecular events underlying the undifferentiated state. In other model organisms, namely *Xenopus* and Zebrafish, Wnt and TGFβ signaling are believed to converge on the induction of primary cell types (Figure 12). In Xenopus embryos, for example, the establishment of the organizer has been shown to result from the dorsally localized coincidence of three events: stabilization

# Figure 12
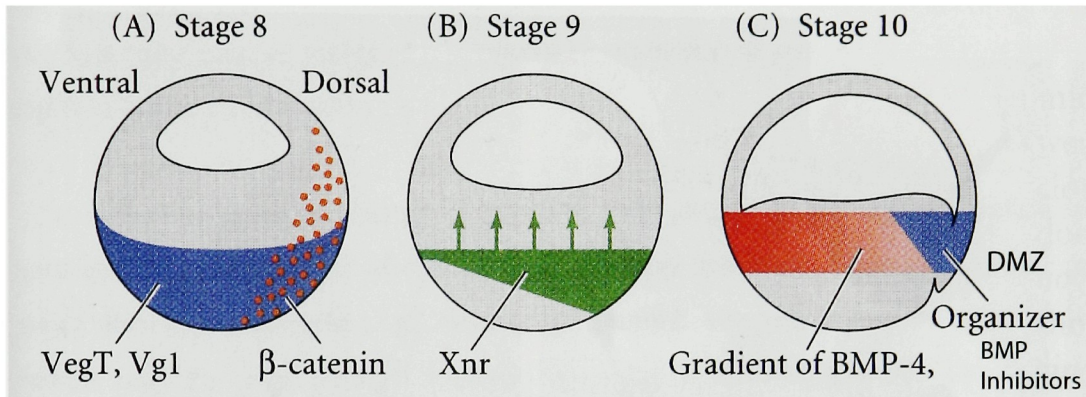


**Figure 12. Model for the formation of Spemann's Organizer by interaction of β-Catenin and TGFβ proteins.** (A) At late blastula stages, Vg1 and VegT are found in the vegetal hemisphere, while β-Catenin is located in the dorsal region. (B) β-Catenin acts synergistically with Vg1 and VegT to activate the Xenopus Nodal-related (Xnr) genes. This creates a gradient of Xnr proteins across the endoderm, highest in the dorsal region. (C) High concentrations of Xnrs at the dorsal marginal zone (DMZ) result in the establishment of dorsal mesoderm and expression of inhibitors including chordin, noggin and follistatin that block the action of BMP. In the regions of chordin and noggin expression, BMPs are totally prevented from binding and these tissues become Spemann's Organizer.
(Adapted from Scialli, **Developmental Biology**, 6th Edition, 2001)

of β-catenin; Smad2/3 activation by Xnr proteins; and inhibition of Smad1/5 activation by the BMP inhibitors *chordin, noggin* and *cerberus* (Scialli, 2003). Input from both Smad2/3 and Wnt signaling has been shown to be required for the expression of the BMP inhibitory organizer genes (Xanthos et al, 2002). Undifferentiated hESCs exhibit the same reciprocal character with respect to Smad activation shown here, with Smad2/3 signaling being active and Smad1/5 signaling being inhibited. In light of BIO's ability to maintain the undifferentiated state of hESCs and the dependence of this ability on active Smad2/3 signaling, it is tempting to speculate that the molecular basis of pluripotent hESC identity may be rooted in a conserved mechanism of primary cell fate specification evident in lower vertebrates.

Embryonic stem cells are defined by their ability to self renew indefinitely and give rise to all cell types of the embryo, yet they are present for a relatively narrow window of the mammalian life cycle. Maintenance of pluripotency in cultured hESCs results from the integration of multiple signaling inputs to retain this identity through indefinite passages. In demonstrating a requirement for TGFβ signaling, we have defined one of the necessary inputs for the maintenance of pluripotency of hESCs. However, the means by which Smad2/3 activation has its effect are unclear. It remains to be seen what targets of Smad2/3 activation are involved in mediating the maintenance of the undifferentiated state; and the manner in which Wnt signaling and Smad2/3 activation collaborate to mediate pluripotency, if at all.

# CHAPTER 2
## Characterization of a New Human Embryonic Stem Cell Line

## Summary

Data on gene expression profiles and optimal culture conditions for human embryonic stem cells are limited, indicating a need for analysis of newly and independently derived cell lines. We have derived a new hESC line, RUES1, and have here characterized its marker expression, functional characteristics and gene expression profiles. We find that RUES1 shares marker expression and functional characteristics with other lines. Additionally, we have also compared RT-PCR data generated from RUES1 to a list of marker genes shown previously by microarray analysis to be consistently enriched in hESCs. While RUES1 shares a marker expression profile with other cell lines overall, we also find differences in the expression levels among gene subclasses. Genes related to nuclear functions were most similar, regardless of culture conditions, while those related to metabolic and cell cycle function were least similar when compared with data sets from previous studies. We also find that genes with unknown functions show very high correlations, suggesting that these genes may play prominent roles in stemness and are fertile ground for future exploration. Finally, we show that signaling related genes maintain similar levels of expression when compared to other cell lines, but only when analyzed under similar culture conditions.

**Introduction**

Embryonic stem cells are a population of cells derived from blastocyst stage mammalian embryos that can be maintained for indefinite passages in tissue culture. In addition to their capacity for perpetual self-renewal, these cells are unique among cultured mammalian cell lines in that they have the ability to differentiate into all adult cell types as routinely assayed *in vitro* in embryoid bodies and *in vivo* in teratomas. The initial derivation (Thomson et al, 1998), and the growing number of reports (reviewed in Hoffman and Carpenter, 2005) on the derivation of new human embryonic stem cell lines (hESCs) has generated great enthusiasm for the promise they provide for cell-based therapies and regenerative medicine.

Several barriers currently limit the migration of hESCs from cell culture to the clinic. Foremost is the common method of hESC derivation and maintainance, whereby the human inner cell mass (ICM) is isolated and cultured in the presence of xeno-derived cells and/or proteins. These methods raise the prospect of disease transfer across species and therefore preclude the use of most existing hESC lines in the clinic. For this reason, many efforts are being made to derive new hESC lines in conditions that are completely free from non-human influences and progress has been made toward this end (Xu et al., 2005; Ludwig et al., 2006). To limit the variability introduced by co-culture techniques, many recent efforts have been focused on establishing chemically defined culture media that are able to maintain the undifferentiated state of hESCs. For example, an input from the Wnt pathway on hESC pluripotency by the GSK3$\beta$ inhibitor, BIO, has recently been described to maintain the undifferentiated state for limited passages in the absence of MEF-conditioned medium (Sato et al, 2004). And a high concentration of basic FGF, acting through BMP inhibition, has been shown to be sufficient for maintenance of hESC pluripotency in the absence of MEF-conditioned medium (Xu et al, 2005); an activity that is also sufficient in completely human derived conditions (Ludwig et al, 2006). Yet hESCs are still most commonly cultured on MEFs or in the presence of MEF-conditioned medium, and alternative culture methods must be further scrutinized to ensure their reliability.

While most hESC lines share marker expression and differentiation potential, subtle differences have even been demonstrated between independently derived hESC lines. For example, culture conditions that use serum instead of serum-free conditions have been shown to lead to altered gene expression patterns in a single hESC line (Skottman et al., 2005). However, genetic differences can also lead to gene expression differences between lines even when maintained under common culture conditions (Abeyta et al., 2004; Skottman et al., 2005). A recent report reanalyzing the raw data from several published microarray studies of hESCs has defined a common set of enriched genes that traverse several commonly used cell lines cultured under different conditions (Suarez-Farinas et al., 2005). Yet, as these studies were limited to a small number of cell lines, there is still a great need to identify markers that consistently identify the undifferentiated state in a variety of unique cell lines cultured under different conditions.

In order for them to realize their full potential, protocols for the maintenance of hESCs must be standardized so that consistent and predictable results can be obtained for their clinical or scientific use. Toward this end, we report the derivation and characterization of a new hESC line, RUES1, and evaluate its molecular phenotype relative to data from existing hESC lines. We isolated the ICMs from frozen-thawed blastocysts that were obtained with informed consent and in excess of clinical need, and derived an embryonic stem cell line that showed the classic hESC phenotype under commonly used culture conditions. We subjected this new line to rigorous molecular analysis to confirm the phenotype, demonstrated formation of embryoid bodies and teratomas containing derivatives of all three primary germ layers, and compared the expression profile of genes known to be consistently enriched in hESCs (Suarez-Farinas et al., 2005) between our new line and existing lines. This work highlights one set of molecular markers that can be used to consistently identify the similarities in the undifferentiated phenotype of hESCs maintained under standard conditions and another set that are sensitive to culture differences.

**Results**

**Derivation of RUES1**

We isolated an hESC line on mouse embryonic fibroblasts by immunosurgery from 10 thawed blastocysts that had been frozen at day six of *in vitro* development after *in vitro* fertilization (Thomson et al., 1998; Cowan et al., 2004). Although the number of attempts was low, the ability to derive lines appeared to correlate with the quality of the thawed blastocyst. Following the grading system developed by the Cornell University program (Veeck, 2003), 4(40%) of the blastocysts were deemed to be of grade 2BB or higher with a distinguishable ICM composed of tighly packed cells (Figure 13A). The remaining blastocysts lacked a distinguishable ICM and appeared to have fewer cells overall with incomplete blastocoel expansion indicating delayed development or poor health. Of the four good quality blastocysts, three resulted in a primary outgrowth (Figure 13B and C). One of these outgrowths expanded with continued culture and gave rise to colonies with tightly packed cells with a high nuclear to cytoplasm ratio (Figure 13D). These could be maintained on MEFs (Figure 13E) by manual dissection (Mitalipova et al., 2005; Oh et al., 2005) or transferred to and maintained on Matrigel coated plates (Figure 13F) in MEF conditioned medium (Hoffman and Carpenter, 2005) for more than 30 passages. This line was named RUES1. Karyotype analysis revealed that the line is male [46, XY] and most cells had a normal karyotype (26/30) after 6 passages (Figure 13G). The infrequent abnormal karyotypes included trisomies of chromosomes 7, 17, and 19. However, as other chromosomes were lost and we did not find multiple cells with these three trisomies, we believe this may represent random instabilities in this culture. Time lapse video-microscopy established that the RUES1 cell cycle is about 30 hours (data not shown). This is equivalent to the rate of most hESCs, reported to be about 24 to 36 hours, with a range of 12 hours to 72 hours (Cowan et al. 2004).

# Figure 13
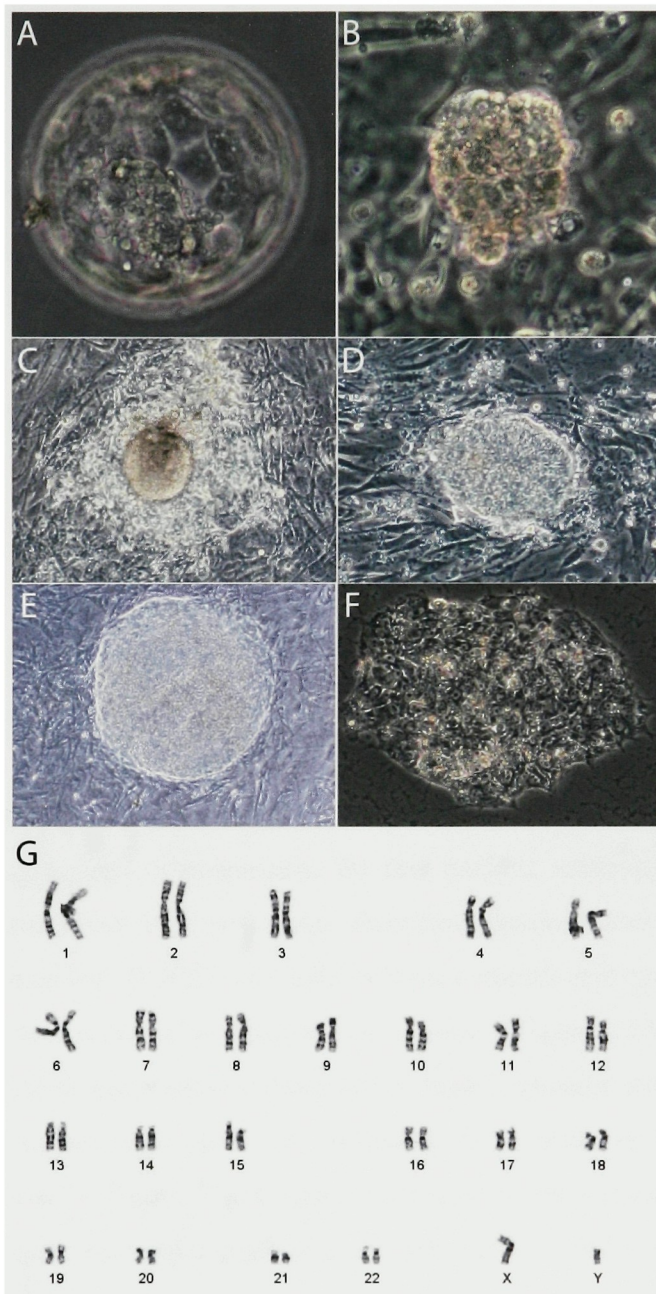


**Figure 13. Derivation and marker expression of RUES1.** RUES1 was derived from a 6 day old blastocyst (A). The ICM isolated by immunosurgery is shown (B). This ICM attached to the MEF feeder layer an produced a primary outgrowth (C). A colony of small ICM-like cells with a high nuclear to cytoplasmic ratio resulting from an outgrowth from the inner cell mass of this embryo is shown at passage 1 (D) and passage 3 (E). Some colonies were transferred at passage 3 to Matrigel-coated plates in MEF conditioned medium and imaged at passage 9 (F). An example of a normal karyotype is shown (G).

**RUES1 marker expression**

We next examined the expression of markers of pluripotency in culture when RUES1 was grown either on MEFs or on Matrigel using culture medium conditioned by MEFs (Figure 14). Under these conditions, RUES1 expressed previously described molecular markers of pluripotency (Hoffman and Carpenter, 2005). The markers Oct-3/4 (POU5F1, Figure 14A and D), SSEA4 (Figure 14B and E), TRA-1-60 (Figure 14C), and alkaline phosphatase (Figure 14F) were readily detected. By real-time RT-PCR, expression of Oct-3/4, Nanog, and Cripto-1 (Figure 14G) were also detected. These data demonstrate that RUES1 is similar to previously reported cell lines in origin, growth properties, and marker expression.

**Differentiation potential of RUES1**

A common method used to assay differentiation potential of hESCs involves culture of hESC aggregates in suspension. By this method, embryoid bodies are formed in which hESC derivatives with molecular identities representative of all three primary germ layers are contained. RUES1 was able to form complex and cystic embryoid bodies when aggregated and cultured in suspension *in vitro* (Figure 15A). Embryoid bodies could be maintained in suspension culture for at least 5 months and during this period, beating cardiac myocytes were observed, indicating the presence of functional mesoderm differentiation (arrow in Figure 15B). After prolonged *in vitro* culture and reattachment to adhesive substrates, embryoid bodies generated multiple cell types indicative of the three embryonic germ layers (Figure 15C-F). Neural cell types were evident in outgrowths from the EBs (data not shown). Immunostaining for Nestin and Neurofilament Heavy Chain (NFH) confirmed the presence of ectoderm in these cultures (Figure 15C); staining for Desmin indicated the presence of mesoderm (Figure 15D); and staining for HNF3β demonstrated the presence of endoderm derivatives (Figure 15E). We also found an early marker of trophectoderm, Cdx2, in EB cultures (Figure 15F). These data indicate that markers of germ layer derivatives can be induced *in vitro*.

# Figure 14
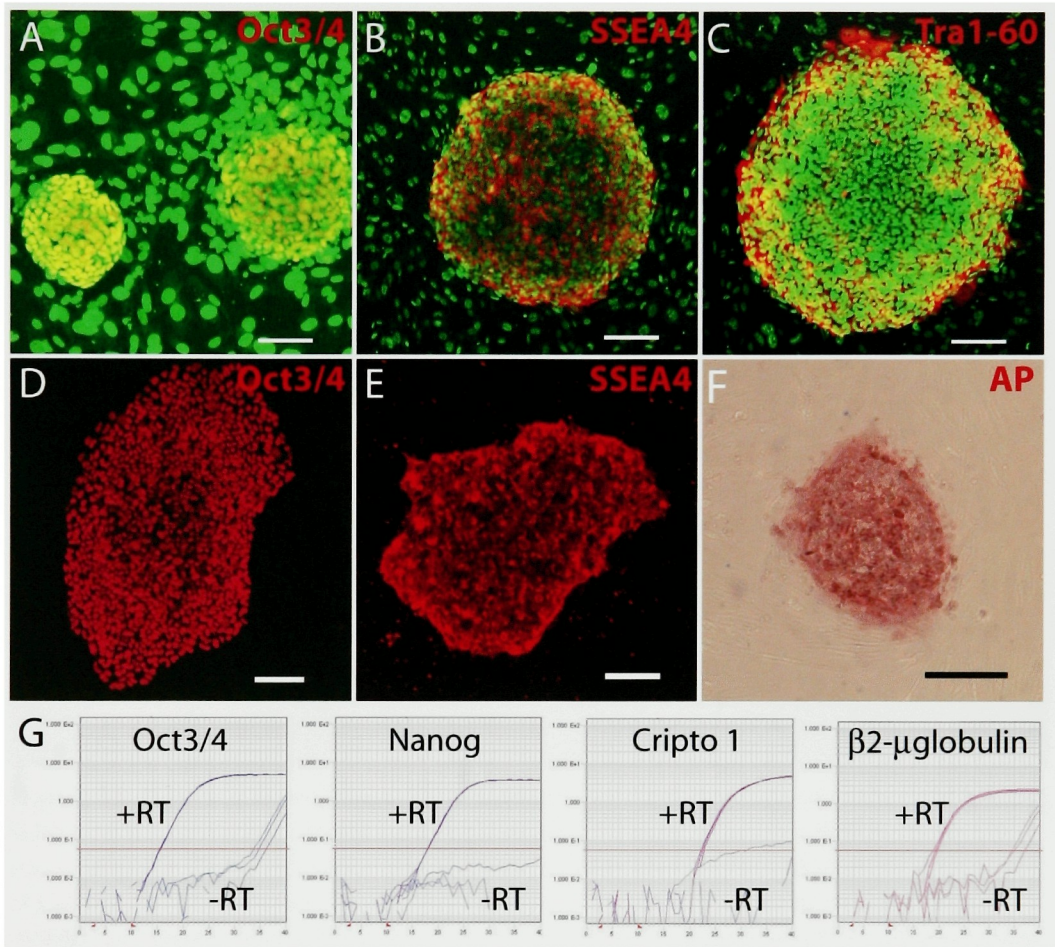


**Figure 14. Pluripotent Marker Expression in RUES1.** Colonies on MEFs (A, B, C, F) and on Matrigel (D,E) were analyzed for the pluripotency markers Oct-3/4 (A,D), SSEA4 (B,E), TRA-1-60 (C) by immunofluoresence. Colonies on MEFs were also positive for Alkaline phosphatase (F) by cytochemistry. Pan nuclear counterstain is shown in green; markers are shown in red. (G) Real-time RT-PCR analysis of pluripotency markers. Shown are amplification plots of relative fluorescence vs. cycle number for Oct-3/4, Nanog, and Cripto-1. β2-microglobulin as an amplification control. The no-RT controls are indicated for each primer. Red lines indicate the threshold cycle of amplification used to determine the level of expression. Note that the no-RT controls show low background amplification at the threshold cycle. Scale bars are 50μm.
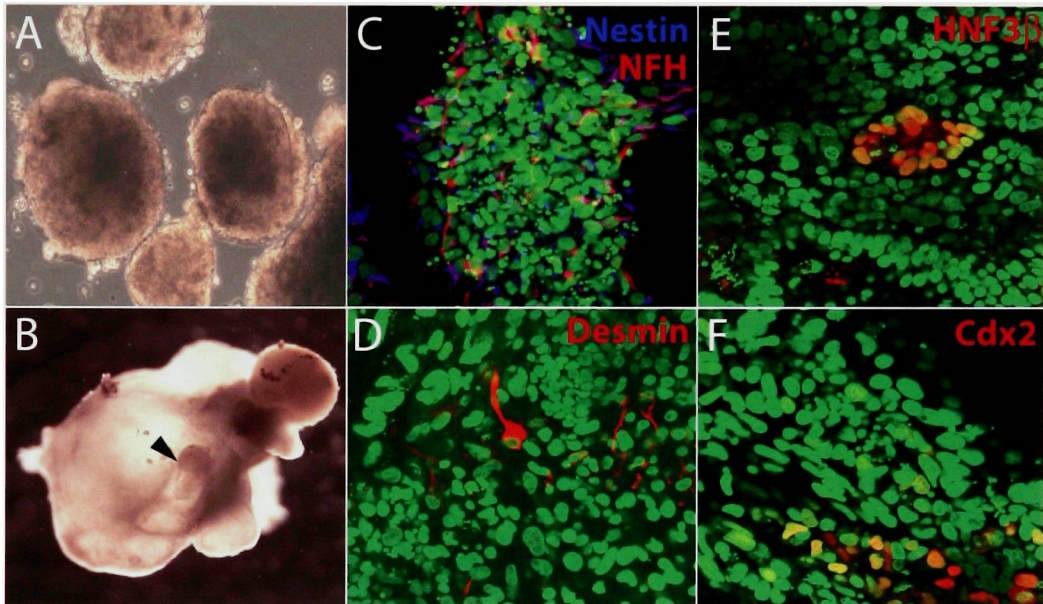
# Figure 15



**Figure 15. Figure 3. Germ layer differentiation of RUES1 hESCs in embryoid bodies.**
RUES1 hESCs generate complex aggregates after 14 days of in vitro differentiation in suspension(A). These subsequently formed complex embryoid bodies during 2 months of culture (B). The arrow in B indicates an area of contracting cardiac muscle after 2 months of culture, indicating mesoderm differentiation. When plated on adhesive substrates, the EBs generated multiple differentiated cell types, including neural tissue (C). The neural cell types can be propagated in vitro and stain for markers of Ecotderm: nestin (C, blue) and Neurofilament Heavy Chain (C, red). Mesoderm, marked by Desmin (D, red), and Endoderm, marked by HNF3β (E, red) as well as Trophectoderm, marked by Cdx2 (E, red), can also be found in EBs. All nuclei are shown in green.

Another functional quality of hESCs is their ability to form teratomas, or disorganized masses of varied differentiated cell types, when injected intramuscularly into immuno-compromised mice. To further demonstrate the differentiation potential of RUES1 we generated teratomas in SCID-beige mice and analyzed them for tissue derivatives of the three embryonic germ layers (Figure 16). Several teratomas were analyzed including a single teratoma from which we could identify neural tissue and retinal pigmented epithelium, representing ectoderm (Figure 16A and B), bone, cartilage and squamous epithelium (Figure 16C, D and E), representing mesoderm and glandular tissue (Figure 16F), representing endoderm. We used immunofluorescent staining for germ layer markers on another teratoma to verify these results. We identified areas of neuroepithelium that stained positively for Nestin, NFH, and TuJ1 (Figure 16G-I); mesodermal tissue that stained positively for Desmin and Muscle MHC (Figure 16J and K); and endoderm tissue that stained positively for AFP and HNF3β (Figure 16L and M); we also identified trophectoderm, as marked by Cdx2 (Figure 16N). Taken together, the results presented above establish that RUES1 is a bonafide hESC line meeting the current criteria of prolonged undifferentiated proliferation while maintaining the ability to differentiate into trophectoderm and germ layer derivatives.

**Comparison of marker gene expression**

We have recently reported the identification of a set of genes that are consistently enriched in undifferentiated hESCs across several independent microarray studies (Suarez-Farinas et al., 2005). We verified for enrichment of 91 of these markers in RUES1 hESCs by real-time RT-PCR. RUES1 hESCs maintained undifferentiated on Matrigel in MEF conditioned medium were compared to those maintained under adherent conditions in non-conditioned medium for 24 days to assay differentiation ability. Table 1 shows the fold-enrichment of these markers in undifferentiated RUES1 hESCs. We find that 88/91 (97%) of these genes were significantly enriched in undifferentiated RUES1 hESCs, of which 83 genes (94%) were enriched by 0.5 (log2 scale) or better and 49 (56%) were enriched by more than 2-fold by RT-PCR. SERPINB9, a proteinase inhibitor,

# Figure 16
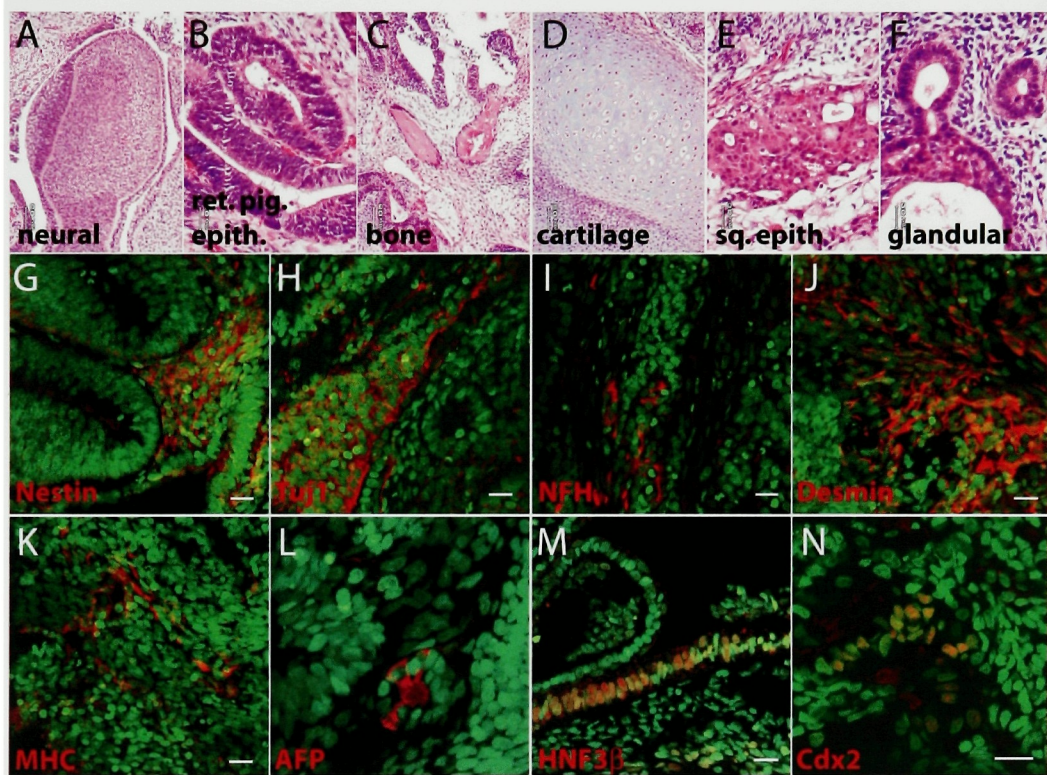


**Figure 16. Germ layer differentiation of RUES1 hESCs in teratomas.** RUES1 hESCs at passage 11 were also injected intramuscularly into SCID/beige mice and allowed to develop for 6 weeks to generate teratomas. (A-F) Histopathology of RUES1 teratoma. Examples of tissues from three germ layers are shown. Ectoderm: neural tissue (A) and retinal pigmented empithelium (B), Mesoderm: bone (C) and cartilage (D) and squamous epithelium (E). Endoderm: glandular tissue (F). Germ layer markers were verified by immunofluoresence on cryosections of the teratoma (G-N). Examples of Ectoderm (Nestin (G, red), Tuj1 (H,red) and NFH (I, red)), Mesoderm (Desmin (J, red) and Muscle MHC (K, red)), and Endoderm (AFP (L, red) and HNF3β (M, red)) are shown. In addition, Trophectoderm (Cdx2 (N, red)) is present. (All nuclei are shown in green. Scale bars in G-N - 50 μM.)

**Table 1.** Real-time RT-PCR analysis of genes consistently enriched in undifferentiated hESCs.

| Symbol | Description | UniGene | FCH* | p value |
|---|---|---|---|---|
| **Signaling factors** | | | | |
| EBAF | endometrial bleeding associated factor (Lefty A) | Hs.25195 | 38.19 | 3.22E-05 |
| STC1 | stanniocalcin 1 | Hs.25590 | 5.48 | 1.26E-04 |
| PROM1 | prominin 1 | Hs.370052 | 4.77 | 1.69E-05 |
| FZD5 | frizzled homolog 5 | Hs.152251 | 4.13 | 1.56E-06 |
| CRABP1 | cellular retinoic acid binding protein 1 | Hs.346950 | 3.76 | 1.14E-05 |
| TRIP | TRAF interacting protein | Hs.21254 | 3.11 | 8.06E-07 |
| TRIP8 | thyroid hormone receptor interactor 8 | Hs.442675 | 2.94 | 1.16E-04 |
| SLC16A1 | Solute carrier family 16 member 1 | Hs.75231 | 2.88 | 2.58E-06 |
| SEMA6A | semaphorin 6A | Hs.443012 | 2.85 | 1.61E-04 |
| SIRT1 | Sirtuin 1 | Hs.31176 | 2.62 | 3.55E-04 |
| THY28 | thymocyte protein thy28 | Hs.13645 | 2.59 | 2.34E-04 |
| CXADR | coxsackie virus and adenovirus receptor | Hs.79187 | 2.42 | 2.37E-04 |
| HOMER1 | Homer homolog 1 | Hs.129051 | 2.24 | 2.45E-03 |
| BCOR | BCL6 co-repressor | Hs.186424 | 1.97 | 8.25E-04 |
| FABP5 | fatty acid binding protein 5 | Hs.408061 | 1.86 | 3.77E-05 |
| HMGB3 | high-mobility group box 3 | Hs.19114 | 1.80 | 4.52E-05 |
| RAB3B | RAB3B, member RAS oncogene family | Hs.123072 | 1.78 | 3.22E-02 |
| PODXL | podocalyxin-like | Hs.16426 | 1.60 | 1.02E-03 |
| CD24 | CD24 antigen | Hs.375108 | 1.58 | 2.43E-04 |
| ACVR2B | Activin A receptor, type IIB | Hs.23994 | 1.51 | 1.76E-02 |
| GULP1 | GULP, engulfment adaptor PTB domain containing 1 | Hs.107056 | 1.46 | 4.16E-05 |
| KCNN2 | potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2 | Hs.98280 | 1.36 | 1.86E-02 |
| BMPR1A | bone morphogenetic protein receptor, type IA | Hs.2534 | 1.24 | 3.75E-02 |
| PELI1 | Pellino homolog 1 | Hs.7886 | 1.08 | 3.54E-01 |
| **Nuclear factors** | | | | |
| PIM2 | pim-2 oncogene | Hs.80205 | 6.34 | 2.25E-06 |
| POU5F1 | POU domain, class 5, transcription factor 1 | Hs.249184 | 4.71 | 1.27E-04 |
| ZNF74 | zinc finger protein 74 | Hs.127476 | 3.29 | 3.61E-06 |
| PSIP1 | PC4 and SFRS1 interacting protein 1 | Hs.351305 | 2.39 | 4.43E-05 |
| SNRPF | small nuclear ribonucleoprotein polypeptide F | Hs.105465 | 2.39 | 1.34E-04 |
| ADPRT | ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase) | Hs.177766 | 2.34 | 8.92E-04 |
| SOX2 | SRY (sex determining region Y)-box 2 | Hs.816 | 2.27 | 1.04E-04 |
| M96 | metal response element binding transcription factor 2 | Hs.31016 | 2.24 | 6.44E-05 |
| RNF138 | ring finger protein 138 | Hs.180403 | 2.06 | 6.22E-04 |
| SNRPD2 | small nuclear ribonucleoprotein D2 polypeptide 16.5kDa | Hs.424327 | 2.05 | 8.63E-04 |
| ZNF43 | zinc finger protein 43 | Hs.419763 | 1.98 | 3.31E-05 |
| RBMX | RNA binding motif protein, X-linked | Hs.380118 | 1.98 | 1.05E-03 |
| SMC6L1 | SMC6 structural maintenance of chromosomes 6-like 1 | Hs.424559 | 1.90 | 1.87E-04 |
| HNRPC | heterogeneous nuclear ribonucleoprotein C (C1/C2) | Hs.476302 | 1.75 | 5.55E-03 |
| LSM3 | LSM3 homolog, U6 small nuclear RNA associated | Hs.111632 | 1.71 | 6.62E-04 |
| JADE1 | PHD protein Jade-1 | Hs.12420 | 1.64 | 3.94E-04 |
| TCERG1 | transcription elongation regulator 1 (CA150) | Hs.300052 | 1.63 | 4.62E-03 |
| NASP | nuclear autoantigenic sperm protein | Hs.446206 | 1.60 | 3.37E-03 |
| ZNF198 | zinc finger protein 198 | Hs.315241 | 1.50 | 2.19E-02 |
| DATF1 | death associated transcription factor 1 | Hs.438300 | 1.44 | 5.78E-04 |
| CPSF6 | cleavage and polyadenylation specific factor 6, 68kDa | Hs.64542 | 1.32 | 1.17E-03 |

## Metabolic, structural and cell cycle related factors

| | | | | |
|---|---|---|---|---|
| UCHL1 | ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) | Hs.76118 | 3.77 | 1.13E-06 |
| UBE2C | ubiquitin-conjugating enzyme E2C | Hs.93002 | 3.25 | 4.45E-05 |
| DNAJB5 | DnaJ (Hsp40) homolog, subfamily B, member 5 | Hs.237506 | 2.96 | 1.96E-07 |
| PRPS1 | phosphoribosyl pyrophosphate synthetase 1 | Hs.56 | 2.70 | 2.44E-04 |
| PSMD11 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11 | Hs.443379 | 2.63 | 1.15E-05 |
| NUDT1 | nudix (nucleoside diphosphate linked moietyX)type motif1 | Hs.413078 | 2.52 | 6.75E-05 |
| CTH | Cystathionase (cystathionine gamma-lyase) | Hs.19904 | 2.30 | 8.95E-06 |
| CA14 | carbonic anhydrase XIV | Hs.192491 | 2.22 | 3.41E-05 |
| GFPT2 | glutamine-fructose-6-phosphate transaminase 2 | Hs.30332 | 2.21 | 1.00E-04 |
| PPAT | phosphoribosyl pyrophosphate amidotransferase | Hs.311625 | 2.18 | 1.43E-04 |
| MRPL42 | mitochondrial ribosomal protein L42 | Hs.331202 | 2.13 | 2.41E-03 |
| UGP2 | UDP-glucose pyrophosphorylase 2 | Hs.417361 | 2.12 | 2.24E-06 |
| UNG | uracil-DNA glycosylase | Hs.78853 | 2.06 | 1.36E-04 |
| PP591 | FAD-synthetase | Hs.118666 | 1.84 | 7.27E-03 |
| DNAJB6 | DnaJ (Hsp40) homolog, subfamily B, member 6 | Hs.181195 | 1.78 | 1.11E-03 |
| TNPO3 | transportin 3 | Hs.412527 | 1.68 | 6.66E-05 |
| ATP5H | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d | Hs.155728 | 1.66 | 3.49E-03 |
| GYG2 | glycogenin 2 | Hs.380757 | 1.65 | 1.69E-02 |
| GCNT2 | glucosaminyl transferase 2, I-branching enzyme | Hs.934 | 1.65 | 3.12E-02 |
| ASMTL | acetylserotonin O-methyltransferase-like | Hs.458420 | 1.64 | 4.31E-04 |
| MRPS27 | mitochondrial ribosomal protein S27 | Hs.376200 | 1.58 | 4.49E-04 |
| SERPINB9 | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9 | Hs.104879 | 0.42 | 4.64E-04 |
| ACTN3 | actinin, alpha 3 | Hs.445037 | 3.44 | 4.73E-06 |
| VDP | vesicle docking protein p115 | Hs.325948 | 1.32 | 5.09E-02 |
| PTTG1 | pituitary tumor-transforming 1 | Hs.350966 | 4.94 | 2.48E-05 |
| CKS1B | CDC28 protein kinase regulatory subunit 1B | Hs.374378 | 2.81 | 3.04E-07 |
| CCND1 | cyclin D1 (PRAD1) | Hs.371468 | 2.35 | 6.87E-05 |
| CDC25C | cell division cycle 25C | Hs.656 | 1.91 | 2.01E-01 |
| CDC7 | CDC7 cell division cycle 7 | Hs.28853 | 1.49 | 3.26E-06 |
| BUB1 | BUB1 budding uninhibited by benzimidazoles 1 homolog | Hs.287472 | 1.47 | 6.32E-03 |

## Unknown factors

| | | | | |
|---|---|---|---|---|
| FLJ12505 | hypothetical protein FLJ12505 | Hs.96885 | 3.97 | 4.27E-05 |
| FLJ10156 | hypothetical protein FLJ10156 | Hs.404323 | 2.71 | 8.73E-05 |
| FN5 | FN5 protein | Hs.416456 | 2.67 | 2.08E-04 |
| C21orf45 | chromosome 21 open reading frame 45 | Hs.49932 | 2.64 | 7.22E-04 |
| KIAA0116 | KIAA0116 protein | Hs.254717 | 2.62 | 1.01E-06 |
| DLG7 | discs, large homolog 7 (Drosophila) | Hs.77695 | 2.51 | 3.43E-05 |
| CGI-30 | CGI-30 protein | Hs.440776 | 2.27 | 9.15E-06 |
| FAM29A | family with sequence similarity 29, member A | Hs.54617 | 2.17 | 4.68E-04 |
| KIAA0095 | KIAA0095 gene product | Hs.295014 | 2.03 | 3.93E-05 |
| P15RS | hypothetical protein FLJ10656 | Hs.300906 | 1.96 | 2.80E-05 |
| FLJ20343 | hypothetical protein FLJ20343 | Hs.171044 | 1.80 | 6.98E-05 |
| BMS1L | BMS1-like, ribosome assembly protein (yeast) | Hs.10848 | 1.80 | 7.71E-04 |
| C6orf111 | chromosome 6 open reading frame 111 | Hs.414993 | 1.79 | 4.06E-02 |
| NIF3L1 | NIF3 NGG1 interacting factor 3-like 1 (S. pombe) | Hs.21943 | 1.64 | 7.15E-03 |
| FLJ20530 | hypothetical protein FLJ205300530 | Hs.370888 | 1.50 | 1.10E-03 |
| FLJ11323 | hypothetical protein FLJ11323 | Hs.378766 | 1.37 | 2.54E-02 |

*Fold enrichment by Real-time RT-PCR on undifferentiated RUES1 hESCs compared to differentiated RUES1 hESCs calculated by the Comparative Ct method. Primers sequences are available upon request.

was the only tested gene significantly enriched in the differentiated RUES1 hESCs, whereas it has previously been shown to be enriched in undifferentiated hESCs (Suarez-Farinas et al., 2005). However, as reported previously, this list of genes does not exclusively include the most enriched genes but also those that are weakly, but significantly, and consistently regulated with differentiation. Therefore, we also correlated the fold-enrichment (log2 scale) by real-time RT-PCR to the M-values (log2 fold-change) for the microarray studies previously reported to determine the degree of fit between RUES1 marker expression and that of other hESC lines. As shown in Figure 17, the degree of fit between RUES1 gene expression and the mean M-values for three previously reported microarray studies was good with positive slopes and correlation coefficients (Figure 17A, blue triangles and line). The correlations were greatly improved when RUES1 is compared to the microarray study data reported by Sato et al. (Sato et al., 2004), presumably because the culture conditions used in that study were identical to our own (Figure 17A, red squares and line). These data indicate that RUES1 hESCs are very similar in their gene expression to previously derived lines and this similarity is increased when compared to another cell line (H1) maintained under similar culture conditions.

As the degree of fit improved when RUES1 hESCs were compared to previous hESC lines maintained under similar culture conditions, we asked if any differences existed between functional classes of genes that could distinguish RUES1 hESCs from other cell lines. The lists of enriched genes were sorted into four functional classifications (presented in Table 1) and compared between RUES1 and the Sato study alone, or between RUES1 and the mean values for all expression studies. The mean log2 RT-PCR fold-change was not significantly different among the groups (by ANOVA, p=0.4368). The nuclear factor classification maintained the greatest degree of fit between RUES1 gene expression and both the Sato study alone and the mean for the all studies (Figure 17C). Genes categorized as Metabolic, structural and cell cycle related did not show significant correlation between RUES1 and either data set (Figure 17D). One member in the structural subgroup, ACTN3, did show similar levels of expression among comparisons, however, its inclusion did not affect the overall results. When signaling related factors were compared, enriched genes and their levels of expression were only

# Figure 17
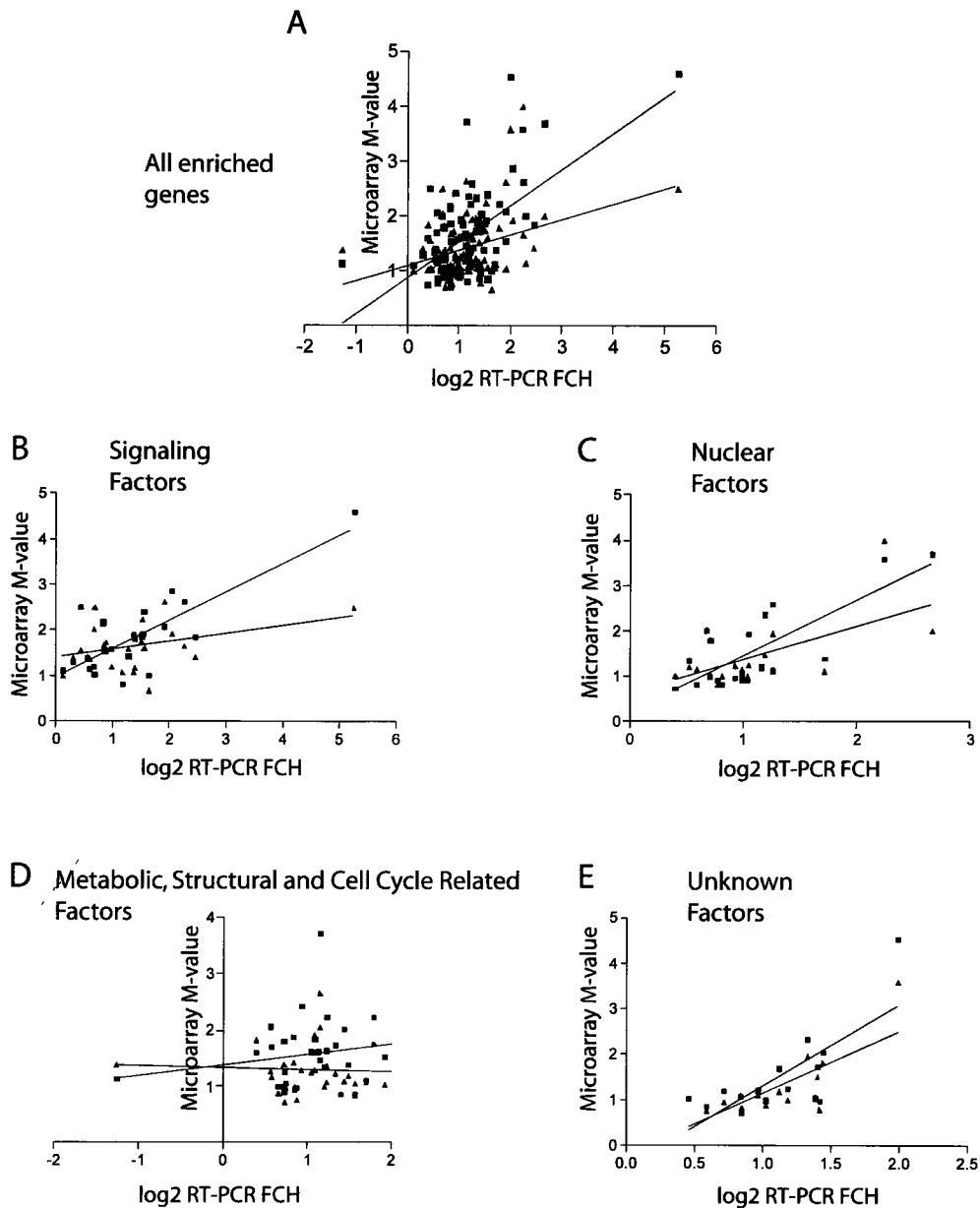


**Figure 17. Real-time RT-PCR analysis of consistently enriched genes.** Shown are linear fits between real-time RT-PCR analysis and microarray data from previously published studies for genes significantly enriched in RUES1 HESCs by real-time RT-PCR (A-E). For all plots, the log2-fold change by RT-PCR vs. the M-values reported in our previous study (Sato et al, 2004), which had identical culture conditions to this study are shown with red squares and regression lines. The log2-fold change by RT-PCR vs. the mean M-values reported previously (Suares-Farinas et al, 2005) are shown with blue triangles and regression lines. (A) Fits for all significant genes compared to Sato study alone (r=0.6096, p<0.0001, R2=0.3717) and mean for all microarray studies (r=0.3514, p=0.0008, R2=0.1235). (B) Fits for Signaling factors compared to Sato study alone (r=0.7919, p<0.0001, R2=0.6271) and mean for all microarray studies (r=0.3114, p=0.1481, R2=0.09697). (C) Fits for Nuclear factors compared to Sato study alone (r=0.0.7740, p<0.0001, R2=0.5990) and mean for all microarray studies (r=0.5777, p=0.0061, R2=0.3337). (D) Fits for Metabolic, structural and cell cycle related factors compared to Sato study alone (r=0.2033, p=0.2994, R2=0.04133) and mean for all microarray studies (r=-0.01186, p=0.9522, R2=0.0001407). (E) Fits for Unknown factors compared to Sato study alone (r=0.7522, p=0.0008, R2=0.5658) and mean for all microarray studies (r=0.7425, p=0.0010, R2=0.5513).

similar between hESC lines that were maintained under similar culture conditions (Figure 17B). A significant degree of fit was obtained when RUES1 signaling related factors were compared to the Sato study alone but was not significant when compared to the mean for the previous studies. This analysis suggests that differences in cell line and/or culture conditions are negligible to the expression levels of enriched nuclear factors between hESC lines, though the expression levels of metabolic and cell cycle gene expression varies considerable between RUES1 and other cell lines. Furthermore, the enrichment of signaling related factors is only conserved between hESC lines maintained in similar culture conditions. And surprisingly, genes classified as having unknown functions showed good correlation in both comparisons (Figure 17E), suggesting that these genes might have consistent and important functions to the maintenance of stemness across cell line and culture conditions. Taken together, these data suggest that differences in culture conditions may result in the use of different signaling pathways that converge on common nuclear factors to maintain pluripotency.

## Discussion

Much remains to be understood about the basic biology of hESCs and the differences among hESC lines. To date, several labs have reported the derivation of over 90 hESC lines (reviewed in Hoffman and Carpenter, 2005). These lines have been maintained under a variety of culture conditions and have been characterized by limited *in vitro* and *in vivo* functional assays. In this study, we add to this growing list a new hESC line, RUES1, that is capable of robust expansion and retains pluripotent differentiation potential. We derived the new hESC line under commonly used conditions and show that it can be maintained in culture media typically used to culture hESCs. We show that a list of marker genes previously found to be consistently expressed in hESCs, regardless of cell line or culture conditions, consistently marks this new line. These data suggest that RUES1 possesses functional and molecular similarities to other reported lines. However, upon examination of signaling related factors within this list, some variation between this line and previously published lines is evident. Further analysis of the diversity among hESC lines may help uncover details of their biology.

The availability of culture conditions devoid of animal products or the need for co-culture will be necessary for the efficient clinical use of hESCs and recent progress has been made in removing these obstacles (Xu et al., 2005; Ludwig et al., 2006). Two culture systems used to date have ostensibly acted through different pathways, Wnt activation and FGF signaling, to maintain the pluripotency of hESCs. Relatively high doses of FGF have been shown to be sufficient to maintain hESCs in commercial serum-replacement medium containing bovine serum albumin (Xu et al., 2005) and in a commercial medium containing only human-derived or synthetic products (Li et al., 2005). And the small molecule inhibitor of GSK3β, BIO, when used in the commercial serum-replacement medium has been shown to maintain hESC pluripotency for limited passages in the absence of CM by activating the canonical Wnt pathway (Sato et al., 2004). We found that RUES1 behaves similarly to existing hESC lines in either of these conditions, maintaining Oct-3/4 expression similarly to CM.

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Gene expression in a cell reflects its environment. In this study, metabolic, and cell cycle genes, while significantly enriched, showed the most variability between existing hESC lines. This might be expected, as metabolic and cell cycle genes are sensitive to day to day changes in the local culture environment. Alternatively, these differences could represent genetic differences. For example, while it is expressed in this and other (Suarez-Farinas et al., 2005) hESC lines, ACTN3, a highly specialized form of alpha-actinin and a component of the fast glycolytic fibers in skeletal muscle, is not expressed in a significant proportion of the human population due to genetic polymorphism (MacArthur and North, 2004). However, expression level differences might also be due to the young age or low passage number of RUES1 relative to the other cell lines.

As most published studies rely on the use of a relatively small number of markers of the pluripotent state (Brivanlou et al., 2003), the possibility of heterogeneity or even multiple states of functional stemness among these cultures remains. Efficiently guiding stem cell differentiation to an endpoint of specific cell therapy will require a more detailed understanding of the molecular and cellular nature of the pluripotent state. Several recent studies have attempted to define these markers and have identified a set of markers, shown to be consistently enriched in undifferentiated hESCs, that are robust to differences in cell line and culture conditions (Suarez-Farinas et al., 2005). Additionally, as genetic differences between hESCs lines (Abeyta et al., 2004; Skottman et al., 2005), compounded by growth in different culture conditions, could mask important underlying heterogeneity or functional states, it will be necessary to compare marker gene expression across a number of lines cultured in similar conditions. Few comparisons of the expression of such marker genes among lines have been reported. Here we have shown that different marker gene subclasses, while consistently expressed, vary in their expression levels between cell lines and culture conditions. Nuclear related factors were expressed similarly when compared to other cell lines, regardless of culture conditions, while signaling related markers maintained similar levels of expression only when compared to cell lines maintained in similar conditions. This suggests that different signaling pathways may converge on common nuclear factors to maintain pluripotency in

hESCs. Further work will be needed to establish how varied signal transduction pathways may be integrated by a common set of nuclear factors to mediate hESC pluripotency. And the correlation between enriched genes of unknown function between multiple hESC lines, regardless of culture conditions, provides fertile ground for the study of uncharacterized factors that may have a role in hESC maintenance. Our derivation of a new hESC line, RUES1, and the future derivation and culture of additional hESC lines in strictly defined conditions will allow for a comparative examination of the molecular bases that underlie hESC pluripotency.

# CHAPTER 3

Generation of Embryonic Chimeras by Engraftment of Human Embryonic Stem Cells into Mouse Blastocysts

## Summary

In addition to their potential for cell based therapies in the treatment of disease and injury, the broad developmental capacity of human embryonic stem cells offers potential for studying the origins of all human cell types. To date, the emergence of specialized cells from hESCs has commonly been studied in tissue culture or in teratomas, yet these methods have stopped short of demonstrating the ESC potential exhibited in the mouse (mESCs), which can give rise to every cell type when combined with blastocysts. Due to obvious barriers precluding the use of human embryos in similar cell mixing experiments with hESCs, human/non-human chimeras may need to be generated for this purpose. Our results show that hESCs can engraft into mouse blastocysts, where they proliferate and differentiate *in vitro* and persist in mouse/human embryonic chimeras that implant and develop in the uterus of pseudopregnant foster mice. Embryonic chimeras generated in this way offer the opportunity to study the behavior of specialized human cell types in a non-human animal model. Our data demonstrate the feasibility of this approach, using mouse embryos as a surrogate for hESC differentiation.

**Introduction**

Embryonic stem cells are a population of self-renewing, pluripotent cells that are derived from the inner cell mass of mammalian blastocyst stage embryos, and are able to differentiate into all the cell types of the adult (Rossant et al., 2001). In recent years, hESCs have generated tremendous enthusiasm for the promise they provide of both revolutionizing cell-based therapies and regenerative medicine, as well as providing a vehicle for the study of early human embryology. While clinical application of hESCs does not necessarily depend on their ability to mimic natural development, the capacity for hESCs to model human embryogenesis, either as a whole or in part, depends largely on the ability of these cells to faithfully parallel their cognate population in a developing human embryo.

Since their discovery, hESCs have often been defined as such by their similarity to mESCs, and many of the efforts directed toward hESC biology have been extrapolated from years of research done on mESCs (Smith, 2001). While mouse and human ESCs have been shown to share similarities in their fundamental biology, there is also an appreciation that major differences exist between ESCs derived from the two species (Sato et al;, 2004; James et al., 2005). These disparities underscore the necessity for an independent and comprehensive examination of both the fundamental biology of hESCs, as well as their capacity to give rise to adult cell types.

To date, hESC differentiation has mostly been assayed by two means: Formation of embryoid bodies and teratomas, both of which contain representative cell types from all three primary germ layers (Conley et al., 2004; Przyborski, 2005). Yet while the timing of gene expression in differentiating mouse embryoid bodies can mirror embryonic gene expression (Keller et al., 1993; Leahy et al., 1999) neither mESCs nor hESCs have been shown to undergo axial morphogenesis within embryoid bodies. And though hESCs can give rise to relatively organized tissue rudiments within teratomas (Przyborski, 2005), they differentiate in response to an environment that does not reflect the developmental context of embryogenesis. For these reasons, engraftment of hESCs

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into an embryonic environment may be better suited to the study of specialized human cell types in a live animal model.

Numerous studies have demonstrated the viability and developmental potency of human stem cells within interspecies chimeras: Human mesenchymal stem cells injected into e11.5 rat embryos have been shown to give rise to complex functional structures of the kidney (Yokoo et al., 2005); hESCs injected into an organogenesis stage chick embryo have been shown to proliferate and contribute to neural cell types (Goldstein et al., 2002); and recently, when hESCs were injected directly into the lateral ventricle of e14 fetal mouse brains, they were shown to give rise to functional human neurons within the adult mouse brain (Muotri et al., 2005). In the present study, we use the mouse blastocyst as a host for engraftment of hESCs. The advantages of using the blastocyst stage mouse embryo as a host for engraftment of hESCs are twofold: first, like the ICM and mESCs, hESCs are theoretically capable of differentiating to any cell type; second, engraftment into mouse embryos at the blastocyst stage allows for integration at a point that is relatively close in developmental timing.

As these types of experiments are currently prohibited by the material transfer agreements attached to the NIH-registry cell lines, none of these hESC lines could have been used. We therefore isolated a new hESC line, RUES1, from human blastocysts donated from IVF clinics (described in detail above.) Using both unmodified and genetically marked RUES1 we showed first that RUES1 injected into mouse blastocysts proliferated, intermingled and differentiated along with host cells in cultured blastocyst outgrowths. Strikingly, hESCs that engrafted to mouse embryos localized to their niche of origin, the ICM, despite a hundred million years of evolutionary distance. When chimeric blastocysts were implanted transiently into pseudopregnant foster mice, most of the resultant embryos were developmentally abnormal, though RUES1 derivatives persisted in rare embryos that proceeded through gastrulation and displayed normal morphology at e8. This study establishes the feasibility of adapting classic embryonic stem cell mixing experiments for use with hESCs. These approaches can be extended to take advantage of the large collection of mutant mice for use as host, and genetically

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modified and/or diseased hESCs as graft, to address both basic embryological properties of hESCs as well as shed light on their potential application for cell based therapies.

## Results

### Mouse and human ESCs can combine and differentiate in mosaic embryoid bodies

Fundamental differences in their biology notwithstanding, the functional qualities of mESCs and hESCs are very much the same; they are both derived from the same embryonic stage and have the ability to form embryoid bodies and teratomas (Smith, 2001; Leahy et al., 1999; Przyborski, 2005). Yet, mouse and human ESCs also show significant differences. For example, cell cycle length and the signaling factors that mediate self-renewal have been shown to be different between the two cell types (Sato et al., 2003; 2004; James et al., 2005). The fact that hESCs are grown on top of MEFs in culture experiments clearly demonstrates that embryonic cell types from the two species can coexist. But factors secreted by MEFs are important for the maintenance of self renewal in hESCs, so it is possible that paracrine signaling between mouse cells and hESCs within embryonic chimeras could affect the differentiation process of one or both cell types. Before undertaking experiments to generate embryonic chimeras, we aimed to ensure that hESCs could survive and differentiate when combined with their mouse cognate cell type. To test this, RUES1 hESCs and mESCs grown on matrigel were dissociated by dispase and trypsin, respectively, and combined to form mosaic embryoid bodies (Figure 18). Equal numbers of human and mouse ES cells (n= 5000 of each cell type) were mixed and cultured together in suspension for seven days. Mosaic embryoid bodies were then immunocytochemically examined for contribution of RUES1 cells using an antibody that specifically recognizes a human nuclear antigen. RUES1 cells were detected in significant number in the mosaic embryoid bodies, though they were always underrepresented relative to mouse cells (data not shown). hESCs differentiated, along with the mESCs, into derivatives suggesting the three primary embryonic germ layers in mosaic embryoid bodies. Markers of ectodermal (neurofilament heavy chain and Sox2 in Figure 18B-D), mesodermal (Desmin in Figure 18F-H) and endodermal (HNF3β in Figure 18J-L) were exhibited by both human and mouse cells. Similar contribution of human cells to mosaic embryoid bodies and subsequent differentiation was also obtained when another hESC line, HUES#6 (Cowan et al., 2004) was used (data not shown).

# Figure 18
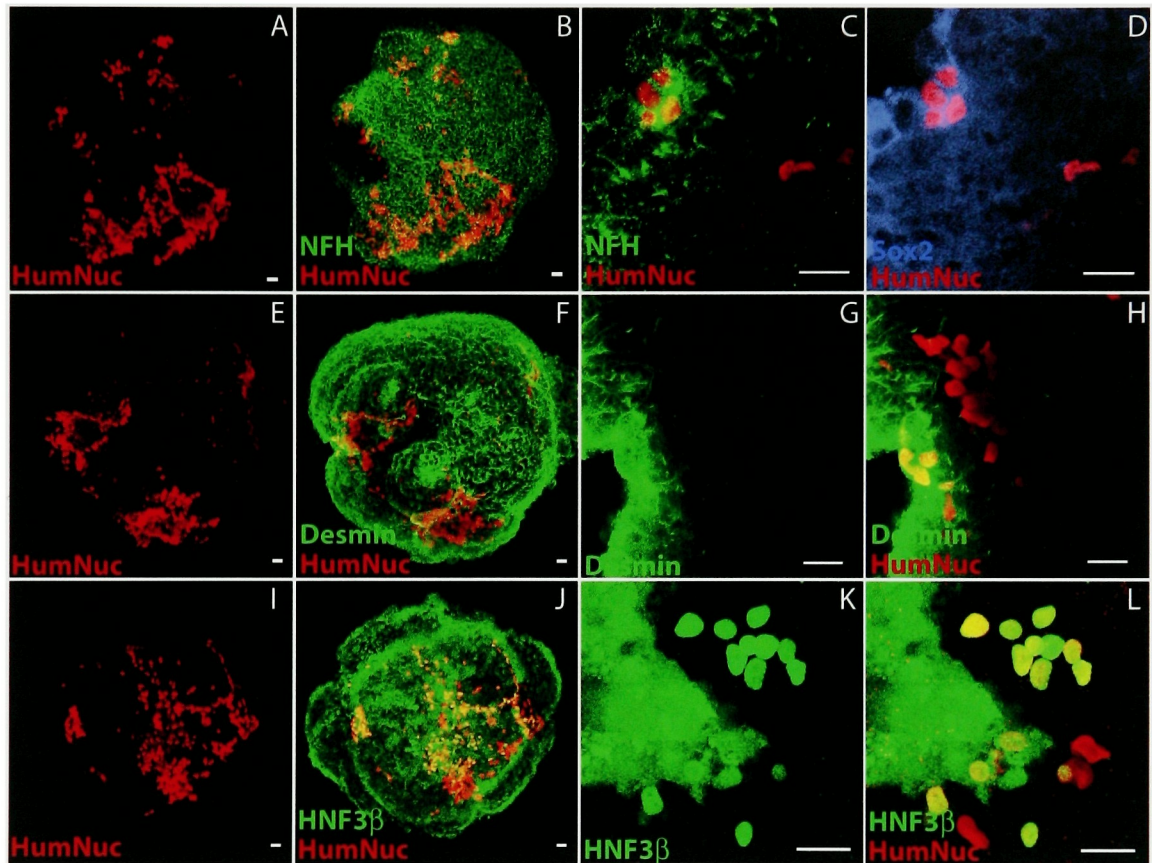


**Figure 18. Mosaic embryoid bodies generated from hESCs and mESCs show differentiation of both cell types to derivatives of the three primary germ layers.** Equal numbers of human (dispase-dissociated) and mouse (trypsin-dissociated) ESCs (n=5000) were mixed in suspended culture for 6 days and resultant EBs were assayed immunohistochemically for the presence of human cell nuclei (Red) and germ layer markers for ectodermal derivatives, including neurofilament heavy chain (Green in B, C) and Sox2 (Blue in D); the mesoderm marker desmin (Green in F-H); and the endoderm marker HNF3β (Green in J-L). A, B, E, F, I and J show whole EBs; C, D, G, H, K and L show single cell resolution. Scale bars – 20 μm.

## hESCs incorporate and differentiate in mouse blastocyst outgrowths

mESCs are derived from blastocyst stage embryos, but co-culture of mouse and human ESCs in mosaic embryoid bodies does not necessarily indicate how hESCs might behave in a bona-fide embryonic environment. In order to assess the ability of hESCs to proliferate, integrate and differentiate in mouse embryos, we injected e3.5 blastocysts with 10-15 cell clumps of RUES1 and cultured the embryos *in vitro* for six days (Figure 19). These experiments described below were designed to minimize hESC input into host embryos, in accordance with policies in place at the Rockefeller University, and are also in line with guidelines recommended by the National Academy of Sciences (http://books.nap.edu/catalog/11278.html). Figure 19A and B shows the injection protocol.

As RUES1 hESCs do not tolerate trypsin-passaging, two independent means of RUES1 dissociation, trypsin and micro-dissection into cell clumps, were compared for their ability to integrate into host blastocysts. In each case a total of 10 to 15 cells, either as individual cells or in clumps, were microinjected into the blastocoel cavity of a mouse blastocyst. When trypsin dissociated cells were compared to cell clumps, micro-dissected colonies showed the best quality and quantity of contribution (data not shown). Other available cell lines have shown poor recovery from trypsin-passaging in tissue culture (Amit et al., 2000), and this enzymatic treatment may account for poor contribution of trypsin dissociated RUES1 hESCs to mouse blastocysts. But another cell line HUES#6 (Cowan et al., 2004), which is routinely trypsin-passaged in cell culture, also showed poor contribution (data not shown). For this reason, the embryonic chimeras in these experiments were generated by injection of manually dissociated clumps.

In order to determine whether human cells would proliferate and mix with the mouse host, injected blastocysts were cultured on Matrigel™ for 6 days *in vitro* (Figure 19C-I). RUES1 derivatives persisted in 14% of cultured chimeric blastocysts after 5 days and resultant embryonic outgrowths showed a complex and disorganized three-dimensional structure with human cells present in significant numbers (>500 nuclei in

# Figure 19
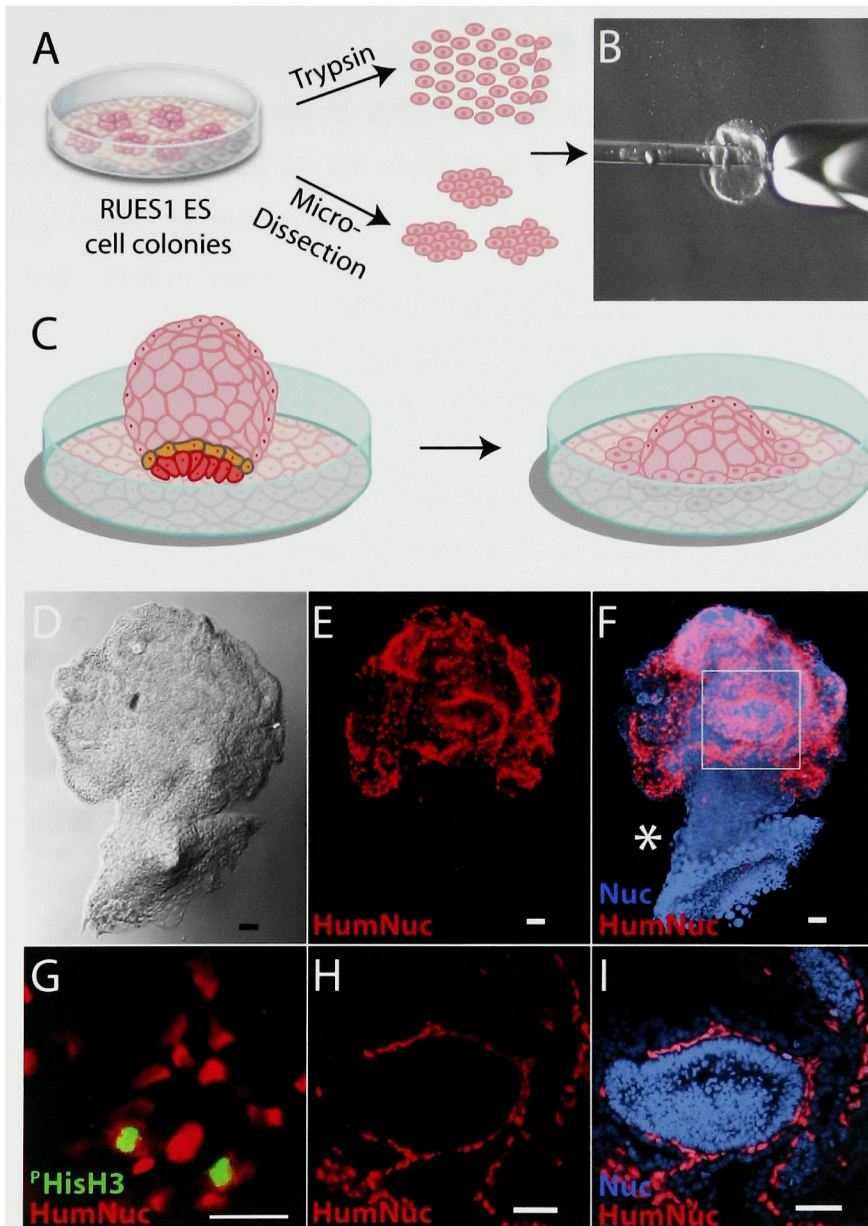


**Figure 19. hESCs survive, proliferate and incorporate into cultured mosaic embryos.** hESCs were dissociated enzymatically by trypsin or manually by micro-dissection and injected into the blastocoel of e3.5 mouse embryos. The injection scheme is shown in panels A and B. hESC-Injected embryos were cultured in vitro on matrigel coated tissue culture plastic (C) for 6 days. Resultant outgrowths showed complex three dimensional structure and human cells were present in significant numbers (D-I). Panel G shows human cells near the end of mitosis; Phoso-HistoneH3, which is phosphorylated in the context of mitosis, is shown in green. The inset in panel F is magnified as a single optical slice in panels H and I, which show intermingling of human cells with the host. Human nuclei are represented in red in E – I. SytoxGreen nuclear counterstain is shown in blue. Scale bars – D, E, F, H and I – 50 μm; G – 20 μm.

some cases) (Figure 19D-I). RUES1 derivatives were predominantly concentrated in the suspended body of the outgrowth, while the "stalk" by which the outgrowth adhered to the extra cellular matrix (star in 19F) was devoid of human cells. Cells were actively proliferating, as evidenced by the co-localization of Phospho-HistoneH3 (Gurley et al., 1978) and human nuclear antigen (Figure 19G). Relative to host cells, however, human nuclei were underrepresented in all chimeric outgrowths (Figure 19F and data not shown). This evidence established that hESCs could proliferate and intermingle with their mouse embryonic counterparts in cultured blastocyst outgrowths.

Mouse embryonic fibroblasts have commonly been used to maintain the

undifferentiated state of hESCs, so it is possible that the mouse embryonic environment may impede the differentiation of hESCs. In order to address this, we examined whether human cells within the outgrowths expressed markers of the differentiated state. Human cells derived from all three germ layers were detected (Figure 20E-J). Furthermore, we concluded that all the human cells were differentiated, as no RUES1 nuclei were positive for the pluripotency marker Oct3/4. In fact, a cluster of Oct3/4 positive mouse cells that was retained in one outgrowth provided a valuable internal control for the absence of Oct3/4 in human cells (Figure 20A-D).

**Stable expression of a genetic marker in RUES1**

In order to localize RUES1 derivatives within chimeric blastocysts and cultured outgrowths, we performed immunocytochemistry on our samples using an antibody specific for human nuclear antigen. While these methods were adequate for our purposes, secondary detection by immunofluorescence can result in signal artifacts that arise from non-specific binding of primary or secondary antibodies. To avoid this possibility in our subsequent experiments, we set out to generate hESCs that stably express green fluorescent protein (GFP). Because methods commonly used to generate stably expressing mESCs by lipofection were not as effective in hESCs (data not shown), we used lentiviral transduction to stably integrate GFP into RUES1 (Figure 21). We first transfected HEK 293 cells with a lentiviral vector containing eGFP and used the super-

# Figure 20
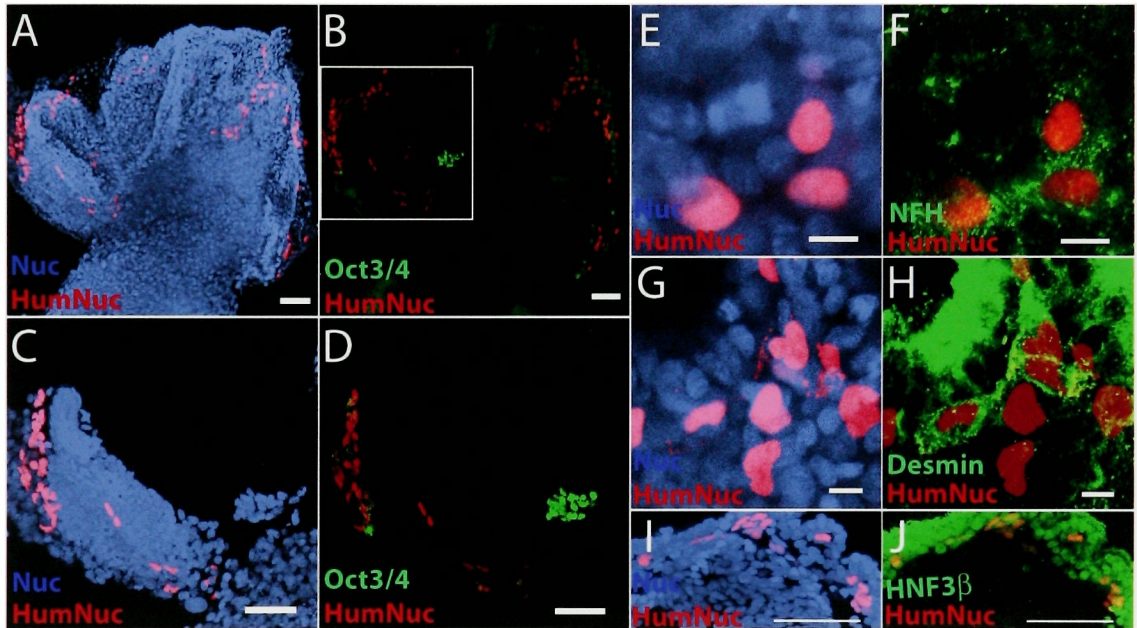


**Figure 20. hESCs differentiate into three primary germ layer derivatives within mosaic outgrowths.** hESCs were injected into e3.5 mouse blastocysts and cultured for 6 days on Matrigel. Resulting outgrowths were fixed and processed immunohisto-chemically using antibodies specific for Oct3/4 (Green in B and D), neurofilament heavy chain (Green in F), desmin (Green in H) and HNF3β (Green in J). Human nuclei are represented in red and nuclear counterstain in blue. Panels C and D represent a magnified view of the inset in B. Scale bars - A-D, I and J – 50 µm; E - H – 10 µm).
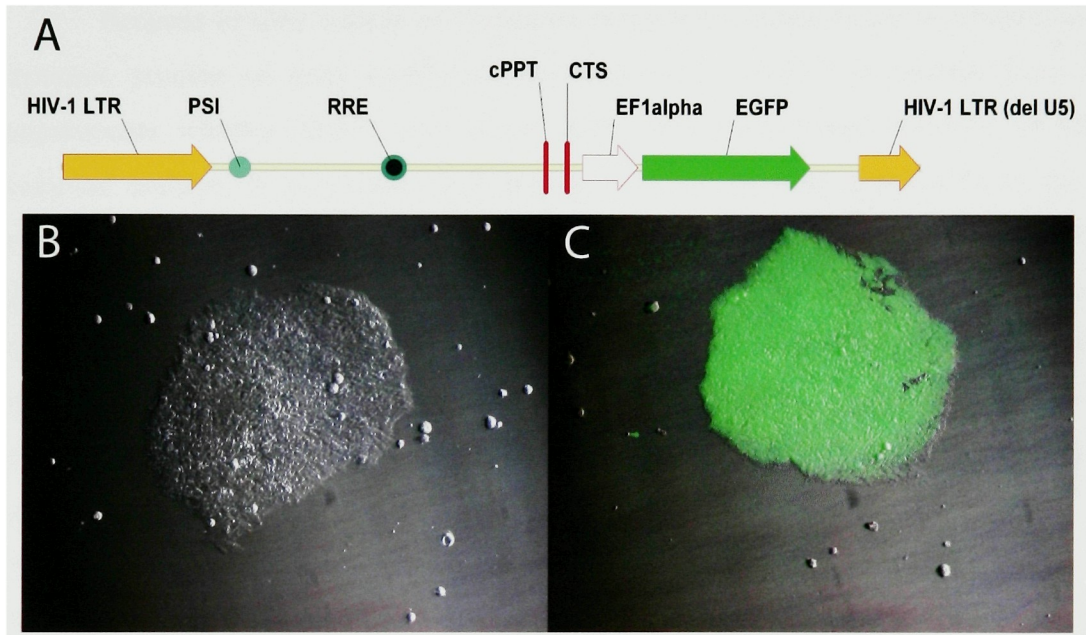
# Figure 21



**Figure 21. eGFP transduction of RUES1 by lentivirus.** (A) Schematic map of the pTrip-eGFP vector (Sirven et al., 2000; Sirven et al., 2001). (B and C) show RUES1 hESCs viewed with DIC (panel B), and with a composite of DIC and fluorescence in RUES1 transduced (at 5 X 10₅ ifu/ml) with pTrip-eGFP vector, after two rounds of manual passaging.

natant from these cells to infect RUES1 at 5 X $10^5$ infectious units/ml. After manually selecting for regions of strong GFP expression through two passages, homogenous, GFP-expressing RUES1 colonies were obtained (Figure 21B and C). GFP expression remained stable in these hESCs for more than 10 passages.

**hESCs can maintain their pluripotency within the mouse ICM niche**

Because *in vitro* culture of blastocyst outgrowths cannot begin to recapitulate the dynamic process of early embryonic development *in vivo*, it is unclear from these experiments whether hESCs and mouse ICM derivatives would combine to form a coherent embryo. It is possible that human cells are growing ectopically in the host embryo and only become intermingled with mouse cells by virtue of their proximity. This possibility prompted us to ask whether RUES1 would integrate into and maintain the identity of the host ICM shortly after injection. In order to address this, we repeated the injection protocol and examined the expression of Oct3/4 after 24 hours of culture. Figure 22 shows that RUES1 cells that incorporated generated a small niche of Oct3/4 positive cells among their Oct3/4 mouse counterparts in the ICM (Figure 22C, D, G and H). In contrast, we consistently observed that hESCs that did not incorporate into the ICM were Oct3/4 negative and showed unhealthy nuclear morphology (arrow in Figure 22C). In these settings we never found contribution of human cells to the trophectoderm. Human cells that integrated into host ICM maintained Oct3/4 levels and were negative for the trophectoderm marker Cdx2 (Figure 22F-H). From this evidence, we concluded that the differentiated cells seen in the mosaic outgrowths originated from the hESCs that engrafted into the host ICM.

**hESCs aggregated with blastomere stage mouse embryos engraft into ICM**

A common alternative to blastocyst injection for the generation of embryonic chimeras entails the aggregation of ESCs with pre-compacted blastomere stage embryos (Zeilmaker, 1973; Nagy et al., 1993). Given the technical difficulty and physical stress involved in the injection of hESC clumps into mouse blastocysts, adapting aggregation

# Figure 22
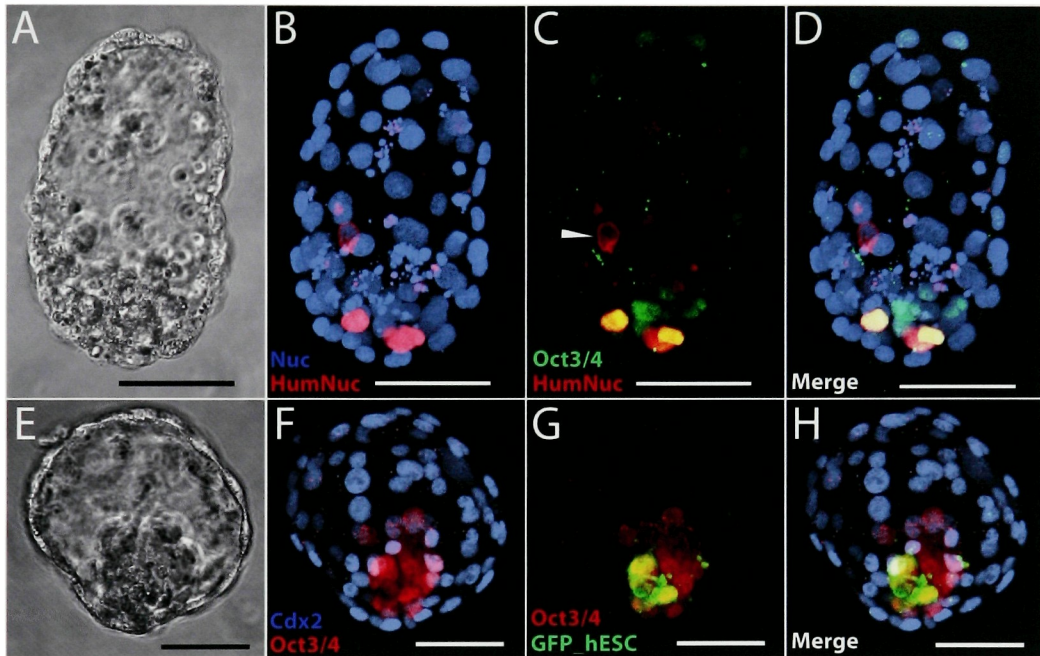


**Figure 22. RUES1 hESCs integrate into host ICM and retain pluripotent identity.**
Embryonic chimeras were generated by blastocyst injection (A-H) and fixed 24 hours
post injection for immunohistochemical analysis. (A-D) Human cells detected by anti-
human nuclear antibody (red in B-D) that integrated into the host ICM showed healthy
nuclear morphology (blue in B and D) and maintained Oct3/4 (green in C and D). Human
cells that did not incorporate into host ICM exhibited unhealthy, apoptotic morphology
and did not retain Oct3/4 (arrowhead in C). (E-H) GFP expressing hESCs (green in G and
H) were negative for the trophoectoderm marker Cdx2 (Blue in F and H) in embryonic
chimeras generated by blastocyst injection. Nuclear counterstain is shown in blue in B
and D. Scale bars – 50 μm.

protocols to suit hESCs would not only allow for an increase in the scale of chimera generation, but would ensure reduced trauma to engrafted hESCs. Furthermore, prior to compaction, mammalian embryos have yet to make the cell fate distinction between ICM, which gives rise to the embryo proper, and trophectoderm, which mediates invasion of uterine epithelium during implantation and gives rise to extraembryonic tissues. While mESCs do not normally differentiate to trophectoderm lineages *in vitro*, hESCs have demonstrated this potential (Xu et al., 2002b). Hence, engraftment of hESCs into mouse blastomere stage embryos provides a means of testing the ability of hESCs to take on dual cell fates (trophectoderm vs. ICM) concomitantly with the cells of the host. To address these questions, we combined dispase-dissociated RUES1 colonies with pre-compaction embryos in conical bottomed wells followed by mild centrifugation (Schematic shown in Figure 23A). After 48 hours, an average of 39% of resultant blastocysts contained hESCs in three independent experiments. Engrafted cells were localized to the ICM of a vast majority (97%) of embryonic chimeras that formed, and they retained the Oct3/4 positive identity of adjacent mouse cells (Figure 23B-E). As was the case for RUES1 hESCs injected into mouse blastocysts, human cells did not exhibit the trophectoderm marker Cdx2 in successfully aggregated embryos (Figure 23D-F). However, in rare cases, aggregation resulted in chimeric blastocysts in which hESCs were not localized to the host ICM (arrow in Figure 23B). In these chimeras, some human cells retained Oct3/4 expression, but surprisingly, a minority exhibited Cdx2 expression (arrowhead in Figure 23G), though at a reduced level relative to host trophectoderm (Figure 23G-I); and upon further differentiation in outgrowths after six days, Cdx2-positive human cells were also evident (data not shown). From these observations, we concluded that hESCs preferentially localized to host ICM in embryonic chimeras generated by blastocyst injection or morula aggregation, though hESCs showed the capacity, in some cases, for differentiation toward trophectoderm fate.

# Figure 23
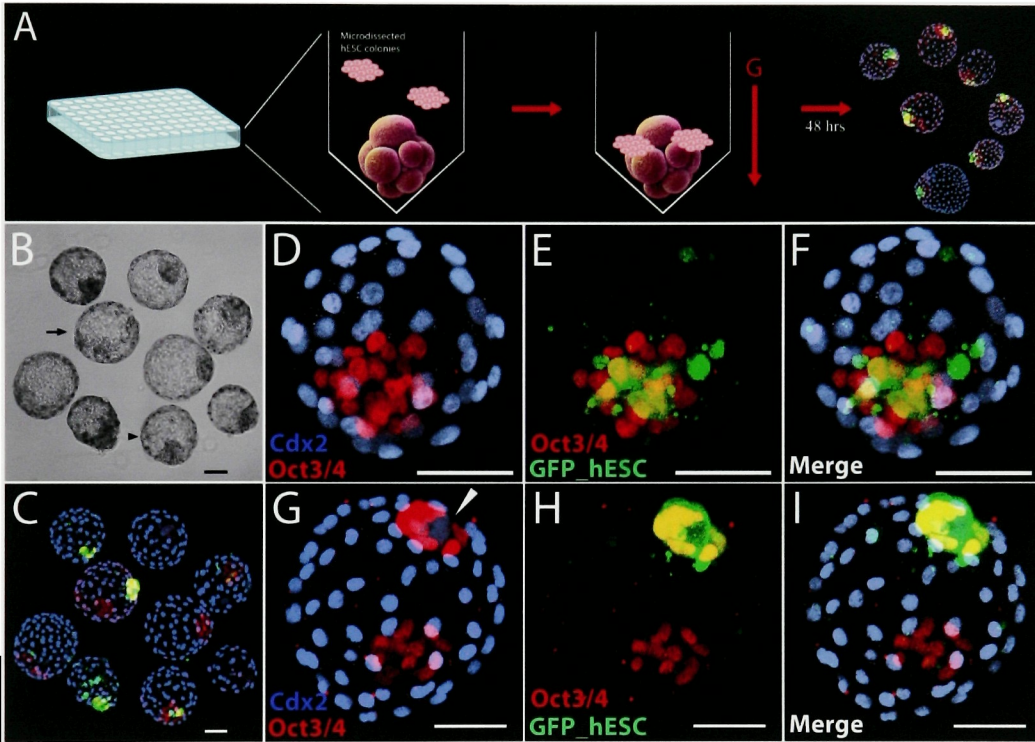


**Figure 23. RUES1 hESCs engraft into blastomere stage mouse embryos, localize to host ICM and retain pluripotent identity.** Embryonic chimeras were generated by aggregation (A-I) and fixed 48 hours post aggregation for immunohisto-chemical analysis. A schematic describing the aggregation protocol is shown in (A). (B-F) A majority of embryonic chimeras generated from morula aggregation showed localization of hESCs (green in C, E and F) to host ICM with retention of Oct3/4 (red in C-F) and absence of Cdx2 (blue in C, D and F). (G-I) Rare embryos contained hESC derivatives (green in H and I) that were positive for Cdx2 (arrowhead in G). Panels D-F and G-I show magnified views of embryonic chimeras indicated by the arrowhead and arrow, respectively, in panel B. Scale bars – 50 μm.

## hESCs persist in implanted embryonic chimeras *in vivo*

Given the strikingly disparate developmental schedules for mouse and human embryogenesis, it is unexpected that embryonic cell types from the two species could be combined within chimeras to form a coherent embryo. To determine whether embryonic chimeras generated by blastocyst injection would give rise to developmentally viable embryos *in vivo*, we implanted hESC-injected blastocysts into the uterus of pseudopregnant foster mice and retrieved them at embryonic day 8 (Figure 24). Of 28 chimeric embryos that were implanted, 24 formed deciduae that contained embryos. 13 of these embryos were phenotypically normal and did not contain GFP-positive RUES1 derivatives (Figure 24A, left); 7 embryos were developmentally delayed and did not contain RUES1 derivatives (Figure 24A, right); and 3 embryos contained GFP-positive RUES1 derivatives, but showed aberrant morphology (Figure 24B and C). Strikingly, one embryo was morphologically identical to normal littermates, but contained 10 GFP-positive hESC derivatives localized to the prospective foregut endoderm and neuroepithelium (Figure 24D-H). The persistence of human cells in implanted chimeras was supported by a separate data set, in which human cells were shown by immunocytochemistry to persist in rare embryos, specifically in the anterior neural folds of embryonic chimeras at e8.5 (Figure 24I-K). From these experiments, we concluded that hESCs can persist in embryonic chimeras implanted *in vivo* and furthermore, that they can be maintained in an embryo that proceeds normally through gastrulation.

# Figure 24
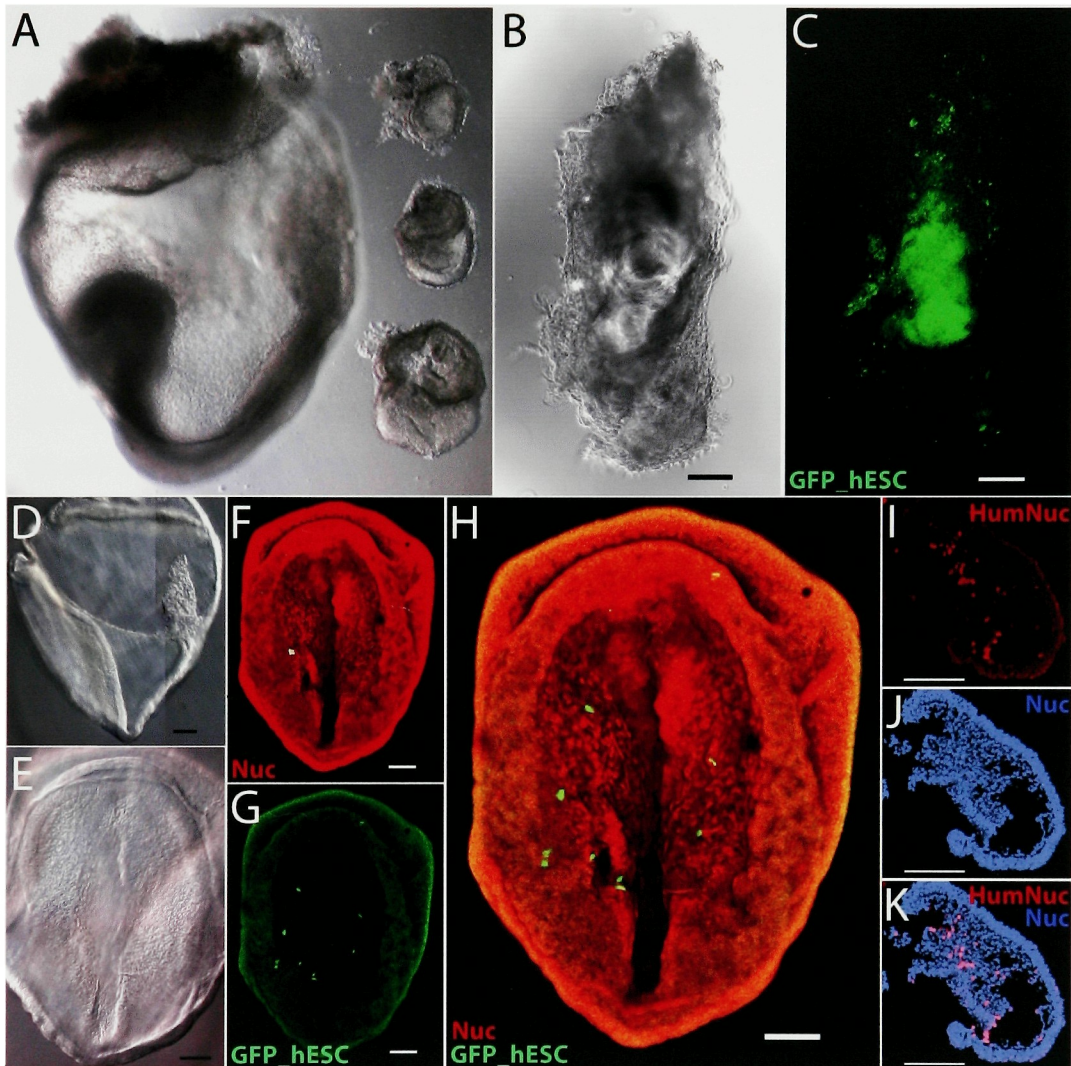


**Figure 24. hESC-derivatives are retained in embryonic chimeras following implantation in vivo.** hESC-injected blastocysts were implanted into the uterus of pseudopregnant foster mice and recovered after 5 days of development. (A) Examples of implanted blastocysts that resulted in wild type phenotype (left) and aberrant/delayed phenotype without GFP-positive hESC contribution (right). (B and C) Brightfield and fluorescent images of abnormal embryos containing GFP-positive hESC contribution. (D-H) Morphologically normal embryo containing 10 GFP-positive hESC derivatives. (D) saggital view; (E-H) anterior view. (I-K) A section of the neural fold region of an embryonic chimera at e8.5 was labeled by immunocytochemistry with the anti-human nuclear antigen antibody. Multiple positive human nuclei are shown. Nuclear counterstain is shown in red in (F) and (H) and in blue in (J and K); GFP-positive cells are shown in green in (C, G and H); cells that were positive for the anti-human nuclear stain are shown in red in (I and K). Scale bars – 100 μm.

## Discussion

Embryonic cell mixing and recombination experiments between closely related species is a traditional approach of experimental embryology, used for more than a hundred years to understand embryonic processes at the cellular level. The origin of these methods can be traced to experiments in which early embryonic explants were transplanted between frog and newt gastrulae; an approach that allowed the identification of the origins of inductive signals during embryogenesis (Spemann, 1918; Spemann, 1921; Spemann and Mangold, 1923). Later work demonstrated that when presumptive jaw epithelium of a 5 day old chick embryo is recombined with the molar mesenchyme of an e16 mouse molar mesenchyme, a "hen's tooth" is generated (Kollar and Fisher, 1980). This established that the cells of the chick, which have not made teeth for nearly 100 million years, are still competent to respond to an appropriate inducer. In the past few decades, these and other pioneering experiments have contributed greatly to the understanding of vertebrate embryogenesis, but relative to other model species, a similar understanding of our own development has been elusive. hESCs have the potential to resolve this. In this study, we chose to test the capacity of a new hESC line, RUES1, to incorporate into the closely related embryonic environment of the mouse ICM. We showed that hESCs engrafted into pre-implantation stage mouse embryos and proliferated into differentiated human derivatives in the context of host tissue *in vitro*. In addition, we showed that there is biological compatibility between both human and mouse cells in the ICM of mouse blastocysts as well as following implantation into pseudopregnant foster mice. These data establish for the first time that hESCs can integrate into the mouse embryo, validating the potential for non-human embryos to serve as a surrogate environment in which to study hESCs and their derivatives.

Our study complements previous evidence establishing that ESCs have the capacity to integrate into extra-species hosts; mESCs differentiated *in vitro* to motor neurons innervate chick hind legs (Wichterle et al., 2002); and hESCs differentiate into neuronal cell types *in ovo* in the context of the chick embryo (Goldstein et al., 2002) or when injected directly into lateral ventricles of e14 fetal mouse brains (Muotri et al.,

2005). While these studies recombine late stage or stage-mismatched tissue, our embryonic chimeras are recombined from cells of the same stage as the one from which they were derived: the blastocyst. Twenty four hours after injection into mouse blastocysts, hESCs that were not incorporated into the ICM showed fragmented nuclear morphology suggestive of apoptosis or necrosis. And those hESCs that did incorporate into ICM maintained Oct3/4 expression at levels similar to adjacent mouse cells, while those that did not incorporate were negative for Oct3/4. Embryonic chimeras were also generated by aggregation of hESCs with blastomere stage embryos, and 48 hours after aggregation, hESCs were again localized to the host ICM and maintained Oct3/4 levels similar to adjacent host cells.

Localization of hESCs to host ICM is relevant to the ability of embryonic chimeras to implant and develop *in vivo*. Chimeras generated from aggregation of rat and mouse embryos show varied developmental progress depending on the methods used to combine them. When rat and mouse embryos are combined at the blastomere stage, rat cells contribute to the trophectoderm of the chimeras and they fail to implant into mouse uterus, presumably due to an immune response against the foreign rat component (Rossant, 1976). However, when rat ICM cells are injected into blastocyst stage mouse embryos, they do not contribute to trophectoderm, and these chimeras are able to implant and develop as far as early organogenesis stages (Gardner and Johnson, 1973; Gardner and Johnson, 1975). Our observation that injected hESCs almost always engraft into host ICM and do not contribute to host trophectoderm, regardless of their being derived by blastocyst injection or morula aggregation, suggests that chimeras generated by either method should at least be able to implant into the uterus of mouse foster mothers. Indeed, embryonic chimeras generated by blastocyst injection were able to implant and develop within foster mice, though the influence of hESCs seemed to disrupt embryogenesis in most cases.

The generation of interspecific chimeras using mouse pre-implantation embryos was first accomplished in 1973, when chimeric blastocysts were generated from mouse and rat embryos (Mulnard, 1973; Stern, 1973; Zeilmaker, 1973). Since then, embryonic

chimeras have been generated from combinations of mouse and vole (Mystkowska, 1975), the variant mouse species *Mus musculus* and *Mus caroli* (Rossant and Frels, 1980), the variant cow species *Bos taurus* and *Bos indicus* (Williams et al., 1990), and sheep and goat (Fehilly et al., 1984). Not surprisingly, chimeras generated from the more evolutionarily distant species were not viable, presumably due to irreconcilable differences between developmental programs - chimeras between mouse and vole or mouse and rat, for instance, did not come to term and only rarely developed to advanced stages *in utero*. On the other hand, chimeras generated from mixing embryos of closely related species (*Mus musculus* and *Mus caroli*, *Bos taurus* and *Bos indicus*, or sheep and goat) resulted in successful development to adulthood. Considering these results, it seems unlikely that chimeras generated from engraftment of hESCs into mouse blastocysts would develop into viable chimeric embryos. Our results show the majority of embryonic chimeras that implanted and retained hESC derivatives were developmentally abnormal/delayed. But rarely, hESCs persisted in morphologically normal embryos, demonstrating that hESC engraftment is not irreconcilable with mouse embryogenesis. In fact, the differences between mouse and human embryogenesis may account for these rare morphologically normal embryos. In particular, the difference in cell cycle between mouse and human ESCs may explain the relative scarcity of hESC derivatives in our embryonic chimeras at e8: Considering our experimental design, in which hESC contribution was intentionally minimized, combined with the relatively slow pace of hESC proliferation and/or human embryogenesis, it makes sense that hESC derivatives should be underrepresented; and if human contribution is minimized, the relatively brisk pace of mouse development may allow the host cells to out-compete the hESC derivatives, resulting in "pockets" of human cells in a morphologically normal mouse embryo.

The observation that RUES1 localized to the ICM of host blastocysts indicated that hESCs preferentially occupied a niche that parallels that of their origin, the ICM. Yet, the emergence of functional neurons from undifferentiated hESCs injected directly into the lateral ventricles of e14 mouse brains (Muotri et al., 2005) establishes that pluripotent human cells and their derivatives can respond appropriately to the inductive

signals of an evolutionarily distant niche. In our embryonic chimeras that were generated by aggregation of hESCs with blastomere stage mouse embryos, hESCs rarely engrafted "ectopically" into regions of trophectoderm upon blastocyst formation (Figure 23N-P). In these embryos, most of the hESC derivatives retained Oct3/4 expression, but in a subset of the engrafted cells, Oct3/4 was completely lost, and a weak Cdx2 signal was observed. While little can be concluded as to the timing of differentiation in mouse vs. human cells from these experiments, they did provide some indication that human cells were capable of taking on the molecular identity of the niche into which they engrafted. Following from our work, it is feasible that mouse/human chimeras could be generated in which hESCs are engrafted into pre-implantation stage mouse embryos and distributed throughout the host anatomy through gastrulation. This may allow for chimeras in which hESC derivatives are "seeded" into an array of developmental niches within a viable mouse, which would be of considerable value for the modeling of human development and disease in live animals.

While these experiments are in line with ethical guidelines set forth by the National Academy of Sciences (http://books.nap.edu/catalog/11278.html), we purposefully restricted our analysis to early developmental stages and minimized hESC engraftment in order to affirm the feasibility of these assays. As such, the current study is lacking in some respects. First, in order to further explore the utility of embryonic chimeras as a vehicle for examining the emergence and behavior of human cell types, we must characterize the extent to which human cells can contribute to a viable mouse-human chimera. Allowing progression of chimeras to later developmental time points would indicate whether hESC derivatives are capable of integrating functionally into host anatomy. Second, in the rare instances in which hESC derivatives persisted in morphologically normal chimeras, the observed GFP-positive cells could have been a result of cell fusion rather than persistence of bona-fide human cells. Given the rarity of this phenotype and the scarcity of presumed human cells, it is difficult to rule out the possibility of cell fusion without increasing the scale of the experiments and allowing further proliferation of hESC derivatives within chimeras left to develop to later stages. The efficiency of blastomere stage embryo aggregation with hESCs allows for a scalable

platform whereby embryonic chimeras can be generated in which hESC input is varied by number and/or differentiation state.

Regardless of whether human cells can accommodate the spatiotemporal signaling environment and/or developmental schedule of mouse embryogenesis in any or all instances, the generation and culture of mouse/human chimeras *in vitro* may at least allow for a study of the murine embryonic explant's influence on hESC differentiation, yet engraftment of hESC derivatives into live chimeric animal models would be a much more valuable tool. Provided that hESCs can be reconciled with mouse embryogenesis *in vivo*, engrafting hESCs into host anatomy before gastrulation may provide an accessible platform for studying the emergence of many human cell types; and with the expansion of available hESCs to include genetically diseased lines, mouse/human chimeras may allow us to elucidate the bases of disease by examining the behavior of such hESC lines in live animal models. In these ways, the advances reported here provide a foundation for future work towards an understanding of human development and disease.

# MATERIALS AND METHODS

## Reagents

Human Activin A, Activin RIB/ALK-4/Fc Chimera, Activin RIIB/Fc Chimera, Cripto-1 and Bmp4 were purchased from R&D. Mouse LIF (Esgro®) was purchased from Chemicon International. The TGF-β inhibitor SB-431542 (SB) was purchased from Tocris. The GSK-3β inhibitor, 6-bromoindirubin-3'oxime (BIO) was generously provided by Laurent Meijer. Pronase, Demecolcine solution, and guinea pig complement were purchased from Sigma. Dispase, Collagenase and basic FGF were purchased from Invitrogen. Primary antibodies used included phospho-Smad1/5, phospho-Smad2, and phospho-Ser CDKs substrate (Cell Signaling); Smad1/5 (Upstate); Smad2/3 and Oct3/4 (Signal Transduction Laboratories); hNanog (R&D); SSEA4, Tra-1-60 and Nestin (Chemicon); β-tubulinIII/Tuj1 and α-Tubulin (Sigma); Alpha-1-Fetoprotein and Human Placental Alkaline Phosphatase (DAKO); HNF3b, Sox2, Oct3/4 (Santa Cruz); MuscleMHC/MF20 (Developmental Studies Hybridoma Bank); Neurofilament Heavy Chain, PhosphoHistoneH3 and Desmin (Abcam); and Cdx2 (Biogenix). Secondary antibodies used were donkey anti-mouse, anti-goat or anti-rabbit conjugated to AlexaFluor488, AlexaFluor555 and AlexaFluor647 (Molecular Probes). DNA nuclear counterstains used were SytoxGreen, SytoxOrange and ToPro3-iodide (Molecular Probes).

## RUES1 derivation and culture

Blastocysts frozen at day 6 post fertilization were donated with informed consent from embryos in excess of clinical need according to institutional guidelines. Identifying information was removed before receipt of the vials and blastocysts were thawed by stepwise removal of cryoprotectant. Blastocysts were washed 2X in recovery medium and incubated for two hours before immunosurgery to allow for blastocoel expansion and morphological grading. Recovery medium consisted of 10% Plasmanate, 1X non-essential amino acids, 1X essential amino acids, 1X GlutaMAX in M16 medium (Specialty Medium). The blastocyst was treated with 2mg/ml pronase to remove the zona pellucida and then incubated in a 1:10 dilution of anti-human placental alkaline

phosphatase antibody (DAKO). The embryos were washed three times in recovery medium and incubated in a 1:10 dilution of guinea pig complement (Sigma) and monitored for trophectoderm lysis. Lysed trophectoderm was removed by pipetting through a pulled pasteur pipette and isolated ICMs were washed 2X in HUESM medium. HUESM consisted of DMEM supplemented with 20% KSR, 1X non-essential amino acids, 1X essential amino acids, 1X GlutaMAX and 20ng/ml bFGF (Invitrogen). Human LIF was added (12ng/ml) during the initial outgrowth but was excluded from subsequent culture. ICMs were plated on a MEF feeder layer and outgrowths were micro-dissected and transferred to fresh feeder layers for three passages for expansion. Stable culture of RUES1 was maintained as previously described (Thomson et al., 1998; Xu et al., 2005).

**Cell Culture**

hESC line H1 was obtained from WiCell research institute and BGN1 and BGN2 lines were obtained from BresaGen. Human embryonic stem cells were maintained as previously described (Sato et al., 2003). BGN2 Cells were plated on matrigel coated poly-D lysine/laminin Biocoat® coverslips (Becton-Dickinson) and cultured 5 days in the described conditions before fixation and immunofluorescence. RUES1 derivation and culture conditions are described above. mESCs were cultured in 15% fetal calf serum and maintained undifferentiated by addition of LIF to culture medium. Mitotically inactivated mouse embryonic fibroblasts were purchased from Specialty Media.

**Immunofluorescence**

Following culture, hESCs, mouse preimplantation embryos, mosaic embryoid bodies, RUES1 embryoid body and teratoma sections, mouse/human embryonic chimeras and mouse/human blastocyst outgrowths were fixed in 4% Paraformaldehyde in PBS, washed in PBS + 0.2% bovine serum albumin (BSA), permeabilized with 0.1% Triton X-100 in PBS/BSA for 20 minutes, and then blocked in 5% donkey serum in PBS/BSA for 2 hours at room temperature. Cells were incubated overnight at 4°C in appropriate dilutions of primary antibodies. After 3 washes in PBS/BSA, cells were incubated with combinations of AlexaFluor488, AlexaFluor555 or AlexaFluor647 conjugated secondary antibodies for 2 hours at room temperature. Cells were then washed with PBS/BSA and stained with

either 25 nM SytoxGreen, SytoxOrange or ToPro3-iodide nucleic acid stain in PBS/BSA. Endogenous Alkaline Phosphatase was assayed in RUES1 following the manufacturer's instructions (Vector Labs). Coverslips, tissue sections and/or whole tissues were mounted in glycerol and imaged using a Zeiss Pascal confocal microscope.

## hESC cell synchronization

BGN1 cell cycle progression was blocked at metaphase by incubation overnight in conditioned medium with 100 ng/ml Demecolcine solution. Cells were washed four times with PBS to release them from metaphase block and then harvested for immunoblotting at 15 minutes and 4 hours post-release.

## Immunoblotting

Cells were lysed with 100 μl 1X lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM $Na_3VO_4$ and complete mini protease inhibitor cocktail (Roche). 20 μg of total protein was loaded for each lane. Membranes were blocked in TBS with 0.1% Tween and 5% milk. Antibodies used were anti-phospho-Smads (Cell Signaling); anti-Smad2/3 (Transduction Laboratories; 1:1000); anti-Smad1 (Upstate); anti-tubulin (Sigma); anti-Oct3/4 (Transduction Laboratories); anti-hNanog (R & D). Primary antibodies were incubated over night and secondary antibodies for two hours. Proteins were detected with ECL (Amersham Biosciences).

## RT-PCR

Cells were lysed directly with 100 μl RNAbee (Tel-Test, Inc.) and total RNA was extracted. 1 μg of total RNA was reverse transcribed to cDNA and 1/20 of the RT reaction was used as PCR template. Radioactive amplification was according to the following conditions: β-actin (f: TGGCACCACACCTTCTACAATGAGC; r: GCACAGCTTCTCCTTAATGTCACGC; 21 cycles); Oct3/4 (f: GAAGGATGTGGT-CCGAGTGT; r: GTGACAGAGACAGGGGGAAA; 19 cycles); Nanog (f: ACCAGAA-CTGTGTTCTCTTCCACC; r: GGTTGCTCCAGGTTGAATTGTTCC; 21 cycles).

## BrdU incorporation and TUNEL assays

The *In situ* Cell Proliferation, FLUOS (cat. No. 1810740) and *In situ* Cell Death Detection, Fluorescein (cat. No. 1684795) kits were obtained from Roche. BGN2 cells were cultured two days in CM and then further cultured in the indicated conditions for 3 days. Following culture, BrdU incorporation and TUNEL staining was assayed according to the protocols described by the manufacturer. Cells were imaged and quantified using a Zeiss Pascal confocal microscope.

## Blastocyst Outgrowth

Pregnant Swiss-Webster mice were sacrificed at embryonic day 3.5 and the uterus was isolated and flushed with warm culture medium to obtain blastocyst stage embryos as described previously (Nagy, 2003). Embryos were incubated in culture medium containing 1 mg/ml pronase and observed continuously until the zona pellucida was digested. Embryos were then rinsed three times in warm culture medium and incubated on gelatin-coated tissue culture plastic in mouse embryonic stem cell medium (Betts et al, 2001) containing either 20 µM SB-431542 or an equivalent dilution of DMSO. Embryos were not disturbed during culture in order to allow attachment and outgrowth. Embryos were fixed in 4% Paraformaldehyde in PBS at 4 days post extraction and processed for immunofluorescence in the same manner as the hESC. RUES1 injected blastocysts and RUES1 aggregated morulae were allowed to recover for 24 or 48 hours, respectively, and cultured on matrigel coated tissue culture plastic. Following 6 days of culture in medium containing 15% fetal calf serum, chimeric outgrowths were fixed and processed for immunofluorescence. All outgrowths were imaged using a Zeiss Pascal confocal microscope.

## Formation of Embryoid Bodies

Embryoid body formation for BGN2 cells was carried out as previously described (Sato et al., 2004). BGN2 cells grown for 5 days in described conditions were harvested using dispase (Invitrogen) and plated on bacterial culture plates. Embryoid bodies were allowed to form by growth in suspension for 7 days and were photographed by light-field

microscopy. Embryoid bodies were then plated on gelatin coated tissue culture grade plastic and further cultured for 7 days in order to allow for terminal differentiation of cell types. Reattached and terminally differentiated embryoid bodies were harvested and processed by RT-PCR in order to assay for the presence of derivatives of primary germ layers. Embryoid bodies were generated from RUES1 hESCs by incubation of cultures in Dispase until colonies detatched from the substrate. This was followed by culture of the aggregates in DMEM supplemented with 20%FCS, 1X penicillin-streptomycin, 1X GlutaMAX (all from Gibco) on non-tissue culture treated Petri dishes coated with a thin layer of agarose to prevent attachment.

## Real time RT-PCR

RUES1 hESCs were grown in feeder free conditions in the presence of MEF-conditioned medium. To induce differentiation, CM was withdrawn and replaced with non-conditioned growth medium for 30 days. Real-time RT-PCR with previously published gene specific primer sets was performed as described (Suarez-Farinas et al., 2005). Briefly, Ubiquitin-C (UBC) and b-2-microglobulin were used as an internal control for normalization. Data were analyzed in triplicate.

## Teratoma formation

To generate teratomas, $1\text{-}2\text{X}10^6$ RUES1 hESCs were injected into the rear leg muscle of SCID/Beige mice. Teratomas were allowed to develop for 4-6 weeks and were then excised and fixed in neutral buffered formalin and analyzed histologically by trained pathologist. Some teratomas were fixed, equilibrated in 30% sucrose and embedded for cryosectioning. Sections were processed immunohistochemically for markers of germ layers as described above.

## Karyotype analysis

RUES1 hESCs were independently karyotyped after 6 passages by G-banding at 450-650 band resolution (Cancer Genetics, Inc.)

## Lentiviral vectors and infections

Supernatants containing infectious particles were collecred 36 hours after calcium phosphate co-transfection of HEK 293 (Graham et al., 1977; Graham and van der Eb, 1973) cells with pTrip (Sirven et al., 2000; Sirven et al., 2001), ps PAX2 (D. Trono Swiss Institute of Technology Lausanne), and pL-VSV-G (Bartz and Vodicka, 1997; Yee et al., 1994). hESCs were infected at 5 X $10^5$ ifu/ml.

## Statistical analysis

Student's t-test was performed in Excel (Microsoft) to access significance of differences in deltaCt values between differentiated and undifferentiated samples in Real-time RT-PCR analysis. For statistical comparisons of fold-change measurements between RT-PCR results and previously published microarray data (Suarez-Farinas et al., 2005), ANOVA as well as Pearson correlation and regression analysis with two-tailed p-values was performed in Prism 3.0 (GraphPad Software, Inc.).

## Blastocyst injections and embryonic outgrowth culture

RUES1 hESCs were manually dissected into 10-15 cells clumps using finely drawn glass Pasteur pipets and injected into embryonic day 3.5 mouse blastocysts flushed from the uterine horns of Swiss Webster mice. hESC clumps were drawn into custom pulled transfer pipets with a 25 μm bore (Eppendorf™) and injected into the blastocoel cavity of mouse embryos. hESC injected blastocysts were either fixed 24 hours post injection, or cultured on Matrigel™ coated tissue culture plastic in culture medium containing 15% fetal bovine serum for 6 days. Resultant embryonic outgrowths were fixed and processed immunohistochemically as described above.

## RUES1 aggregation with mouse blastomere embryos

Embryonic day 2.5 mouse embryos were flushed from the oviduct of superovulated CBA/B6 mice and treated with acid tyrodes to remove their zona pellucidae. In conical bottomed wells of a 96-well plate, one embryo was placed with a dispase-dissociated

hESC clump of ~10-15 cells and the plate was centrifuged briefly in order to combine them (adapted from Nagy, 2003). Embryos were allowed to recover for 48 hours, when they were fixed and processed immunocytochemically as described above. Under these conditions, mESCs aggregated in parallel showed contribution to host ICM after 48 hours.

**Chimeric blastocyst implantation**

Embryonic day 3.5 mouse blastocysts that were injected with hESCs and allowed to recover for 6 hours post-injection were transferred to the uterine horns of pseudopregnant foster mice as previously described. Five days following transfer, implanted embryos were recovered from the uterus of foster mothers and examined for hESC contribution.

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