Tri-lineage differentiation of embryonic stem cells:

Role of Wnt signaling

by

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"It is not birth, marriage, or death,
but **gastrulation**, which is truly the most
important time in your life."
Lewis Wolpert (1986)

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Abstract

Since embryonic stem cells (ESC) and the epiblast share a gene expression profile and an attenuated cell cycle, ESC are an attractive model system to study lineage choice at gastrulation. We have employed mouse ESC as a model system to examine the role of Wnt signaling in the transition from pluripotency to neural precursors and have identified a new function for the *Geminin* gene in mesendodermal differentiation.

Wnt signaling is required for differentiation of both epiblast and ESC to the mesendodermal lineage. To test the hypothesis that blocking Wnt signaling in the absence of BMP signaling would promote differentiation of mESC to neural precursors, we developed a mESC line that inducibly expresses a dominant negative Tcf4 (dnTcf4) protein to block canonical Wnt signaling. Cells expressing the dnTcf4 protein differentiated largely to Sox3 positive neural precursors but were unable to progress to βIII tubulin positive neurons unless Wnt signaling was de-repressed. This cell line will provide an important resource for other investigators studying the role of Wnt signaling in embryonic development.

Geminin has been described as a bi-functional protein that inhibits endoreduplication during cell division and promotes neural differentiation by inhibiting BMP signaling. Geminin also binds transcription factors and chromatin

remodeling proteins in the nucleus to inhibit their function. In mESC over-expression of Geminin induced mesendodermal differentiation and an epithelial to mesenchymal transition (EMT) via increasing Wnt signaling. We hypothesize that Geminin binds to Groucho/TLE proteins in the nucleus thereby dis-inhibiting Wnt target gene transcritption by Tcf/Lef proteins. Germinin is frequently over-expressed in cancer cells and EMT is an integral part of cancer metastasis. These observations make Geminin an attractive candidate for cancer therapy; reduction of Geminin protein could reduce metastasis as well as induce cell death due to endoreduplication. Overall, this work demonstrates that Wnt signaling is required for mesendodermal differentiation, EMT, and the transition from proliferative neural precursor to primitive neurons during lineage allocation of mESC.

Chapter 1

Introduction

Overview of early mouse development

The single cell zygote must make an astounding number of cell fate choices as it progresses to the fully formed embryo/fetus. The first fate choice of the embryo, trophectoderm versus inner cell mass (Figure 1.1 E), and the second fate choice, primitive endoderm versus epiblast (Figure 1.1 G), are made prior to implantation and occur via a combination of asymmetrical/symmetrical cell divisions, cell polarity, and differential gene expression, the control of which is still being vehemently debated (Arnold and Robertson, 2009; Rossant and Tam, 2009; Zernicka-Goetz *et al*., 2009). Shortly after implantation (E 4.5) the embryo elongates along the proximal-distal axis and undergoes cavitation to generate the cup shaped “egg cylinder” (Figure 1.1 H). At this time there are gradients of signaling proteins that control gene expression along the proximal-distal axes. The next crucial step is to break the radial symmetry of the egg cylinder and establish the anterior-posterior axis that precedes the organized differentiation of all the various other cell types. The creation of the axes and breaking of the symmetry of the embryo generate signaling centers including the anterior visceral endoderm (AVE), primitive streak, and node; these signaling centers secrete signaling molecules to pattern the early embryo.

The mouse embryo is radially symmetrical and there are no overt morphological signs of the anterior-posterior axis until the formation of the primitive streak at E6.25 (Figure 1.2). Dividing cells begin to converge at the proximal posterior epiblast border, down-regulate E-cadherin expression, undergo an epithelial to mesenchymal transformation (EMT), and exit the epiblast, first replacing the visceral endoderm (VE) with definitive endoderm (DE), while cells migrating between the epiblast and endoderm give rise to different types of mesoderm based on the timing and anterior-posterior level of the primitive streak that they migrate through. The most posterior portion of the streak, which is also the first to undergo EMT, gives rise to extraembryonic mesoderm. At more intermediate levels, lateral plate, cardiac, and paraxial mesoderm migrate through the streak. Finally, cells that migrate through the anterior end of the node and primitive streak give rise to the notochord and prechordal plate. The portion of the epiblast that doesn't undergo EMT becomes epidermal and neural ectoderm (discussed in detail below). Overall, the process of gastrulation transforms the two-layered egg cylinder into an embryo with three germ layers as depicted in Figure 1.2.

Next, the process of neural induction sets aside the medial portion of the epiblast that will differentiate into the central nervous system. The timing and control of this process is directly linked to the signaling events that establish the AVE, primitive streak, and node and will be discussed in detail below. Decades of research with amphibian embryos led to the "default hypothesis" which proposed that in the absence of bone morphogenic protein (BMP) the epiblast

differentiates into neural ectoderm (Klingensmith *et al*., 1999). The node (the mouse homologue of the organizer) secretes BMP inhibitors including: Noggin, Chordin, and Follistatin, while the head patterning region, the AVE, secretes BMP inhibitors such as Cerberus-like (Cerl) and Wnt inhibitors such as Dickkopf-1 (DKK1), and secreted frizzled related proteins (sFRPs) (Figure 1.3). More recent work in mouse, amphibian, and chicken embryos has called this hypothesis into question and it is likely that control of neural induction is more complex and has yet undiscovered components (Delaune *et al*., 2004; Heeg-Truesdell and LaBonne, 2006; Kuroda *et al*., 2004;Linker and Stern, 2004). The situation in the mouse embryo is more complicated due to the presence of multiple signaling centers and the simultaneous processes of neural induction and gastrulation. Various protein gradients work to establish the neural ectoderm in the anterior of the embryo while inhibiting the signals that induce the primitive streak, thereby positioning it in the posterior of the embryo.

Signaling pathways involved in lineage allocation during early mouse embryogenesis

Pre-implantation development

Lineage allocation in the preimplantation mouse embryo begins with the segregation of the inner cell mass (ICM) of the blastocyst into the epiblast which differentiates into the embryo proper and subjacent primitive endoderm (PE) which gives rise to extraembryonic tissues that play key roles in axis specification. It was initially supposed that ICM cells were uniform and assumed

different cell fates based on undefined positional cues (Dziadek, 1979). More recently it was demonstrated that prior to differentiation, cells of the ICM express either Nanog (epiblast marker) or Gata6 (primitive endoderm marker) in a “salt and pepper” pattern, indicating that the ICM is not a homogeneous population (Chazaud *et al*., 2006). The model proposed based on this work suggested that expression of Nanog versus Gata6 controlled the expression of different cell surface adhesion molecules allowing the cells to physically sort into two distinct populations. Recently, it has been shown that up-regulation of FGF/MAP kinase signaling in the ICM converts the entire population to PE cells while blocking FGF/MAP kinase converts the entire population to epiblast cells (Yamanaka *et al*., 2010). Cells within the ICM express FGF4 (Nichols *et al*., 1998; Yuan *et al*., 1995) and the *FGF4* null mouse completely lacks PE development (Feldman *et al*., 1995), suggesting that signaling within the ICM promotes PE fate (Yamanaka *et al*., 2010).

Proximal-distal axis formation

The next critical event is establishing the proximal-distal axis, followed by the anterior-posterior axis of the embryo. The proximal-distal axis is established by a gradient of transforming growth factor - β (TGF-β) signaling via proteins expressed in both embryonic and extraembryonic layers (Figure 1.4). The TGF-β family member Nodal is expressed throughout the epiblast and in extraembryonic tissues at the egg cylinder stage (Varlet *et al*., 1997). In the absence of Nodal/SMAD2 signaling, embryos die prior to gastrulation and fail to form a

primitive streak (Conlon *et al*., 1994; Waldrip *et al*., 1998; Zhou *et al*., 1993). Activated nodal in the extraembryonic ectoderm (EXE) up-regulates BMP4 expression that induces Wnt3 expression in the epiblast (Ben-Haim *et al*., 2006; Brennan *et al*., 2001). Wnt3 in turn increases nodal protein expression, which activates SMAD2 in the most distal visceral endoderm (DVE) (Brennan *et al*., 2001; Morkel *et al*., 2003). SMAD2 activation in the visceral endoderm stimulates expression of the nodal inhibitors, Cerberus-like (Cerl) and Lefty1, that inhibit nodal signaling to create a proximal-distal nodal gradient positioning the future AVE, node, and primitive streak (Brennan *et al*., 2001). *Lefty1* and *Cerl* double-null embryos form multiple primitive streaks, indicating that inhibition of nodal in the VE is required to properly place the node and primitive streak (Perea-Gomez *et al*., 2002).

Anterior-posterior axis formation

Formation of the anterior-posterior axis occurs via the anterior migration of the distal VE (DVE) to form the anterior visceral endoderm (AVE), thus, setting the position of the node and primitive streak in the posterior of the embryo. In addition to nodal inhibitors, the DVE also expresses several transcription factors including Otx2. DVE migration fails in *Otx2* null mice (Pera-Gomez *et al*., 2001) due to a lack of Otx2 driven DKK1 expression (Kimura-Yoshida *et al*., 2005). Although the mechanism is not fully understood, Kimura-Yoshida *et al*., (2005) demonstrated that the movement is directed by a gradient of Wnt signaling. The DVE migrates towards cells expressing DKK1 in the proximal anterior VE and

away from Wnt signaling in the posterior of the embryo (Kimura-Yoshida *et al*., 2005). If the Wnt gradient is disrupted either by loss of DKK1 expression or over-expression of a Wnt in the anterior region, DVE migration fails. Reduction of Nodal signaling diminishes and in some cases severely limits migration of the AVE (Lowe *et al*., 2001) suggesting, that it is required for AVE formation most likely because the Nodal, BMP, and Wnt3 expression cascade in the proximal posterior of the embryo is required to establish the Wnt gradient in the VE. In the absence of BMP signaling, Wnt3 is not expressed in the epiblast and DKK1 is not expressed in the VE and the AVE fails to migrate (Miura *et al*., 2010). Differential rates of proliferation between anterior and posterior VE controlled by nodal antagonists Lefty1 and Cerl may also play a role in AVE migration (Yamamoto *et al*., 2004). Figure 1.5 depicts the migration of the DVE to form the AVE and establishment of the anterior-posterior axis. Once the DVE has migrated to form the AVE, the anterior-posterior axis of the embryo is set and the primitive streak and node are placed via the restriction of nodal, Wnt, and BMP signaling to the posterior of the embryo.

Gastrulation

Establishing the primitive streak

Gastrulation is the process that produces the three primary germ layers of the embryo. Before the onset of gastrulation at E6.25, the posterior proximal portion of the epiblast where the primitive streak will form expresses Wnt3

(Rivera-Perez and Magnuson, 2005) that activates canonical Wnt signaling (Mohamed *et al*., 2004). In the absence of Wnt3 or its downstream effector β-catenin (Figure 1.2), embryos fail to develop a primitive streak or node and gastrulation fails, and the epiblast remains undifferentiated (Haegel *et al*., 1995; Huelsken *et al*., 2000; Liu *et al*, 1999). Wnt3a is expressed in the primitive streak at the onset of gastrulation on E7.5 (Greco *et al*., 1996; Takada *et al*., 1994); therefore, *Wnt3a* null embryos initiate gastrulation, but lack more posterior primitive streak derivatives such as paraxial mesoderm, have posterior truncations, and form ectopic neural tissue (Yoshikawa *et al*., 1997). Embryos heterozygous for *Lrp5* and null for *Lrp6* (Wnt ligand co-receptors) exhibit reduced Wnt signaling in the epiblast (Kelly *et al*., 2004). *Lrp* mutant mice are characterized by expansion of neural ectoderm, loss of the posterior primitive streak, and expansion of the anterior primitive streak and its derivatives axial mesoderm and DE due to a failure to down-regulate Nodal expression as the primitive streak develops (described in detail below).

Anterior primitive streak derivatives are critical for the differentiation and later pattering of anterior neural ectoderm (Martinez-Barbera and Beddington, 2001). Based on their observations, Kelly *et al*. (2004) concluded that in wild type embryos Wnt signaling inhibits differentiation/formation of neural ectoderm and anterior primitive streak via down-regulation of nodal. Furthermore, *Tcf1*$^{-/-}$*/Lef1*$^{-/-}$ (Tcf/Lef factors are DNA binding proteins that complex with β-catenin, Figure 1.3) embryos form ectopic neural tubes (Galceran *et al*., 1999) while *Tcf4*$^{-/-}$ */Tcf1*$^{-/-}$ exhibit both caudal truncations and hindgut defects (Gregorieff *et al*., 2004). As

predicted by the Wnt signaling mutants, over-expression of a Wnt ligand (Popperl *et al*., 1997) or stabilization of β-catenin (Kemler *et al*., 2004**)** expanded Wnt signaling and induced multiple axes or produced a nearly complete conversion of epiblast to mesendoderm. *Tcf3* null embryos, which actually have increased Wnt signaling due to the normally inhibitory role of Tcf3, also have duplicated nodes and notochords (Merrill *et al*., 2003). Taken together, these data clearly demonstrate that Wnt signaling plays a crucial role in the initiation of gastrulation and concomitant mesendodermal differentiation.

Genes required for mesendodermal differentiation

One of the main targets of Wnt signaling in the primitive streak is the T box transcription factor *Brachyury*, a crucial regulator of mesoderm and endoderm differentiation, which is also expressed in the posterior proximal epiblast prior to gastrulation (Rivera-Perez and Magnuson, 2005). *Brachyury* null embryos die at E10 due to lack of an allantois, a critical extraembryonic structure derived from mesoderm (Hermann 1992). *Wnt3* mutants fail to express Brachyury (Liu *et al*., 1999), *Wnt3a* mutants have reduced Brachyury expression (Yamaguchi *et al*., 1999); further, *Brachyury* promoter analysis demonstrated that it is a direct target of canonical Wnt signaling (Arnold *et al*., 2000; Yamaguchi *et al*., 1999). In turn, Brachyury maintains Wnt3a expression during the late primitive streak stage (Rashbass *et al*., 1994). *Tbx6*, another T box transcription factor is one of the few confirmed downstream Brachuyury targets (Chapman *et al*., 1996; Hofmann *et al*., 2004), so the mechanism of how *T* induces mesoderm

differentiation remains unclear (Wardel and Papaioannou, 2008). One important function of Brachuyry seems to be the control of cell adhesion proteins required for mesendoderm migration through the primitive streak (Wilson *et al*., 1995). The expression of the Nodal target *Eomesodermin* (*Eomes*), another T box transcription factor, also precedes the formation of the primitive streak in the mouse embryo (Ciruna and Rossant *et al*., 1999; Hancock *et al*., 1999) and is required for mesoderm formation by controlling the entry of epiblast cells into the primitive streak (Russ *et al*., 2000). *Mix-like* (*Mixl1*), a homeodeomain gene with unknown function is the only known Eomes target (Pearce and Evans, 1999). *Mixl1* has a critical role in gastrulation and endoderm differentiation, in null embryos the anterior-posterior axis fails to elongate, the node is not specified, and the heart tube and gut fail to form resulting in lethality at E9 (Hart *et al*., 2002). Interestingly, *Mixl1*$^{-/-}$ embryos have an expanded anterior primitive streak, perhaps due to cells being unable to enter the primitive streak as suggested for *Eomes*$^{-/-}$ embryos. While the T box genes *T*, *Tbx6*, and *Eomes* are all clearly necessary and in some cases sufficient for mesendoderm differentiation, the underlying mechanisms are not well understood.

TGF-β signaling in mesendoderm differentiation

Signaling by BMP and Nodal proteins, members of the TGFβ superfamily, is necessary not only to position the primitive streak, AVE, and node, but is also required for germ layer allocation from the epiblast during gastrulation. Studies of mice null for *BMP4* or its receptors, *BMPRIA* and *BMPRII* indicate that BMP

secretion from the extraembryonic ectoderm is required for the formation of the primitive streak and node (Beppu *et al*., 2000; Fujiwara *et al*., 2002; Mishina *et al*., 1995; Winnier *et al*., 1995). Conditional deletion of *Bmpr1a* specifically in the epiblast increased neural ectoderm and endoderm, indicating that BMP signaling normally inhibits their differentiation (Davis *et al*., 2004). Because loss of BMP signaling results in lethality prior to gastrulation, the detailed mechanisms underlying the requirement of BMP during mesendoderm differentiation are not entirely understood (Gadue *et al*., 2005).

During the early streak stage at E6.5, Nodal expression is restricted to the posterior epiblast and primitive streak (Conlon *et al*., 1994; Varlet *et al*., 1997), then by late streak stage, Nodal is expressed only in the node (Lu and Robertson, 2004). Absent Nodal expression, there is a complete failure of primitive streak formation (Conlon *et al*., 1994; Lu and Robertson, 2004) but reduced levels of Nodal protein caused loss of endoderm and anterior patterning defects associated with loss of anterior primitive streak derivatives (Lowe *et al*., 2001).

The DE arises from the anterior end of the primitive streak, incorporates into the VE, and migrates anteriorly. It was hypothesized that the DE displaces the VE from the embryo (Tam *et al*., 1993; Lin *et al*., 1994), but new evidence suggest that there is considerably more integration of the VE into endoderm, particularly of the gut than was previously appreciated (Kwon *et al*., 2008). The level of Nodal signaling, via Nodal ligand and downstream SMAD2 signal transducer, plays a key role in DE formation; anterior endoderm requires a higher

level than posterior endoderm and but if the level of Nodal signaling gets too low, endoderm, but not mesoderm is lost (Liu *et al*., 2004; Lowe *et al*., 2001). Mice null for *Mixl1*, mentioned above as a target of the Nodal target Eomes, lack DE but have increased mesodermal expression of Brachyury and Nodal (Hart *et al*., 2002). One function proposed for Mixl1 is down-regulation of Brachyury and Nodal expression to promote patterning of the nascent mesendoderm (Hart *et al*., 2002). Conditional deletion of β-catenin in the endoderm demonstrated that Wnt signaling is required for DE specification and in its absence there is an expansion of mesoderm (Lickert *et al*., 2002). Therefore, both Wnt and TGFβ signaling are critical to formation of the primitive streak and lineage segregation at gastrulation.

Figure 1.6 summarizes the signaling gradients in the late primitive streak stage (PS) mouse embryo. Taken together, the Wnt, T box, and TGFβ knock-out and over expression data suggest that allocation of epliblast at gastrulation is regulated by gradients of signaling proteins that establish the embryonic axes, position the AVE, node, and primitive streak (by controlling proliferation and gene expression) as well as directing cell fate as cells pass through various regions of the primitive streak. Cells that migrate through the posterior will give rise to extraembryonic and paraxial mesoderm via Wnt and BMP signaling while cells that migrate through the anterior end of the streak will give rise to axial mesoderm and DE via Nodal signaling (Gadue *et al*., 2005).

Epithelial to mesenchymal transformation

Cell movements in gastrulation begin in the primitive streak when cells in the posterior epiblast divide and move to the dorsal midline (Tam *et al*, 2001). Once cells reach the streak, they undergo an epithelial to mesenchymal transition (EMT); the cells loose apical-basal polarity, change shape, loose epithelial cell junctions via down-regulation of E-cadherin expression and up-regulation of N-cadherin, express integrin receptors, and migrate from the epiblast (Hay 1995; Nakaya and Sheng 2008). Figure 1.7 depicts the movements of cells into and through the primitive streak.

FGF signaling plays a biphasic role in gastrulation. FGF signaling up-regulates the expression of the E box transcription factor Snail (Ciruna and Rossant, 2001), a transcriptional repressor that reduces expression of E-cadherin (Batlle *et al*., 2000; Cano *et al*., 2000). FGF signaling then promotes expression of the mesodermal inducing transcription factors Brachyury and Tbx6 (Ciruna and Rossant, 2001). In *FGFR-I* and *FGF8* null mice, mesedodermal cells are unable to leave the primitive streak (Circuna *et al*, 1997; Sun *et al*., 1999; Yamaguchi *et al*., 1994). In the absence of *Snail1*, mesoendoderm is still specified, however, gastrulation fails and cells are unable to leave the primitive streak (Carver *et al*., 2001). Taken together, these data indicate that FGF signaling is one of the crucial pathways for initiating EMT.

Interactions between Wnt and FGF signaling are also involved in the down-regulation of E-cadherin during EMT. In the absence of Wnt signaling, active GSK3β (a regulatory kinase in the Wnt and other signaling pathways,

Figure1.2) phosphorylates Snail1 protein inducing proteosomal degradation, similar to the way GSK3β regulates β-catenin protein degradation (Yook *et al*., 2006; Zou *et al*., 2004). Wnt signaling increases the expression of both related E-box transcription factors Snail1 and Snail2 (Murray *et al*., 2006; Sakai *et al*., 2005) while Snail1 feeds back to maintain Wnt signaling (Stemmer *et al*., 2008). Finally, elevated levels of E-cadherin in *fgfr-1* null mice trap β-catenin at the cell membrane inhibiting its nuclear function and reducing Wnt signaling by Wnt3a (Ciruna and Rossant, 2001). The authors hypothesized that one of the normal functions of FGF signaling during EMT is to down-regulate E-cadherin and free β-catenin for signaling. The end result of gastrulation is the specification of the three germ layers from the epiblast that are then further patterned into the organs and tissues that comprise the body plan.

Neural induction

Neural induction has been proposed to occur by a "default" mechanism that requires the exclusion of Nodal, BMP, and Wnt signals from the prospective neural ectoderm of the embryo (Sasai, 1998). The node and AVE secrete many proteins that inhibit nodal, BMP, and Wnt signaling (Levine and Brivanlou, 2007) including: Lefty, Cer-l, sFRPS, Dkk1, Noggin, Chordin, and Follistatin. Supporting this hypothesis, *Noggin* and *Chordin* double null animals have a dramatic loss of forebrain as well as posterior neural ectoderm (Bachiller *et al*, 2000). A careful examination of *Nodal* null mouse embryos demonstrated an increase in neural ectoderm differentiation concomitant with the loss of

mesendoderm (Camus *et al*., 2006), suggesting that in the absence of posteriorizing signals the epiblast can differentiate into neural ectoderm. However, there is considerable evidence to suggest that while the node seems to be required for the most anterior neural ectodermal structures, there is differentiation of more posterior neural ectoderm in the absence of the node (Klingensmith *et al*., 1999). In fact, *Arkaida* mutant embryos lack both a node and notochord due to a loss of *Foxa2* expression, but initially develop relatively normal headfolds that fail to differentiate into forebrain and midbrain, but do generate hindbrain (Episkopou *et al*., 2001). Clearly, there must be additional as yet unidentified proteins involved in neural induction in the mouse embryo.

Following induction, the neural ectoderm is proliferative and a boundary must be set to maintain the domains of surface ectoderm surrounding the neural ectoderm. A temporally-regulated combination of BMP and Wnt signaling sets this boundary with high levels of BMP and Wnt signaling in the surface ectoderm and low levels in the neural ectoderm (Patthey *et al*., 2009; Steventon *et al*., 2009). The continued expression of BMP protein in the surface ectoderm in close proximity to the neural plate necessitates that BMP signaling continues to be repressed in the neural ectoderm after neural induction.

In *Xenopus* embryos, there are multiple pre-neural genes that are expressed between BMP inhibition and overt neuronal differentiation that are important not only in maintaining BMP repression but also maintaining proliferation of the neural ectoderm and preventing premature differentiation (Sasai, 1998), including the forkhead transcription factor FoxD5 that activates

expression of genes such as *Geminin*, *Sox11*, and *Zic2* (Yan *et al*., 2009). Geminin has been described as an inhibitor of BMP4 gene expression which, when over-expressed, expands the neural plate (Kroll *et al*., 1998) and represses neurogenic bHLH gene expression (Seo *et al*., 2005). In addition to regulating the cell cycle, this protein is likely important in the transition from proliferating precursors to differentiated cells (Seo and Kroll, 2006). Sox11 increases the expression of Sox3 (Yan *et al*., 2009) and also up-regulates Geminin expression as well as increasing neural progenitor number by promoting proliferation and inducing Sox2 expression (Rogers *et al*., 2009). Sox1, Sox2, and Sox3 can all block the activity of bHLH genes to maintain neural precursor populations (Bylund *et al*., 2003). FoxD5 and Notch increase expression of both Geminin and Zic2 that function to inhibit neuronal differentiation and maintain the neural precursor state (Yan *et al*., 2009). Following induction, the neural ectoderm must be carefully patterned with some cells becoming specified as neurons, a process controlled by several neurogenic basic helix-loop-helix transcription factors. Taken together, these results demonstrate a sophisticated and highly redundant cascade that ensures the inhibition of BMP signaling, proliferation, and inhibition of terminal differentiation necessary to specify the complex organ that comprises the vertebrate nervous system.

Embryonic stem cells as a model of lineage choice in development

Because mESC are derived from the pre-implantation blastocyst (on approximately E3.5), they poses several characteristics that make them an

outstanding model system for studying embryonic development. They are unique among cultured cells because they have an apparently limitless capacity to self-renew *in vitro*, as well as being pluripotent - the ability to generate all cell types present in the embryo. The gene expression pattern and cell cycle characteristics of mESC are remarkably similar to that of the epiblast (discussed in detail below), providing a reasonable facsimile of lineage allocation in the mouse embryo even at very early stages. For example, mESC can be de-differentiated and give rise to trophoblast cells by reducing the level of Oct3/4 expression (Niwa *et al*., 2000). When mESC are grown in suspension culture as embryoid bodies (EB), floating aggregates of cells, they spontaneously differentiate into all three germ layers: ectoderm, mesoderm, and endoderm. However, there are significant differences in the organization of the embryo and an EB. Lineage allocation in an EB is far more chaotic, i.e., there is no organized geometry, formation of an AVE, node, or primitive streak. An EB obviously does not receive signals from the uterine environment following implantation (Armant, 2005; Fazleabas *et al*., 2004; Vigano *et al*., 2003). Despite these differences, the ability to alter soluble factors in the EB environment and gene expression in cells forming the EB makes them an attractive model; particularly when combined with conventional mouse genetic technologies.

ESC can be used to test the role of genes and gene products in development free from the constraints characteristic of the complex three dimensional embryo, some of which include: lack of accessibility, the complex growth factor signaling and milieu of the embryo, and the amazing ability of the

embryo to recover from various perturbations, masking subtle effects of knock-outs or transgenes. Alternatively, when a specific gene knock out or transgene is lethal early in development, later developmental events cannot be examined, while mESC can often continue lineage specification and yield further information. Since they can be produced in large quantities, many biochemical assays are possible that would be much more difficult using the vanishingly small sample obtained from early mouse embryos. Discoveries using mESC can be employed to identify targets to test *in vivo* with conventional mouse technologies. Overall, testing gene function in both ESC and the early embryo provide a very powerful system to approach the complexities of development.

Directed lineage differentiation of mESC

A vast array of cell types has been generated from mESC (Giudice and Trounson, 2008; Irion *et al*., 2008, Murray and Keller, 2008). The main challenge with any directed differentiation scheme is to obtain an enriched population of target cells. Since mESC typically grow in clusters, cell-cell signaling can alter cell fate decisions, and maintain undifferentiated cells within the population. The degree of cell to cell contact and/or signaling varies with cell density even within a single tissue culture vessel. Regional variations in cell density often contribute to the high variability in target lineage purity. Despite these challenges, mESC have been differentiated into multiple cells types with the goal of probing gene function in development and also generating clinically relevant cell types for replacement therapies. The most successful paradigms employ promoters from

various lineage markers driving a fluorochrome for fluorescence-activated cell sorting or an antibiotic resistance cassette to help identify, quantify, and purify the desired lineages.

Selection strategies have been used extensively to study mesendoderm differentiation in combination with addition of soluble Wnt ligands or Activin A protein to mESC mimic signaling in the primitive streak. Promoters from mesendoderm restricted genes such as *Brachyury*, *Goosecoid*, or *Foxa2* have been used to drive selectable markers to enrich the desired cell population (Gaude *et al*., 2006; Kubo *et al*., 2003; Tada *et al*., 2005; Yasunaga *et al*., 2005). Once a relatively pure population of either mesoderm or endoderm has been established, more mature cell types such as cardiomyocytes (Shimoji *et al*., 2010), pancreatic beta cells (Boyd *et al*., 2008), or hepatocytes (Kubo *et al*., 2010), have been obtained with additional culture.

Primitive neural ectoderm is another highly desired target cell type for directed differentiation of mESC. Purification of neural ectodermal cells has been achieved using the *Sox1* promoter (Abranches *et al*., 2009) or the *Mapt* (*tau*) promoter (Bibel *et al*., 2004) to drive selectable markers. Deriving clinically pure populations of cells for replacement therapy will surely require selection strategies in combination with directed differentiation using growth factors or genetic manipulations.

The best directed differentiation schemes use embryonic development to inform the necessary genes and growth factors required to produce a given lineage (Murray and Keller, 2008). Factors that promote neural differentiation

from neural precursors such as FGF and EGF have also been used to drive mESC to the neuronal lineage (Nakayama *et al*., 2004). Protocols to generate specific neuronal subtypes such as motor neurons (Wichterle *et al*., 2002), dopaminergic neurons (Cajánek *et al*., 2009), or glial subtypes such as oligodendrocytes (Liu *et al*., 2000) have also been developed. The main challenge remaining with directed differentiation schemes is still the generation of sufficient numbers of pure cells in a particular lineage. A related problem is that highly differentiated cells are typically no longer mitotic while dividing cells can often still generate multiple lineages. The key to this problem may be to create pure populations of unipotent precursors that remain mitotically active, but will only form the target cell type; however, the genetic cascades or growth factors used to drive differentiation of most cell type specific precursors are largely unknown and the protocols to direct their differentiation require further development.

Maintenance of ESC pluripotency

Pluripotency of ESC is controlled at multiple levels including: a poised chromatin state with a large transcriptome, a core set of transcription factors that inhibit differentiation, extracellular signaling molecules, and a unique cell cycle that promotes proliferation and inhibits differentiation. Interestingly, the chromatin in ESC tends have an abundance of trimethylation of Lysine 4 of histone H3 (H3K4) and acetylation of histone 4 (H4Ac) that is associated with Trithorax proteins (Ringrose and Paro, 2007) and generally marks areas of open

chromatin with concomitant gene activity (Azurara *et al*., 2006). The large regions of open chromatin result in widespread gene expression, but expression is at low levels, leading to the suggestion that ESC are “primed” to differentiate (Efroini *et al*., 2008). There is more stochastic transcription in ESC than in differentiated cells or even lineage committed progenitor cells (Efroni *et al*., 2008; Meshorer *et al*., 2006) likely allowing them to respond quickly to signals for differentiation. As ESC begin to differentiate in lineage restricted patterns, genes involved in irrelevant lineages are down-regulated. While histone marks associated with Polycomb group (PcG) proteins (Ringrose and Paro, 2007) and gene inactivation, such as trimethylation of Lysine 27 of histone H3 (H3K27) are more rare, they are concentrated on promoters of lineage specific genes (Azurara *et al*., 2006; Bernstein *et al*, 2006). These promoter regions are also the targets of repression by the core transcription factors Oct3/4, Sox2, and Nanog (Boyer *et al*., 2005 discussed in detail below). Regions of DNA marked with both closed (H3K27) and open (H3K4) chromatin histone marks are considered bivalent and thought to represent the pluripotent state, creating the unique situation in which ESC are poised for differentiation yet held in an undifferentiated state (Azurara *et al*., 2006; Bernstein *et al*, 2006). In essence, the chromatin of lineage specific gene promoters is kept open to enable a rapid response, but PcG proteins repress gene expression to prevent random and uncontrolled differentiation. While epigenetic mechanisms are no doubt important in the regulation of pluripotency, in the absence of key PcG, components self-renewal can continue, albeit with increased expression of

differentiation-specific genes, implying that other mechanisms are involved (Pasini *et al*., 2007).

Core pluripotency transcription factors

Three transcription factors maintain the undifferentiated state of ESC: Oct3/4 (Nichols *et al*., 1998), Sox2 (Avilion *et al*., 2003; Nichols *et al*., 1998), and Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003). Oct3/4 is a POU domain transcription factor that is expressed in the early mouse embryo from the four-cell stage until it is down-regulated during the differentiation of the epiblast (Ovitt and Scholer, 1998). Oct3/4 represses the trophectoderm and promotes the ICM lineage choice by forming a repressive complex with the cdx2 transcription factor (Niwa *et al*., 2005; Ralston and Rossant 2008). In the absence of *Oct3/4*, the ICM fails to form, resulting in early embryonic lethality (Nichols *et al*., 1998). The level of Oct3/4 expression must be tightly regulated; as little as a twofold increase can initiate mesendoderm differentiation while down regulation results in trophoblast differentiation (Niwa *et al*., 2005).

Sox2 is a member of the SRY-related HMG box gene family that is expressed in the morula stage mouse embryo in the entire ICM and epiblast, then restricted to the neural ectoderm (Avilion *et al*., 2003). Like *Oct3/4*, *Sox2* is required for survival of epiblast cells (Avilion *et al*., 2003). When Sox2 is over expressed in mESC, they differentiate predominantly to neural precursors (Kopp *et al*., 2008), while expression of a dominant negative Sox2 in mESC results in trophoblast differentiation (Li *et al*, 2007). Oct3/4 and Sox2 frequently co-

regulate gene expression by binding to adjacent sites (POU/HMG sites) within gene promoters, and can reciprocally regulate their own promoters (Chew *et al*., 2005). Paradoxically, Oct3/4 and Sox2 also induce expression of FGF4 (Kunath *et al*., 2007) that in mESC poises cells for differentiation via Erk/ mitogen-activated protein (MAP) kinase signaling (Burdon *et al*., 1999). Erk/MAP kinase signaling does not promote lineage differentiation per se, but inhibits self-renewal. However, Oct3/4 and Sox2 also regulate vast numbers of genes both positively and negatively within the large ESC transcriptome (Boyer *et al*., 2005). Overall, Oct3/4 and Sox2 function to inhibit the expression of lineage-specific genes and promote expression of pluripotency genes.

Nanog is a homeobox protein, with no known homology to other proteins, discovered by two labs performing screens to identify novel pluripotency factors (Chambers *et al*., 2003; Mitsui *et al*., 2003). The authors named the protein after the mythical Celtic land of the ever young, Tir nan Og, because forced expression of Nanog maintained ESC in an undifferentiated state in the absence of LIF (a cytokine used to maintain ESC pluripotency, see below). In the mouse embryo, Nanog expression is restricted to cells that will become the ICM in the compacted morula, the ICM, and the early epiblast (Chambers *et al*, 2003). *Nanog* null mESC are able to remain pluripotent in the presence of LIF, but they have an increased tendency to differentiate (Mitsui *et al*., 2003; Chambers *et al*., 2007), while Nanog null mouse embryos fail to form an epiblast and instead give rise to only PE (Mitsui *et al*., 2003). Nanog over-expression in mESC under different culture conditions can block the formation of endoderm (Chambers *et*

al., 2003; Hamazaki *et al*., 2006; Mitsui *et al*., 2003), mesoderm (Suzuki *et al*., 2006), and neural ectoderm (Ying *et al*., 2003). Interestingly, Oct3/4 and Sox2 act together to induce Nanog expression (Kuroda *et al*., 2005; Rodda *et al*., 2005) and Nanog can block mESC differentiation even in the presence of active Erk/MAP kinase signaling initiated by Oct3/4+Sox2 (Chambers *et al*., 2003; Ying *et al*., 2003). Nanog occupies many of the same gene promoters as Oct3/4 and Sox2 (Boyer *et al*., 2005), indicating that these three transcription factors work in concert to maintain the balance of pluripotency versus differentiation of mESC (Figure 1.8).

Signaling molecules that maintain mESC pluripotency

Many other growth factors and signaling pathways have been implicated in controlling mESC pluripotency. The best characterized of these is leukemia inhibitor factor (LIF), a member of the ciliary neurotrophic factor (CNTF) family. LIF binds its receptor and gp130 thereby, stimulating phosphorylation and activation of Janus-associated tyrosine kinase (JAK) as well as signal transducer and activator of transcription (STAT). In mESC, STAT3 is activated (Niwa *et al*., 1998; Matsuda *et al*., 1999). Mouse embryos null for *STAT3* die at E6.5, indicating that LIF signaling may not be required for specification and development of the ICM *in vivo* (Takeda *et al*., 1997). One of the most important targets of STAT3 is the basic helix-loop-helix zipper transcription factor c-Myc (Carwright *et al*., 2004). Myc proteins bind 10-15% of all genomic loci in ESC, making it difficult to identify additional downstream direct targets of the pathway

(Eilers and Eisenmann, 2008). However, it is likely that c-Myc activation contributes to the unusual cell cycle characteristics of mESC (below) that inhibit differentiation (Niwa, 2007). Additional mechanisms have been proposed for LIF signaling activity such as cooperation with Nanog to block lineage specific differentiation (Bourillot *et al*., 2009) and up-regulation of Sox2 via Klf4 and Nanog via Tbx3 (Niwa *et al*., 2009).

While LIF is critical in maintaining pluripotency, it requires cooperation with other growth factors found in fetal calf serum (Ying *et al*., 2003). BMP4, a member of the TGF-β signaling family, is one of the serum components thought to play a role in pluripotency. Inhibitor of differentiation (Id) genes are BMP4-stimulated negative regulators of bHLH transcription factors thought to inhibit ESC differentiation by blocking bHLH target gene activation and stimulating proliferation and survival (Yokota, 2001). However, Id genes require STAT3 function to block differentiation (Ying *et al*., 2003). Additionally, BMP4 contributes to blocking MAP kinase pathway activation that promotes differentiation of ESC (Qi *at al*., 2004; Burdon *et al*., 1999). Phosphoinositide 3-kinase (PI3K) is yet another kinase that can be stimulated by growth factors found in serum as well as LIF. Activation of Akt by PI3K also appears to contribute to ES cell self-renewal and pluripotency (Palining *et al*., 2004; Wantanabe *et al*., 2006). It seems likely that many more, yet unidentified, factors combine to maintain the pluripotent state.

Cell cycle and mESC pluripotency

ESC and the epiblast share an attenuated cell cycle that appears to be both inhibitory to differentiation and maintains pluripotency. In fact, LIF independent but TGFβ dependent pluripotent stem cells, that express Oct3/4, Sox2, and Nanog have been derived from the mouse epiblast (Epiblast stem cells - EpiSC) (Brons *et al*., 2007; Tesar *et al*., 2007). Just prior to gastrulation, the mouse embryo expands from 20-25 cells to approximately 660 cells in roughly 1.5 days (Stead, *et al*., 2002 and Ciemerych and Scinski, 2005). During this period of rapid cell division, it is critical that the epiblast remains undifferentiated. ESC and the epiblast spend the majority of the cell cycle in S phase with a very short G1 phase (Burdon *et al*., 2002 and White *et al*., 2005), unlike somatic cells where G1 predominates. These differences not only produce the rapid cycling of the epiblast and ESCs, but also may play an important role in inhibiting differentiation and maintaining multipotency. The most crucial decision point in the cell cycle: i.e. to proliferate, differentiate, quiesce, senesce, or apoptose, occurs at the G1 checkpoint (Blomen and Boonstra, 2007). Spending minimal amounts of time in G1 phase had also been suggested to prevent differentiation by insulating cells from growth factor signaling (Orford and Scadden, 2008; Burdon *et al*., 1999). In fact, many of the mechanisms that maintain ESC pluripotency are directed at keeping the cells rapidly dividing. Moreover, exit from the cell cycle is considered a prerequisite for differentiation due to differential requirements for organization of the cytoskeleton in both processes (Grosshans and Wieschaus, 2000).

MicroRNAs and mESC pluripotency

MicroRNAs (miRNA) are small (20-25 nucleotide) non-coding RNAs that bind 3' untranslated regions of target mRNAs to repress translation and decrease mRNA stability (Gangaraju and Lin, 2009). Various regulatory RNAs are required for early mouse embryo development. Mouse embryos that lack Dicer-1 protein, an enzyme required to cleave precursor miRNAs to small interfering RNAs (siRNAs), die very early in development and fail to specify an epiblast (Bernstien *et al*, 2003). However, the miRNA pathway is globally suppressed early in development (Suh *et al*., 2010), therefore the *Dicer-1* phenotype may be due to endogenous small interfering RNAs (endo-siRNAs), another class of small interfering RNAs. *Dicer-1* deficient mESC remain undifferentiated both *in vitro* and *in vivo*, implying that miRNA regulation is required for differentiation (Kanellopoulou *et al*., 2005). A major function of miRNA regulation in mESC is to control progression through the cell cycle. Without miRNA function, mESC have prolonged G0 or G1 phase, but still fail to differentiate, implying there are mechanisms other than cell cycle control involved (Calabrese *et al*., 2007; Kanellopoulou *et al*., 2005; Wang *et al*., 2007). In fact, several miRNAs target Oct3/4, Sox2, and Nanog (Houbavivy *et al*., 2003; Marson *et al*., 2008; Tay *et al*., 2008), indicating miRNA are part of the mechanism involved in the down-regulation of the core transcription factors required for differentiation. Additionally, miRNAs control expression of proteins important for the epigentic regulation of chromatin configuration in mESC (Benetti *et al*., 2008; Sinkkonen *et al*., 2008). Clearly, the miRNA pathway plays an important role in maintaining

the balance in pluripotency versus differentiation in mouse embryo and mESC in combination with epigenetic regulation, the core transcriptional regulators, and the cell cycle.

Wnt signaling

Like the early embryo, many signaling pathways contribute to the pluripotency versus differentiation of mESC. The Wnt family of secreted ligands bind to frizzled receptors, inhibiting GSK-3β phosphorylation and the destruction of β-catenin, resulting in the accumulation of nuclear β-catenin that binds to Tcf/Lef transcription factors to activate and/or repress gene expression in the nucleus (Blauwkamp *et al*., 2008). There are four Tcf/Lef factors in the mouse genome that act as the downstream transcriptional effectors of the canonical Wnt signaling pathway. All Tcf/Lef transcription factors bind similar promoter sequences and can act as repressors in the absence of Wnt ligand activity (by binding Groucho/TLE family of co-repressor proteins) but can also switch to transcriptional activation when β-catenin enters the nucleus and displaces Groucho/TLE proteins (Brantjes *et al*., 2001; Daniels and Weiss, 2005). Despite their widespread importance in development, few Wnt pathway component null mESC lines have been developed and characterized, these include: *Wnt1* (Cajánek *et al*., 2009), *β-catenin* (Anton *et al*., 2007; Cajánek *et al*., 2009), *LRP6* (Cajánek *et al*., 2009), *TCF3* (Cole *et al*., 2008; Pereria *et al*., 2006) and *Wnt5a* (Bryja *at al*., 2006). The differentiation of Wnt pathway null mESC is discussed in detail below.

Wnt signaling can promote pluripotency

Wnt signaling can have opposing effects during development and differentiation ranging from maintaining proliferation to promoting differentiation likely in cooperation with other signaling pathways. Activation of the canonical Wnt pathway, but not the non-canonical pathway, has been reported to maintain self-renewal of mESC when combined with inhibition of the MAP kinase pathway (Singla *et al*., 2006). One proposed mechanism for maintenance of pluripotency by Wnt signaling is by up-regulating expression of pluripotency factors such as STAT3 (Hao *et al*., 2005) or Nanog in cooperation with Oct3/4 and/or Sox2 (Miyabayashi *et al*., 2007; Takao *et al*., 2007). Another proposed mechanism that appears to important for not only ESC as well as many tissue specific stem cells is increased proliferation (Kleber and Sommer, 2004; Wang and Wynshaw-Boris, 2004) by promoting expression of proteins important for cell cycle progression such as c-Myc and CyclinD1 (Willert *et al*., 2002). Inhibition of GSK3β by various small molecule inhibitors was suggested as another way to maintain ESC pluripotency by stimulating Wnt signaling (Bone *et al*., 2008; Sato *et al*., 2004; Singla *et al*., 2006), but may require cooperative low levels of LIF/STAT3 signaling to inhibit differentiation (Hao *et al*., 2005; Ogawa *et al*., 2006; Bone *et al*., 2008). Despite considerable data suggesting that Wnt signaling is required for pluripotency, β-catenin null mESC have decreased expression of the stem cell marker genes Rex-1, dppa-4, and dppa-5 and increased expression of FGF5, indicating a transition to a primitive ectoderm or

epiblast like status (Rathjen *et al*., 1999). The expression level of the core transcription factors (Oct3/4, Sox2, and Nanog) is unchanged in these cells indicating that Wnt signaling is not necessarily a requirement for pluripotency (Anton *et al*., 2007).

Tcf3 in pluripotency

The Tcf3 transcription factor also plays a key role in the balance between self-renewal and differentiation that is independent of Wnt signaling, even though Tcf3 is a key component of the Wnt pathway. During early mouse development, Tcf3 acts mainly as a transcriptional repressor of Wnt pathway targets; targeted disruption of *Tcf3* results in formation of multiple primitive streaks indicating hyperactivation of the Wnt pathway (Merill *et al*., 2003). Tcf3 is expressed in the ICM (Tam *et al*., 2008), binds to the promoters of Oct3/4, Sox2, and Nanog as well as co-occupying promoters with many of their targets (Cole *et al*., 2008; Pereria *et al*., 2006; Tam *et al*., 2008). *Tcf3* null and knock-down mESC are resistant to differentiation (Cole *et al*., 2008; Pereria *et al*., 2006). Pereria *et al*. (2006) suggest that Tcf3 acts mainly as a repressor of Nanog expression by its interaction with Groucho/Transducin like enhancer of split (TLE) proteins on the Nanog promoter, thereby enabling the differentiation of mESC. Cole *et al*. (2008) propose that Tcf3 acts to balance pluripotency versus differentiation based on the status of Wnt signaling. Tcf3 inhibits expression of Oct3/4, Sox2, and Nanog when interacting with Groucho proteins and promotes expression of Oct3/4, Sox2, and Nanog when interacting with β-catenin. Tam *et al*. (2008) hypothesize

that Tcf3 acts solely as a transcriptional repressor of Oct3/4 expression and thereby represses Nanog expression. Overall, these data indicate that the Tcf3 protein is a critical component in the transition from pluripotency to differentiation in mESC; however, since the epiblast of *Tcf3* null embryos undergoes differentiation there must be additional mechanisms which control this transition in the early embryo (Merill *et al*., 2003).

Wnt signaling in the differentiation of mESC

As predicted by its crucial role in the primitive streak of the early mouse embryo, Wnt signaling promotes the differentiation of mESC to the mesendodermal lineage (ten Berge *et al*., 2008; Gadue *et al*., 2006; Lindsley *et al*., 2006; Nakanishi *et al*., 2008). Wnt pathway activity increases BMP signaling and thereby promotes mesendodermal differentiation and blocks neural differentiation (Haegele *et al*., 2003). Also consistent with the expression of the Wnt inhibitory molecules DKK-1 and SFRPs in the mouse AVE and node, blocking Wnt signaling has been reported to promote the formation of neural ectoderm (Aubert *et al*, 2003, Cajánek *et al*., 2009; Kong and Zhang, 2009; Verani *et al*., 2007).

Wnt signaling in nervous system development

What has not been effectively addressed in mESC models of neural differentiation is the progression from the initial specification of the neural ectoderm to overt neuronal differentiation later. A single reference suggests that

neuronal differentiation of mESC requires Wnt signaling (Otero *et al*., 2004). As described for specification of neural ectoderm by mESC, neural induction likely requires inhibition of Wnt signaling in conjunction with inhibition of BMP signaling (Heeg-Truesdell and Labonne, 2006; Kuroda *et al*., 2004). Further patterning and differentiation of the nervous system requires both activation of Wnt signaling (Hirabayashi *et al*., 2004; Joksimovic *et al*., 2009; Lie *et al*., 2005; Muroyama *et al*., 2004; Parr *et al*., 1993) and inhibition of Wnt signaling (Houart *et al*., 2002; Lagutin *et al*., 2003) in a time and region dependent manner. During dorsal-ventral and anterior-posterior patterning of the nervous system, gradients of Wnt signaling help establish positional identity (Kim *et al*., 2001; Nordstrom *et al*., 2002; Megason and McMahon, 2002; Muroyama *et al*., 2002). One of the mechanisms by which Wnt signaling controls patterning of the nervous system is through control of neural precursor proliferation (Kalani *et al*., 2008; Horn *et al*., 2007; Wexler *et al*., 2009). Control of the divergent processes of proliferation and differentiation is likely via graded levels of Wnt signaling, i.e., high levels promote proliferation while low levels are required for differentiation (Zechner *et al*., 2003). Cross talk with other signaling pathways including: TGFβ (Chestnutt *et al*., 2004; Falk *et al*., 2008), FGF (Israsena *et al*., 2004), and Notch (Shimizu *et al*., 2008) also play a role in differentiation.

A second mechanism involved in the transition from proliferation to differentiation is increased expression of genes required for neuronal differentiation. For example, the neurogenic bHLH gene NeuroD1 is a direct target of the Wnt signaling pathway (Gao *et al*., 2009; Kuwabara *et al*., 2009).

Wnt signaling is required for the transition from precursor to fully differentiated cell type in other stem cell populations (Naito *et al*., 2006; zur Nieden *et al*., 2009; Ueno *et al*., 2007) indicating that alterations in Wnt signaling are repeated multiple times during the development of a particular tissue.

BMP and TGFβ signaling

BMP signaling has been proposed to maintain pluripotency of mESC by inducing the expression of Id proteins that inhibit differentiation in conjunction with STAT3 (Ying *et al*., 2003), inhibiting MAP kinase (Qi *et al*., 2004), or increasing proliferation by stimulation of Akt and Wnt signaling (Lee *et al*., 2009). However, BMP can also promote mesendodermal differentiation of mESC, as in the mouse embryo (Johansson *et al*., 1995; Park *et al*., 2004). BMP signaling is inhibitory to neural differentiation from mESC (Finley *et al*, 1999, Ying *et al*., 2003), and inhibiting BMP signaling during mESC differentiation promotes differentiation of neural ectoderm (Gratsch and O'Shea, 2002; Tropepe *et al*., 2001).

TGFβ signaling in mESC cultures is activated with Activin A rather than Nodal, but both proteins activate the same receptors and thereby activate SMAD2 (Gadue *et al*., 2005). As predicted by its expression in the primitive streak in the early embryo, Activin A promotes mesendodermal differentiaton of mESC (Johansson *et al*., 1995; Kubo *et al*., 2004; Pfendler *et al*., 2005), while blocking neuronal differentiation (Kubo *et al*., 2004; Pfendler *et al*., 2005). The process of mesendoderm differentation is dose-dependent with high levels of

TGFβ promoting endodermal differentiation and low levels promoting mesodermal differentiation (Kubo *et al*., 2004). Hedgehog signaling has also been probed in mESC differentiation, but appears to play a role in patterning of tissues like mesendoderm following TGFβ-induced specification rather than overt lineage differentiation.

FGF signaling

FGF signaling plays multiple roles in lineage specification of mESC. First, it can induce differentiation of PE cells from mESC (Li *et al*., 2001; Chen *et al*., 2001; Yoshida-Koide *et al*., 2004), as it has been shown to induce differentiation PE cells from the ICM (Chazaud *et al*., 2006). Blocking FGF signaling using a dominant negative receptor or small molecule inhibitor results in the loss of the endodermal layer typically formed around the outside of an EB (Li *et al*., 2001; Li *et al*., 2004; Chen *et al*., 2001). Second, autocrine FGF signaling is required for differentiation of neural ectoderm from mESC (Ying *et al*., 2003). Finally, FGF signaling can stimulate cell division and thereby help maintain pluripotency of mESC (Filipczky *et al*., 2007). Therefore, it is likely that FGF signaling acts to refine differentiation in combination with other pathways rather than direct differentiation to one specific lineage.

Retinoic acid signaling

Many differentiation paradigms include addition of retinoic acid to the culture medium (Bain *et al*., 1996; Guan *et al*., 2001; Okada *et al*., 2004). Not

only does retinoic acid block Wnt signaling by inducing SFRP2 expression and disruption of β-catenin-Tcf/Lef binding by the retinoic acid receptor (Lu *et al*, 2009) but it also stimulates the Erk/MAP kinase pathway to initiate differentiation and block BMP signaling (Lu *et al*, 2009; Stavridis *et al*., 2010). Furthermore, the retinoid X receptor binds to the promoter of many genes, including Sox3 to stimulate expression and induce differentiation of neural precursors (Mojsin *et al*., 2006).

Notch signaling

Cell to cell communication via the Notch signaling pathway has been well-characterized in the process of lateral inhibition, whereby some neural precursors differentiate to neurons, then inhibit their neighbors from attaining the same fate (Artavanis-Tsakonas *et al*., 1995; Greenwald *et al*., 1992). Cultures of mESC are prone to uneven cell densities due to their preference to grow in clusters. Inhibition of Notch signaling with a small molecule inhibitor has been shown to increase the number of cells progressing from neural progenitors to neurons (Abranches *et al*., 2009). However, the Notch pathway impinges on many other binary cell fate decisions (Chiba, 2006). While inhibition of Notch signaling does not induce differentiation of mESC in the presence of LIF, activation of Notch signaling by Notch ligands by Jagged-1 promotes neural differentiation and by Delta-like-4, promotes mesoderm differentiation-both in the presence of LIF (Ramasamy and Lenka, 2010).

Taken together, these results affirm the concept that mESC make an excellent model system to probe the roles of individual signaling pathways in differentiation in a manner reminiscent of the early mouse embryo.

Advantages and disadvantages of ESC for disease therapy

A more complete understanding of roles of the various signaling cascades during embryonic development will eventually allow directed differentiation of ESC for stem cell replacement therapy. The ultimate translational goal for stem cell research is to generate large quantities of cells that can be used safely for transplantation. One very attractive idea would be to simply mobilize endogenous stem cells to repair of replace damaged tissues or organs. However, it has proven very difficult to mobilize stem cells from their niche in sufficient numbers to offer any therapeutic benefit (Goldman, 2004), with the exception of the liver and the bone marrow, both of which have a significant innate ability to regenerate within their native microenvironment (Cottler-Fox *et al*., 2003; Michalopoulos *et al*., 2010).

The major disadvantages of using ESC in cell replacement therapy are their potential to generate teratocarcinomas (malignant tumors with all three germ layers), teratomas (benign tumors with all three germ layers), or simply undergo uncontrolled levels of proliferation that result in graft overgrowth. One of the most scientifically sound ways to approach this problem is to gain sufficient understanding of development necessary to differentiate ESC to the level of the resident tissue stem cell, then implant those cells with a “suicide” transgene (e.g.,

thymidine kinase driven by a Cyclin gene promoter) that could be used to kill cells if they re-enter cell cycle (Fareed and Moolten, 2002). An alternative approach would be to implant primitive cells so that the local microenvironment can program them to the desired cell type. One final caveat in cell replacement therapy is that placing new cells into a diseased environment might yield nothing but dead cells because the diseased tissue/organ is producing a deficient microenvironment e.g., in Alzheimer's disease. Careful studies with ESC may enable co-transplantation of stem cells with supporting cells that promote survival and colonization and thereby, improve patient outcomes.

Reprogramming

By carefully expressing 24 different transcription factors in mouse embryonic fibroblasts (MEFs), Takahashi and Yamanaka (2006) discovered that forced expression of only four factors (Oct3/4, Sox2, Klf4, and c-Myc) was required to reprogram MEFs to a pluripotent state, termed induced pluripotent cells (iPS cells). This offered the potential for transplantation of patient-derived cells, thereby, bypassing host versus graft rejection issues. Additionally, it called into question the need to destroy embryos in order to derive cells for patient therapy, a practice that some people find ethically unacceptable. Current research suggests that in fact iPSC are not ESC and behave differently both *in vitro* and when transplanted (Hu *et al*., 2010). Even if iPS cells are never used clinically, they offer tremendous potential in developing disease-specific human cells for the study of disease progression and pharmacological approaches to treating

disease (Papadeas and Maragakis, 2009). An even more exciting study demonstrated the ability to directly reprogram MEFs and tail fibroblasts from neonatal animals to functional neurons (iN cells) via forced expression of only three transcription factors, thereby, skipping the pluripotent state all together (Vierbuchen *et al*., 2010). While the initial reprogramming results are very enticing, much work remains to be completed in order to fully understand and control the process. In conclusion, it's only by studying pluripotency factors in the embryo and testing their function in ESC that will lead to a more complete understanding of the mechanism of reprogramming.

The work undertaken for this dissertation explores the role of Wnt signaling in tri-lineage differentiation of mouse embryonic stem cells. In Chapter 2, Wnt signaling was blocked during the differentiation of mESC with an inducible dominant negative Tcf4 protein, resulting in increased neural precursor differentiation. However, the mESC derived neural precursors were unable to complete neuronal differentiation unless Wnt signaling was restored. In Chapter 3, over-expression of Geminin protein in mESC was shown to induce an epithelial to mesenchymal transition and mesendodermal differentiation via increased Wnt signaling. Finally Chapter 4 represents a synthesis of this work and consideration of future directions.

List of terms used to describe early mouse development

Blastomeres - Cells that are derived from cleavage of the fertilized egg. Figure 1.1 B-E

Trophectoderm - The outer layer of the blastocyst that becomes the placenta. Figure 1.1 F

Inner cell mass - Cells in the blastocyst that give rise to extraembryonic as Well as all embryonic cells. Figure 1.1 F

Epiblast - Develops from the inner cell mass and gives rise to three germ Layers of the embryo (ectoderm, mesoderm, endoderm). Figure 1.1 G

Primitive endoderm - Extraembryonic tissue that lines the blastocele surface Of the inner cell mass and gives rise to visceral endoderm. Figure 1.1 G

Visceral endoderm - Expansion of the primitive endoderm that remains in contact with the epiblast and extraembryonic ectoderm. When initially positioned at the distal pole = DVE When position at the anterior pole = AVE, a signaling region. Figure 1.1 H

Extraembryonic ectoderm - Derived from the trophoblast, this structure is involved with establishing the proximal-distal axis of the embryo.
Figure 1.1 H

Definitive (embryonic endoderm) - The interior germ layer, which forms the gut tube becomes liver, lung, pancreas, stomach, and intestinal tract.

Mesoderm - The middle germ layer, it forms blood and vasculature, muscle, bone, cartilage, and connective tissues.

Ectoderm - The exterior germ layer, it forms skin, central nervous system and neural crest.

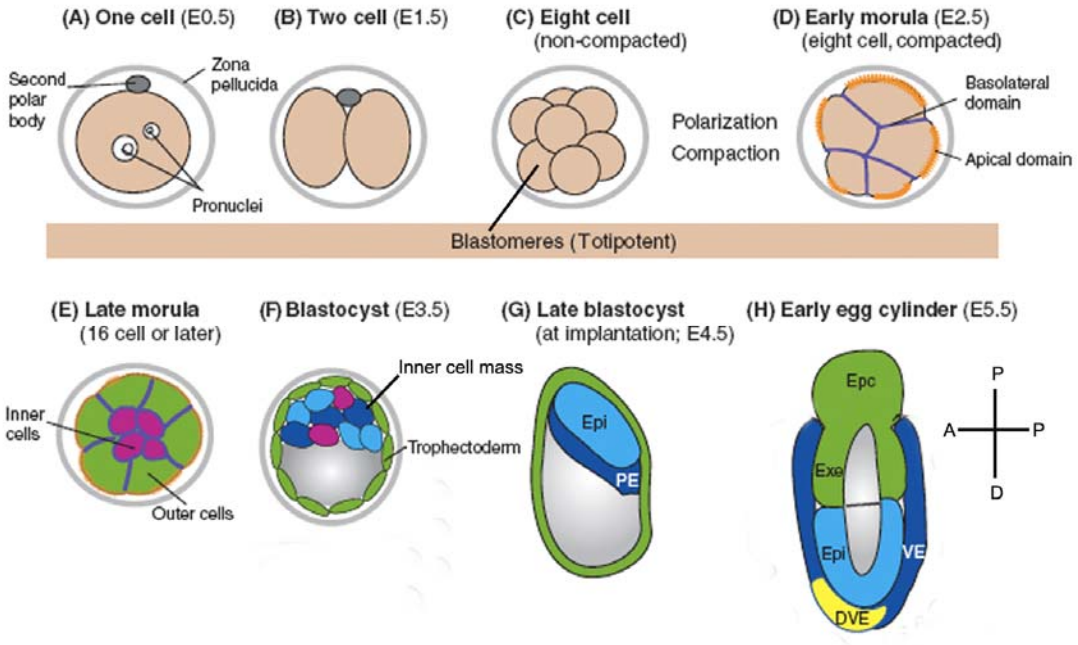


Figure 1.1
Cell lineage specification from zygote to egg cylinder.
The development of the preimplantation mouse embryo starts with division of the blastomeres and compaction, followed by the first lineage choice of trophoblast (green cells in E) versus inner cell mass (purple cells in E+F). The inner cell mass then divides into epiblast (Light blue) versus primitive endoderm (dark blue) before the elongation of the egg cylinder and establishment of anterior-posterior axis and molecular specification of the distal visceral endoderm (DVE). Image modified from Rossant and Tam, 2009.

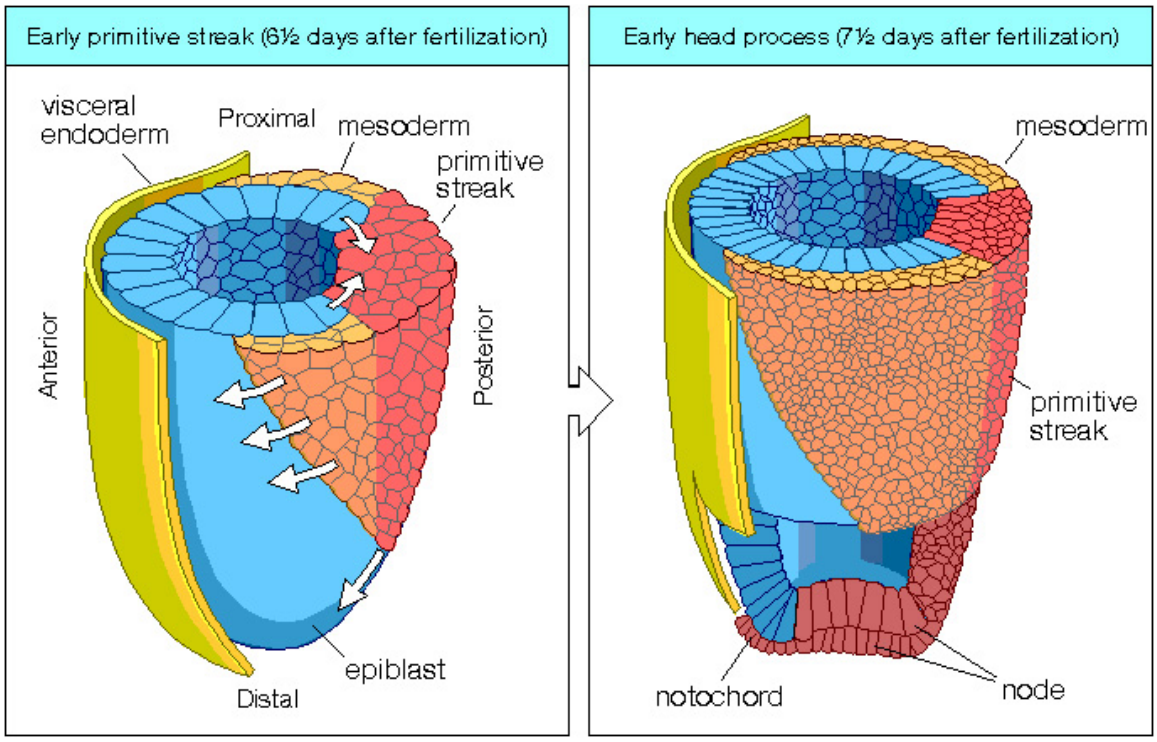


Figure 1.2
Cell movements at gastrulation.
Cells from the primitive streak delaminate from the epiblast and migrate giving rise to different derivatives depending on where they initiate migration and the timing of their migration. Mesoderm migrates first from the proximal/posterior region of the streak and spreads out in over the epiblast. Endoderm migrates slightly later and from the distal/anterior end of the primitive streak and the node and pushes the majority of the primitive endoderm up and out of the embryo into the extra-embryonic region. Image from Wolpert, 2007.

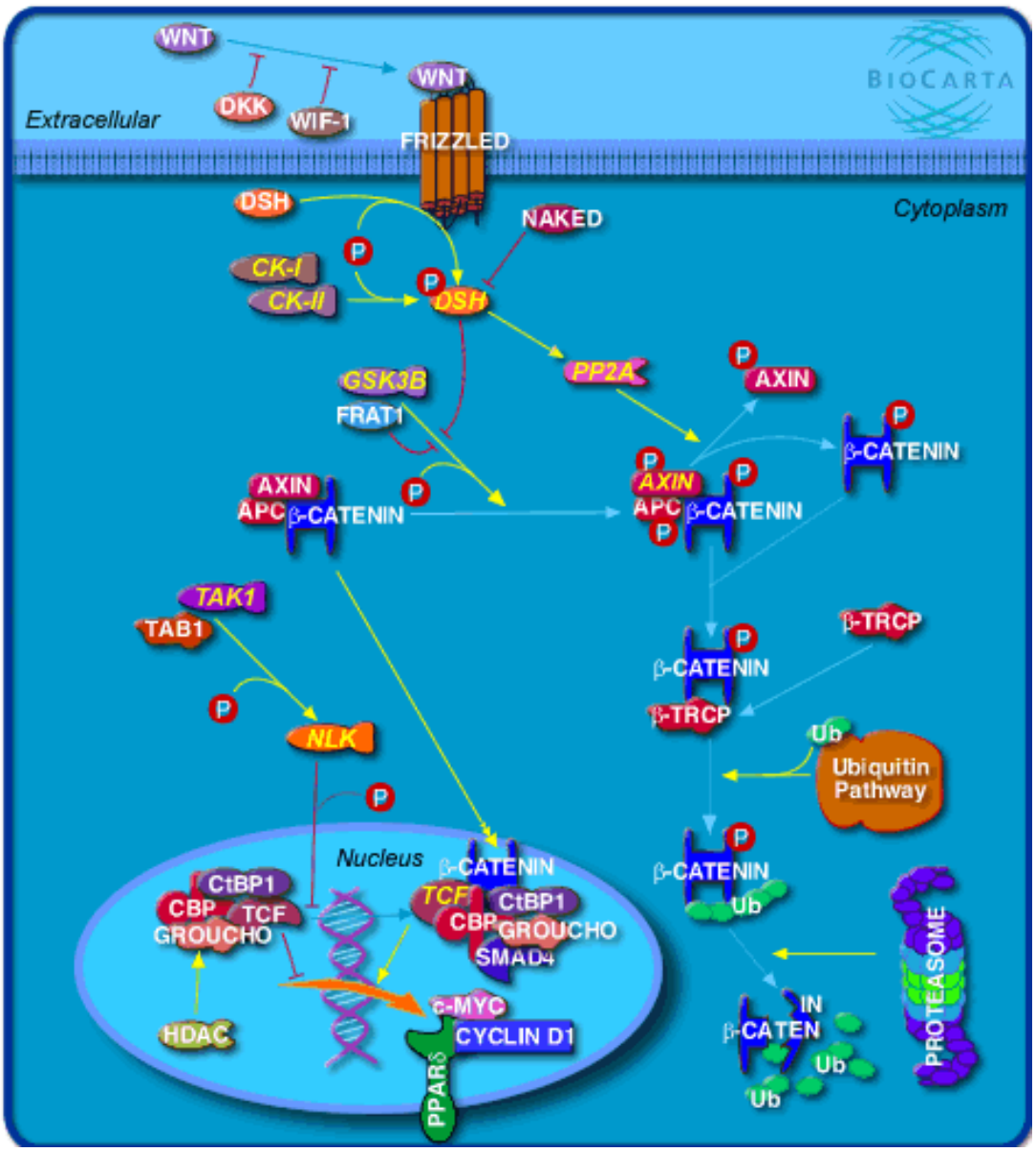


Figure 1.3
The Wnt pathway
Wnt ligands bind Frizzled receptors resulting in inhibition of GSK3β mediated phosphorylation and destruction of β-catenin by the proteasome. Stablilized β-catenin enters the nucleus and distupts Groucho (Transducin like enhancer of split -TLE)/Tcf/Lef protein interaction and binds Tcf/Lef protein complexes and induces transcription of Wnt targets (Pathway diagram courtesy of BioCarta Inc.)

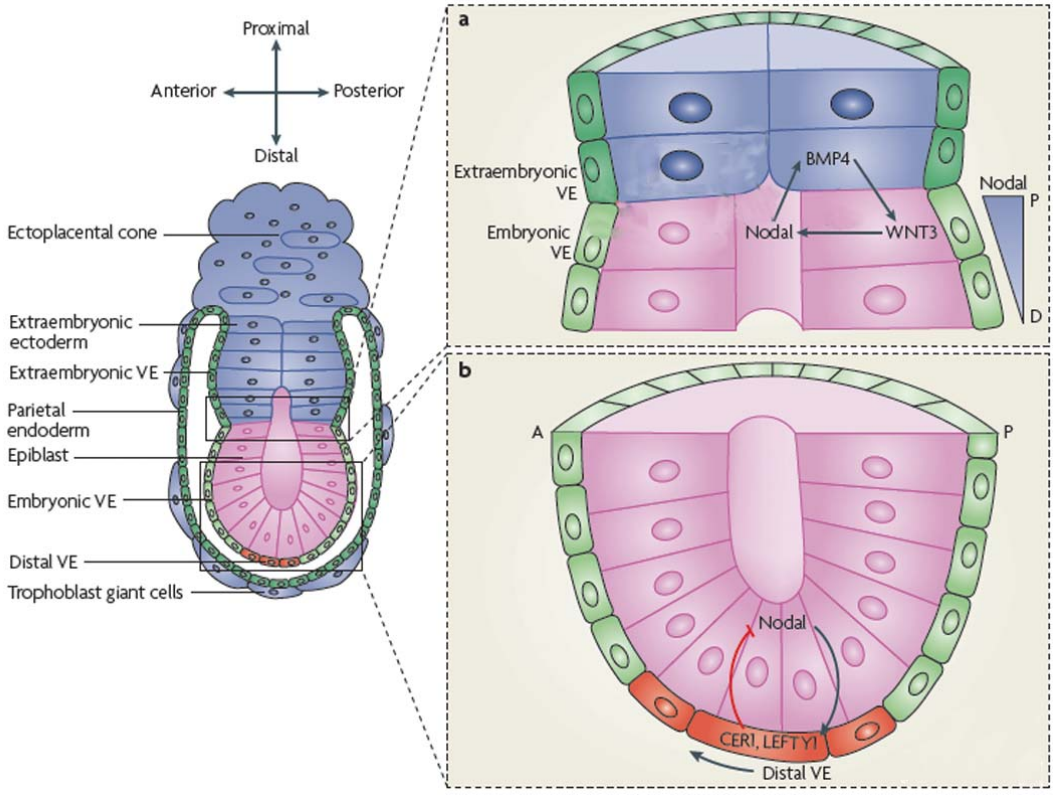


Figure 1.4
A Nodal gradient establishes the proximal-distal axis of the mouse embryo.
The TGFβ family member Nodal is expressed in the entire epiblast and extraembryonic ectoderm. Nodal stimulates BMP4 expression in the extraembryonic ectododerm that initiates Wnt 3 expression in the posterior epiblast. Wnt 3 then increases Nodal expression resulting in the activation of SMAD2 in the distal visceral endoderm (DVE) inducing expression of the Nodal inhibitors Lefty and Cerburus-like and the creation of the proximal-distal Nodal signaling gradient. Image modified from Arnold and Robertson, 2009.

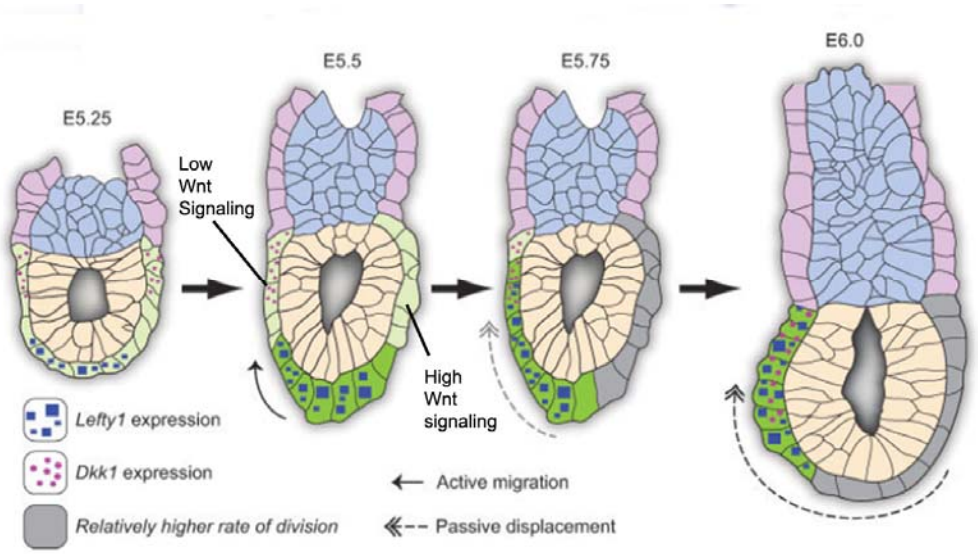


Figure 1.5
The DVE migrates forming the AVE and generating the anterior-posterior axis.
Several mechanisms have been proposed to regulate the anterior movement of the distal visceral endoderm (DVE). The cells potentially migrate towards anterior DKK-1 expression or away from the posterior of the embryo where there is a high level of Wnt signaling. In addition, higher rates of cell division in the posterior might push the DVE towards the anterior side of the embryo. Image modified from Srinivas, 2006.

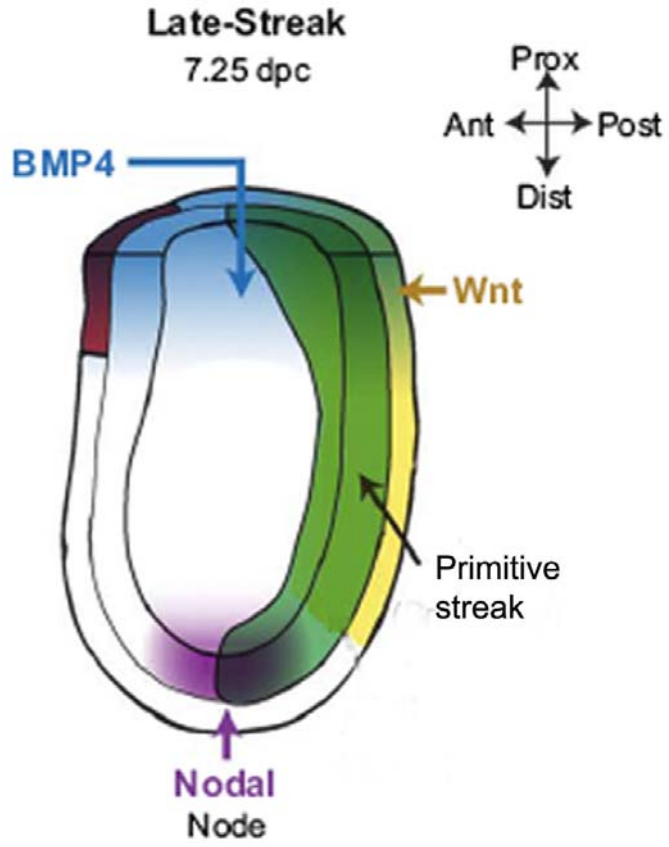


Figure 1.6
Signaling gradients in the late streak stage mouse embryo.
Schematic representation of the signaling gradients in the mouse embryo that establish the proximal-distal and anterior-posterior axes, and drive target gene expression during lineage allocation of the epiblast at gastrulation. Image modified from Gadue *et al*., 2006.

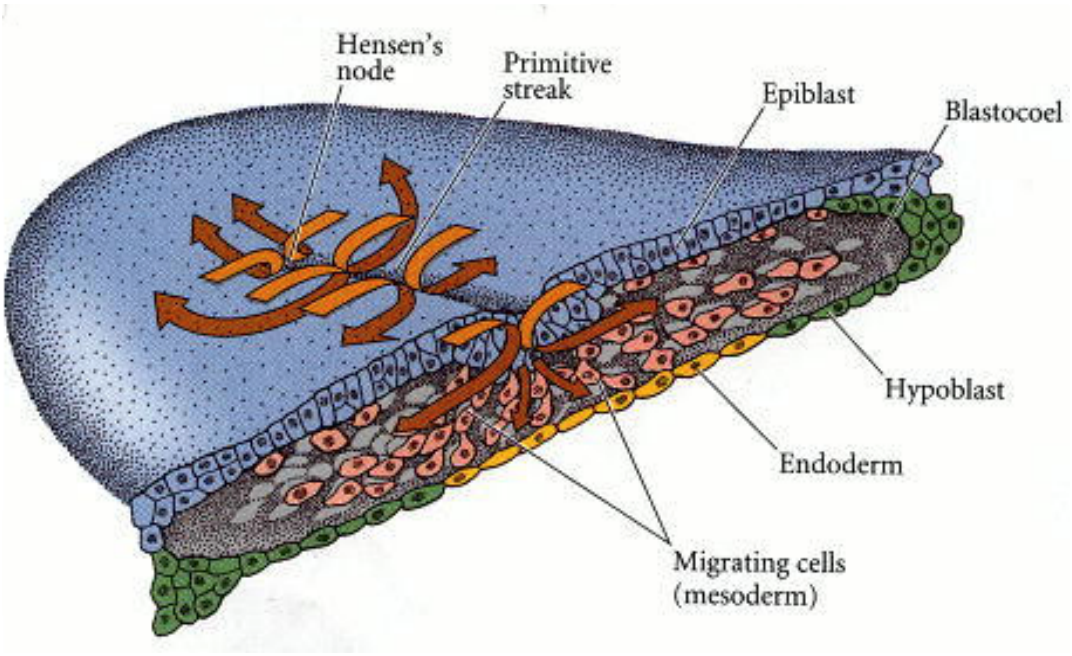


Figure 1.7
Cell movements in gastrulation.
This schematic demonstrates the cell movements during avian gastrulation, a process very similar to the human and mouse. Epiblast cells are pushed into the midline, delaminate from the epithelium, and migrate through and away from the primitive streak, giving rise to mesoderm and endoderm during gastrulation. The remaining epithelial cells form epidermal ectoderm (lateral) and neural ectoderm (midline). Gilbert, 2006.

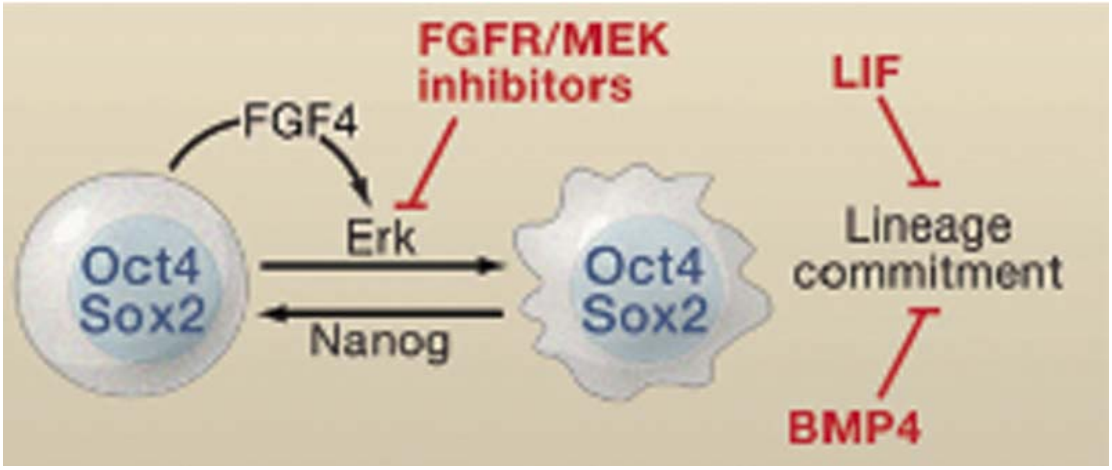


Figure 1.8
Transcription factor network that maintains mESC pluripotency.
Oct3/4 and Sox2 co-activate FGF4 expression that will lead to mESC differentiation that is inhibited by Nanog expression. Image from Silva and Smith, 2008.

Chapter 2

Dynamic changes in Wnt signaling are required for neuronal differentiation of mouse embryonic stem cells

Introduction

Mouse embryonic stem cells (mESC) are derived from the inner cell mass of the pre-implantation blastocyst; accordingly, they have the ability to form all the tissues of the embryo (Evans and Kaufman, 1981; Martin 1981). There is currently great interest in understanding the molecular mechanisms involved in maintaining pluripotency as well as in achieving controlled differentiation of ESC to facilitate cell replacement therapy for the resolution of human disease. Additionally, ESC can be used as a model of lineage choice in development, providing a systematic simplification of this extraordinarily complex and otherwise inaccessible process. Pluripotency of mESC, in the absence of a feeder layer, can be maintained by leukemia inhibitory factor (LIF) signaling through Stat-3 (Smith *et al*., 1988; Williams *et al*., 1988). Blocking or stimulating other signaling pathways has also been suggested to be sufficient to maintain mESC pluripotency either with or without LIF, including: the BMP, PI3 kinase/AKT, MAP-ERK kinases, and Wnt pathways (Lee *et al*., 2009; Paling *et al*., 2006; Qi *et al*., 2004; Watanabe *et al*., 2006: Ying *et al*., 2003). Conversely, manipulation of

these pathways has also been reported to promote lineage specific differentiation of mESC. Untangling these opposing results requires rigorous and likely reversible control of each independent pathway individually and in combination.

The Wnt family of secreted ligands bind to frizzled receptors, inhibiting GSK-3β phosphorylation and the destruction of β-catenin, resulting in the accumulation of nuclear β-catenin that binds to Tcf/Lef transcription factors to activate and/or repress gene expression (Blauwkamp *et al*., 2008). Wnt signaling has been implicated in many diverse and seemingly opposite processes such as self-renewal and proliferation versus differentiation of developing as well as adult tissues. In the case of ESC, there are conflicting reports regarding the role of Wnt signaling in maintaining pluripotency versus promoting differentiation. Many authors have suggested that Wnt pathway activation is sufficient to maintain self-renewal of mESC (Miyabayashi *et al*., 2007; Sato *et al*., 2004; Singla *et al*., 2006; Takao *et al*., 2007), but may require cooperative low level LIF/Stat3 signaling to inhibit differentiation (Bone *et al*., 2009; Hao *et al*., 2005; Ogawa *et al*., 2006). In some contexts, Wnt pathway activation promoted rather than inhibited differentiation of mESC (Gadue *et al*., 2006; Lindsey *et al*., 2006; Nakanishi *et al*., 2008; Otero *et al*., 2004; ten Berge *et al*., 2008). Adding to the confusion, several other groups have demonstrated a role for the Tcf3 transcription factor in maintaining the balance between self-renewal and differentiation (Cole *et al*., 2008), independent of the status of Wnt signaling via its ability to act as a transcriptional repressor (Pereira *et al*., 2006; Tam *et al*., 2008; Yi *et al*., 2008). Certainly, nuances in experimental design and differences between mouse

strains have contributed to the variability in these results, but cannot explain the dramatically different conclusions of these studies.

Wnt signaling plays a role in the specification, proliferation, or differentiation of nearly every tissue in the embryo (Arce *et al*., 2006; Chien *et al*., 2009; van Amerogen and Nusse, 2009). During gastrulation, Wnt signaling is critically involved in establishing the primitive streak and promoting the epithelial to mesenchymal transformation required for mesendodermal differentiation from the epiblast (Doble and Woodgett, 2007; Maretto *et al*., 2003; Mohamed *et al*., 2004; Sinner *et al*., 2004; Yamaguchi *et al*., 1999), thereby, controlling tri-lineage differentiation. Furthermore, differentiation of neural ectoderm both in the embryo as well as during mESC differentiation has been reported to result from inhibition of Wnt signaling (Aubert *et al*., 2002; Cajánek *et al*., 2009; Haegele *et al*., 2003; Kelly *et al*., 2004; Kemler *et al*, 2004; ten Berge *et al*., 2008; Verani *et al*., 2007). Based on these observations, it has been postulated that inhibition of Wnt signaling during mESC differentiation indirectly promotes neural lineage specification by inhibiting mesendodermal differentiation (Aubert *et al*., 2002). However, loss of the mesendoderm lineage does not guarantee default differentiation to neural ectoderm (Linker and Stern, 2004) since there is first a requirement for BMP signal inhibition to induce pan-neural differentiation, followed by subsequent signaling to establish neuronal and glial lineages. In fact, there is considerable evidence suggesting that Wnt pathway activation is required not only for pattering of the nervous system but also for proliferation and differentiation at multiple steps during development (Houart *et al*., 2002; Lagutin

et al., 2002; Kalani *et al*., 2008; Kuwbara *et al*., 2009; Muroyama *et al*., 2003; Vanderhaeghen, 2009; Yu *et al*., 2007; Zechner *et al*., 2003; Zechner *et al*., 2007).

To begin to decipher the sequential roles of Wnt signaling in lineage differentiation of mESC, we created a tetracycline inducible (Masui *et al*., 2005) dominant negative Tcf4 (dnTcf4) expressing cell line. Using these cells, it is possible to block and then relieve the repression on Wnt signaling during mESC differentiation. Blocking Wnt signaling induced differentiation of Sox3 positive neural precursors that could only progress to Tuj1 positive primitive neurons when Wnt signaling was de-repressed. These results are consistent with observations in embryos null for *β-catenin* (Haegel *et al*., 1995; Huelsken *et al*., 2000), LRP5/LRP6 co-receptors (Kelly *et al*., 2004), or Wnt ligands (Liu *et al*., 1999; Yoshikawa *et al*., 1997) that lack mesendoderm differentiation. There is then a requirement for active signaling for the differentiation of proliferating progenitors to mature neurons (Gao *et al*., 2009; Hirabayashi *et al*., 2004; Israsena *et al*., 2004; Kuwabara *et al*., 2009; Lie *et al*., 2005; Muroyama *et al*., 2002). Thus, it is clear that simple blockage of Wnt signaling is sufficient to inhibit tri-lineage differentiation at gastrulation producing a default (neural) ectoderm, but further differentiation to a mature neuronal phenotype requires sequential signaling/patterning.

To test the hypothesis that Wnt signaling is required to complete neuronal differentiation during development *in vivo*, we used shRNA targeting β-catenin to reduce Wnt signaling during postimplantation development and observed an

increase in the differentiation of Sox-3 positive neural precursors but a decrease in the conversion to mature neurons, as well as defects of embryonic axis elongation, neurulation and neural tube closure, that phenocopy the null embryo.

Materials and methods

mES cell culture and differentiation

Undifferentiated mESCs were maintained in 0.1% gelatin coated tissue culture flasks in complete media composed of DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) with 5 ng/ml LIF (Chemicon). Neural-permissive culture conditions were achieved by plating cells at low density ($1.6x10^4$ to $2.6x10^4$ cells per cm^2) in gelatincoated 12 well plates in 20% Neural basal medium (Invitrogen), 80% Ham's F12 medium (Invitrogen) with N2 and B27 salts (Invitrogen), 1 □M retinoic acid (Sigma), and 0.5% Knock-out serum replacement (Invitrogen). To form embryoid bodies (EBs), cells were plated at $1x10^6$ cells per 6cm dish in non-adherent tissue culture dishes in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) without doxycycline for 4 days then transferred to gelatin coated 12 well dishes for an additional 2 days of culture. Cells were maintained at 37^oC with 5% CO_2.

Inducible dnTcf4 mESC line

We obtained the MGZRTcH2 mESC cell line and corresponding exchange vector from Dr. Shinji Masui (Masui *et al*., 2005). The MGZRTcH2 Tet-off cell line has a tetracycline-regulatable transactivator (tTA), a tetracycline response element followed by the minimal promoter of the human cytomegalovirous (hCMV*-1) immediate early gene, a hygromycin resistance cassette, directional loxP sites, and an IRES-Venus (yellow fluorescent protein) cassette knocked into the endogenous ROSA26 promoter. The corresponding exchange vector replaces the hygromycin resistance cassette with the desired cDNA in addition to adding a puromycin resistance cassette. When the exchange vector is correctly enzymatically recombined into the ROSA26 locus, the resulting clones are no longer hygromycin resistant but become puromycin resistant, allowing for both positive and negative selection and ensuring the identification of cell lines with random incorporation of the exchange vector.

The dnTcf4 cDNA was a kind gift from Dr. Eric Fearon at the University of Michigan (Kolligs *et al*., 1999). We added a Kozak's translation initiation sequence to the 5' end of the cDNA and subcloned it into the pPthC exchange vector (Masui *et al*., 2005). We created a second exchange vector with no insert to generate control cell lines (MG). The dnTcf4 and control exchange vectors were co-transfected with pCAGGS-CRE into MGZRTcH2ES cells. Selection of inducible dnTcf4 and MG control cells was carried out using 2□g/ml puromycin and negative selection of improperly targeted colonies was carried out using with 100□g/ml hygromycin. Correctly targeted colonies were expanded in complete

media with 1μg/ml doxycycline (Sigma) to inhibit expression of the transgene and 2μg/ml puromycin to maintain selection. Inducible expression of dnTcf4 was verified by western blot, semi-quantitative RT-PCR, and TOPflash assay.

Fluorescence activated cell sorting (FACS)

Cells were harvested with trypsin, triturated to a single cell suspension, washed with FACS buffer (PBS with 1% donkey serum and 0.02% sodium azide), and fixed with 2% paraformaldehyde (PFA) for 10 minutes at 4^{o}C. Cells were permeabilized with 0.2% saponin in FACS buffer for 10 minutes at 4^{o}C, incubated with rabbit antibodies against Sox3 (Mike Klymkowski, University of Colorado, 1:1000) for 30 minutes at 4^{o}C followed by 3 washes with FACS buffer with saponin. Anti-rabbit CY5 secondary (Jackson Immunoresearch, 1:200) was used for detection followed by 3 washes with FACS buffer with saponin. Analysis was performed on a FACSCalibur from Becton Dickinson.

Immunohistochemistry

ES cells were fixed with 2% PFA for 15 minutes at room temperature and washed twice with PBS. Fixed cells were incubated with 10% donkey serum and 0.5% TritonX-100 in PBS for 30 minutes followed by overnight incubation at 4^{o}C with primary antibody (mouse anti-βIII tubulin/Tuj1 Chemicon 1:500; rabbit anti-Sox3, Mike Klymkowski, University of Colorado 1:1000; goat anti-Oct3/4, Santa Cruz, 1:500; rabbit anti-Foxa2, Upstate, 1:500, goat anti-brachyury, Santa Cruz, 1:500). Cells were washed in PBS and incubated with secondary antibodies

(1:400) conjugated to CY3 or FITC (Jackson Immunoresearch) for 2 hours at room temperature. Nuclei were visualized with Hoechst 33258 (Sigma). Images were obtained using with a Leica DM inverted fluorescence microscope and Olympus DP70 camera with associated software. Quantitative analysis of neuronal differentiation was performed with NIH Image J software (Version 1.4). Results are expressed as mean number of Tuj1 positive pixels divided by Hoechst positive pixels. Ten microscopic fields from triplicate cultures in four biological replicates were analyzed for each cell line. Data were averaged and analyzed using Students t test.

Quantitative and semi-quantitative RT PCR

RNA was harvested with Trizol (Invitrogen) following the manufacturer's protocol and genomic DNA was removed by DNAse digestion (Sigma). Complete DNA digestion was confirmed by semi-quantitative PCR using primers for β-actin before reverse transcription. A 0.5 to 1.0 μg RNA sample was used for reverse transcription with Verso RT (Thermo Scientific) using random nonamers (Invitrogen) following the manufacturer's protocols. For quantitative PCR, cDNAs were diluted 1:3 and 1□l was used per reaction with Abgene SYBR green master mix. All primer pairs were rigorously screened to eliminate primer dimer and reaction conditions were optimized to result in reaction efficiencies between 90% and 110%. Quantitative PCR results were calculated and statistical analysis was performed with REST2008 software (Pfaffl *et al*., 2002). Semi-quantitative PCR was performed as described for the genomic DNA test

above. Primer sequences and detailed reaction conditions are available upon request.

Western blot

ES cells were lysed in Laemmli buffer (Bio-Rad), cell debris was pelleted by centrufugation, and protein loaded onto 12% polyacrylamide SDS PAGE gels then transferred to a PVDF membrane. The membranes were blocked with 5% milk powder/TBST (Tris buffered saline and 0.1% Tween 20) and incubated with goat anti-Tcf4 (1:500; Santa Cruz) and mouse anti-β-actin (1:10,000 Sigma) primary antibodies in 5% milk powder/TBST overnight at 4^{o}C. Membranes were washed in TBST and incubated for 1 hour at room temperature in donkey anti-goat (1:1000) and donkey anti-mouse (1:10,000) horseradish peroxidase conjugated secondary antibodies (Jackson Immunoresearch) in 5% milk powder/TBST. Membranes were developed with Pierce Supersignal West Pico chemiluminescent substrate.

Top/Fopflash assay

MG control and dnTCF4 cells were grown in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) without doxycycline for 48 hours to induce transgene expression. Cells were transfected with TOPFlash or FOPFlash plasmids (Millipore) using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. A Renilla plasmid (pRL-CMV, Promega) was co-transfected as a

transfection control. After 24 hours, cells were lysed with passive lysis buffer and subjected to the dual-luciferase assay (Promega) according to the manufacturer's directions. Samples were read with a Veritas microplate luminometer (Turner Biosystems). Data are expressed as the ratio of TOPFlash relative to Renilla over FOPFlash relative to Renilla.

Tail-vein injection of pregnant dams

Wnt reporter mice (Mohamaed *et al*., 2004) were obtained (from Dr. Phil Gage, University of Michigan) and bred to generate time pregnant females. On day 5 post-coitum, pregnant females were injected via the tail vein with 200 ml Ringer's saline containing 10 μg of two separate plasmids containing a short hairpin RNA against β-catenin or a mutated short hairpin RNA that does not target any sequence in the mouse genome (Figure 2.6). To harvest embryos, dams were euthanized by cervical dislocation prior to removal of embryos from uteri and decidua. Extra-embryonic membranes were removed and embryos photographed using a Wild stereomicroscope and then allocated for analysis by scanning electron microscopy, X-Gal staining, O-Nitrophenyl -□-D-Galctopyranosidase (ONPG) analysis (quantification of β-Galactosidase activity), flow cytometery, or immunohistochemistry as described in detail below.

Scanning electron microscopy

Embryos were fixed for 30 minutes in 1% gluteraldehyde, washed, and stored in PBS at 4^{o}C. They were dehydrated through graded alcohols followed

by hexamethyldisilazane. Embryos were oriented on stubs, sputter coated with gold palladium, viewed, and photographed in an Amray scanning electron microscope.

X-Gal staining

Embryos were harvested and photographed as described above. To genotype individual embryos, a portion of the amnion/chorion was removed and placed into 25 μl alkaline lysis buffer (25 mM NaOH and 0.2mM EDTA) then incubated for 30 minutes at 95^{o}C to release genomic DNA (Truett *et al*., 2000). The lysis buffer was neutralized with 25 μl of 40 mM Tris-HCL and 1 μl of each sample was used for genotyping PCR performed as described in Mohamed *et al*., 2004. Embryos were fixed for 15 to 30 minutes at room temperature in 0.1 M phosphate buffer (3.74 g monobasic sodium phosphate and 10.35 g dibasic sodium phosphate in 1 L water pH 7.3), 5 mM EGTA pH 7.3, 2 mM MgCl, and 0.2% glutaraldehyde then washed 3 times for 5 minutes in wash buffer (0.1 M phosphate buffer pH7.3, 2 mM MgCl, 0.01% deoxcholate, and 0.02% NP-40). Embryos were stained for 15 minutes to 1 hour at 37^{o}C in stain buffer (0.1 M phosphate buffer pH7.3, 2 mM MgCl, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% deoxcholate, 0.02% NP-40, and 1 mg/ml X-gal).

ONPG assay

Embryos were harvested, photographed, and embryonic membranes genotyped as described above. Embryos were lysed in 25 □l of PM-2 buffer

(20mM NaH_2PO_4, 80mM Na_2HPO_4, 0.1mM $MnCl_2$, 2mM $MgSO_4$, 40mM β-mercaptoethanol pH7.3) by 5 cycles of freeze thaw then passed through a 12 gauge needle 5 times. Cellular debris was pelleted by centrifugation at 14,000 x g for 15 minutes at 4°C. Protein concentration was determined by the Bradford protein assay (BioRad). Total embryo protein was added to 400□l of PM-2 buffer and samples were incubated at 37°C for 15 minutes. Next, 100ml of prewarmed ONPG solution (4mg/ml O-Nitrophenyl -□-D-Galctopyranosidase, Sigma, dissolved in PM-2 buffer made fresh for each assay) was added to each tube, assay start time was noted, and protein incubated at 37°C until a yellow color was visible. Finally 250□l Na_2CO_3 was added to stop the reaction, a notation of assay stop time was made, and the absorbance was read at 420nm in a spectrophotometer (BioRad SmartSpec 3000). β-galactosidase activity was calculated with the equation: Units = (380 x A_{420} / time)/ total protein. BSA protein dissolved in PM-2 buffer was used as a negative control and PM-2 buffer was used as the blank in the spectrophotometer.

FACS analysis of neural precursors and neurons

On E11, shRNA and mutated hairpin control embryos were harvested as described above then embryonic membranes and central nervous system dissected free of heart, liver, gut and branchial arch tissues. Embryos were digested with trypsin for 15-20 minutes at 37°C then triturated to a single cell suspension before fixation and analysis as described above for ESC. Each

embryo sample was divided in half and incubated with either rabbit anti-Sox3 (1:1000) or mouse anti-NeuN (Chemicon 1:500) prior to FACS analysis.

Embryo immunohistochemistry

Embryos were harvested and photographed as described above. Selected embryos were embedded in OCT (EMS) frozen in isopentane and sectioned at 8μm using a Microm cryostat. Sections were fixed with 2% paraformaldehyde for 15 minutes at room temperature then washed in PBS. Sections were permeabilized with 0.2% TritonX-100 in PBS then blocked with 10% donkey serum, 0.2% TritonX-100, and 0.05% sodium azide. Sections were incubated overnight at 4^{o}C in rabbit anti-Sox3 (1:1000) and mouse anti-NeuN (1:500). Sections were washed in PBS and incubated with secondary antibodies conjugated to CY3 or FITC (1:200 Jackson Immunoresearch) for 2 hours at room temperature. Nuclei were visualized with Hoechst 33258 (Sigma). Sections were examined and photographed in a Leitz DM microscope and Olmypus camera.

For whole mount immunohistochemistry, selected embryos were fixed in 2% PFA for 30 minutes at room temperature then washed in PBS. Embryos were dehydrated in 30%, 50%, and 80% methanol: DMSO (1:4) then incubated in methanol/DMSO/30% hydrogen peroxide (4:1:1) for 4 hours at room temperature to block endogenous peroxidase activity. Embryos were rehydrated in 50% and 15% methanol/PBS and cut in half before proceeding to increase antibody penetration. Embryos were further permeabilized and non-specific

binding blocked by incubation in PBSMTX (2% milk powder and 0.1% TritonX-100 in PBS) for 2 hours at room temperature. Embryos were incubated overnight at 4^{o}C in rabbit anti-Sox3 (1:2000) and mouse anti-NeuN (Chemicon 1:1000) then washed 2 hours at 4^{o}C followed by 3 hours at room temperature in PBSMTX. Embryos were incubated over night at 4^{o}C in secondary antibodies (1:200) conjugated to horseradish peroxidase (Jackson Immunoresearch) then washed 2 hours at 4^{o}C followed by 3 hours at room temperature in PBSMTX. Embryos were washed in PBT (0.2% BSA, 0.1% TritonX-100 in PBS) for 20 minutes followed by incubation in 0.3mg/ml diaminobenzidine (Sigma) with 0.03% hydrogen peroxide in PBT for 40 minutes at room temperature. Embryos were washed in PBT and photographed using a Wild dissecting scope and a Nikon camera.

Results

Inducible expression of the dnTcf-4 protein blocks Wnt signaling in mESC

To explore the role of Wnt signaling in multi-lineage differentiation of mESC, a cell line was created that inducibly expresses a dominant negative Tcf4 (dnTcf4) protein (Tet-off) and thereby, inhibits canonical Wnt signaling (Figures 2.1 and 2.2). A total of 24 dnTcf4 and 24 MG control (puromycin resistant cells that inducibly express only venus yellow fluorescent protein) lines were cloned and expanded for further study. Proper targeting of all chosen lines was verified by loss of hygromycin resistance. Several lines of each type were selected for

further study based on doxycycline regulated dnTcf4 protein and/or Venus protein expression (data not show). In the presence of doxycycline, the dnTcf4 cell line grows in a fashion similarly to the control cell line (data not shown). Control (MG) and dnTcf4 (dn4) cells express all four Tcf/Lef transcription factors (Figure 2.2). The dnTcf4 mRNA is highly expressed within 24 hours of doxycycline withdrawl and is three-fold down-regulated when doxycycline is added back to the culture medium for an additional 24 hours. Neither doxycycline exposure nor expression of the dnTcf4 affected expression of either native Tcf4 or the three other Tcf/Lef transcription factors. The dnTcf4 protein is detectable at six days of doxycycline withdrawl and disappears completely following reintroduction of doxycycline for 48 hours (Figure 2.2).

The dnTcf4 protein reduced canonical Wnt signaling approximately 40 fold in the TopFlash luciferase assay (Figure 2.2), but after reintroduction of doxycycline for 24 hours, the levels of Wnt signaling were restored to nearly normal. More importantly, the dnTcf4 inhibited Wnt signaling even in the presence of a Gsk3β inhibitor (Chir99021, Stemgent) previously shown to promote Wnt signaling (Finaly *et al*., 2004). These data indicate that the dnTcf4 transgene is rapidly expressed in response to doxycycline withdrawl, and functions to inhibit Wnt signaling.

Regulated expression of the dnTcf4 protein promotes neural differentiation of mESC in a monolayer assay

To explore the role of Wnt signaling in neuronal differentiation of mESC, MG control and dnTcf4 expressing cells were plated in a serum free monolayer differentiation assay in neural permissive media. When Wnt signaling was abrogated throughout the culture period by removal of doxycycline from the medium, there was a dramatic increase in the number of Sox3 positive cells after as little as 4 days (Figure 2.3), but there was little neuronal differentiation even if the cells were kept in culture as long as 8 days. When the dnTcf4 was expressed early in the culture period (days 1 to 3) then down-regulated by addition of doxycycline during the second phase of differentiation (days 3-6) to permit Wnt signaling, there was a striking increase in the number of β□□□ tubulin positive neurons in the cultures. FACS analysis indicated that there was a statistically significant increase in Sox3 positive cells in MG versus dnTcf4 cells in all conditions tested (Figure 2.4). Because FACS sorting of Tuj1 positive cells resulted in high levels of background signal that did not accurately reflect differentiation, to quantify the number of primitive neurons in these cultures we carried out IHC of β□□□ tubulin, then used ImageJ software to determine the mean number of green (β□□□ positive) pixels in each condition. To control for the effects of cell number, we also measured mean pixels in the blue (Hoechst) channel. Data are presented as mean number of Tuj1 pixels/mean number of Hoechst pixels. Consistent with our IHC results, ImageJ analysis indicated that maximal differentiation of primitive neurons occurred when Wnt signaling was

initially inhibited, then released for the final differentiation of precursors to neurons (Figure 2.3).

Blocking Wnt signaling increases neural precursor differentiation and decreases mesendoderm differentiation in an EB assay

To investigate the role of Wnt signaling in mESC in a more complex three-dimensional multi-lineage differentiation assay, we employed an embryoid body (EB) differentiation assay. Cells were plated in non-adherent tissue culture dishes (in the absence of LIF and doxycycline but with serum) promoting the formation of EBs and thereby inducing differentiation. Cells were grown as spheres for four days then replated into adherent tissue culture dishes for an additional two days before fixation for immunhistochemistry (IHC) or harvesting of RNA.

Axin2, a well-characterized canonical Wnt target gene (Hughes and Brady 2006; Jho *et al*., 2002; Leung *et al*., 2002; Yan *et al*., 2001), was down-regulated after 6 days in culture (Figure 2.5), confirming a functional Wnt signaling blockade. The block in canonical Wnt signaling caused a delay or stalling of differentiation with increased expression of the mESC and epiblast markers Oct3/4 and Fgf5 (Figure 2.5). Decreased Sox-17 expression and a trend toward decreased T expression indicated a reduction in mesendodermal differentiation. Primitive neural ectoderm differentiation increased as marked by increased Sox3 expression. However, the dnTcf4 expressing cells were unable to progress to immature β□□□-tubulin positive neurons in the continued Wnt blockade. When

doxycycline was reintroduced in these cultures midway through differentiation, β□□□-tubulin positive neurons did not differentiate possibly because of the overly crowded conditions within each EB (data not shown). These data indicate that in the absence of Wnt signaling, there is a general delay in differentiation, perhaps due to the elimination of the mesendodermal choice, as well as an increase in neural precursors and a decrease in mesendoderm differentiation.

β-catenin knock-down affects post-implantation survival and gastrulation

The shRNA constructs used in these experiments are illustrated in Figure 2.6. and prior to use in pregnant dams, we verified that each construct efficiently decreased β-catenin protein in mESC (Figure 2.6). To examine the role of Wnt signaling in lineage differentiation *in vivo*, we delivered shRNAs targeting β-catenin via the tail vein on E5.5 of gestation. Knock-down avoids the peri-implantation lethality of conventional knock-out approaches and results in graded knock-down of gene expression allowing for the correlation of phenotype and gene expression. Reduced β-catenin expression was often embryo lethal, increasing the number of resorptions observed from 5.6% in control litters to 25.9% on E7. Since Wnt signaling is required to position the primitive streak and for EMT at gastrulation, we examined the displacement of primitive endoderm (PE) by newly formed (embryonic) definitive endoderm (DE) using scanning electron microscopy (SEM). On E7, gastrulation was fully underway in embryos exposed to the scrambled hairpin (controls) with the boundary marking PE and DE moving 75% of the way to its final position at the embryonic—extra-

embryonic boundary (Figure 2.7). Control embryos had typically expanded in the proximal-distal, but not anterior-posterior axis at this stage of development. In embryos exposed to shRNA β-catenin, gastrulation was slowed, DE typically progressing 1/4 to 1/3 of the way along the proximal-distal axis (Figure 2.7). Occasionally, newly formed mesendodermal cells piled up at the node in β-catenin targeted embryos, remaining as an ectopic cluster, rather than migrating anteriorly to replace PE and form new mesoderm.

When we examined β-galactosidase expression in control "Wnt indicator" mice, the X-gal reaction product clearly marked the primitive streak at the posterior pole of the embryo (Figure 2.7). In β-Catenin targeted embryos at this stage, there was a range in β–gal expression from slight to undetectable levels (Figure 2.7), confirming knock-down, and presumably inhibition of Wnt signaling, to near normal levels.

By E8.0 of development, control embryos had well-developed headfolds, were expanded in the proximal-distal plane; AP axis expansion was underway. The neural folds were elevating and invagination of the optic vesicles was just beginning in the cephalic region (Figure 2.7).

Gene targeted embryos were characterized by extremely shortened PD axes, often with an expanded AP axis and shortened posterior region giving a "rocking horse" appearance (Figure 2.7). The anterior neural folds were slightly elevated, but were characteristically flattened in the midbrain region; the border between neural ectoderm and epidermal ectoderm was wavy rather than even. The allantois appeared elongated, the first branchial arch reduced and

abnormally displaced posteriorly (ventrally). Neural tube closure defects were common in the midbrain, where there was a very characteristic eversion of neural tissue. However, in extremely shortened embryos, the entire neural tube had often failed to close throughout its extent.

Wnt signaling activity increased significantly by E8.5, with strong β-gal expression throughout the cranial neural ectoderm, especially high in midbrain and hindbrain regions in control embryos. X-gal staining was also high in the posterior neuropore and regressing primitive streak, as well as throughout the closed neural tube. Signaling was also strong in the first branchial arch, in neural crest forming the extra-ocular muscles, surrounding the otic vesicles and in the cardiac neural crest (Figure 2.7). In targeted embryos, there was a consistent reduction in the expression of β-gal—particularly in the first branchial arch, midbrain and heart (Figure 2.7).

To quantify Wnt signaling activity in control versus β-catenin shRNA embryos, we used the O-nitrophenyl-beta-D- galactopyranoside (ONPG) assay in Wnt indicator mice. In control embryos (6 litters, 40 embryos) signaling was significantly higher 0.29 +/- 0.22 versus 0.129 +/- 0.04 ($p \leq 0.03$, Sudents t test) than in β-Catenin shRNA treated embryos (8 litters, 59 embryos), suggesting that shRNA knock-down persisted in surviving embryos.

Immunohistochemisty and FACS analysis

Since Wnt signaling appears to be required for neuronal differentiation of proliferating precursors, we examined the conversion of neural precursor cells to

neurons using immunohistochemistry and FACS analysis in control versus β-catenin shRNA exposed embryos. There was a slight increase in Sox3 positive cells in whole mount as well as in sections through the neural tube (Figure 2.7) in β-catenin shRNA exposed embryos compared with control embryos exposed to a mutated shRNA. Immunohistochemistry with a NeuN antibody demonstrated a clear decrease in differentiation of immature neurons which were beginning to stratify within the neural ectoderm in conjunction with reduced Wnt signaling. To quantify these changes, we dissected the CNS from E10.5 embryos, dissociated each embryo to a single cell suspension, divided each embryo into two equal samples, and stained embryos for Sox3 or NeuN. Cell number in individual embryos was quantified in FACS analysis, 40 scrambled and 43 β-catenin shRNA exposed embryos. Data were averaged and compared using student's t test. There was a significant increase in Sox3 positive cells in embryos exposed to the β-catenin shRNA compared to control embryos (20.72+/-6.31 versus 26.54+/-7.22, $p \leq 0.004$) and a corresponding decrease in NeuN positive cells (7.76+/-1.57 versus 6.17+/-1.30, $p \leq 0.004$), confirming that *in vivo* as *in vitro*, Wnt signaling is required at multiple stages of neuronal differentiation, in this case in the conversion of neural precursors to primitive neurons.

Discussion

Morphogenesis of the vertebrate embryo requires precise control of lineage differentiation that in turn necessitates highly regulated control of multiple

interconnected signaling pathways. One pathway that is critical for early embryogenesis is signaling by the Wnt family of secreted molecules, receptors, and intracellular signal transducers. Considerable evidence from gene targeting experiments has established a role for Wnt signaling in lineage specification and cell type specific differentiation of many lineages. However, much research regarding the role of Wnt signaling in neuronal specification during mouse embryogenesis and mESC differentiation has produced conflicting results, suggesting that canonical signaling can both promote differentiation (Hirabayashi *et al*., 2004; Kuwabara *et al*., 2009; Lie *et al*., 2005; Muroyama *et al*., 2004; Otero *et al*., 2004; Vanderhaeghen, 2009) and under other conditions inhibit differentiation (Aubert *et al*., 2002; Cajánek *et al*., 2009; Haegele *et al*., 2003; Horn *et al*., 2007; Kong and Zhang, 2009; Verani *et al*., 2007; Wexler *et al*., 2009; Zechner *et al*., 2003).

To probe the role of Wnt pathway activation in lineage differentiation, we derived a tetracycline regulated dnTcf4 expressing mES cell line in which Wnt signaling could be tightly controlled. There are four Tcf/Lef factors in the mouse genome that act as the downstream transcriptional effectors of the canonical Wnt signaling pathway. All four transcription factors bind similar promoter sequences and can act as repressors in the absence of Wnt ligand activity (by binding Groucho/TLE family of co-repressor proteins) but can also switch to transcriptional activation when β-catenin enters the nucleus and displaces Groucho/TLE proteins (Brantjes *et al*., 2001; Daniels and Weiss, 2005). Rather than using extracellular inhibitors, we chose to employ a dnTcf4 protein, which

lacks the β-catenin binding domain, since this should block all transcription downstream of the Wnt pathway. In several stable lines, the dnTcf4 transgene was inducible as demonstrated at the mRNA and protein level; the dnTcf4 mRNA was rapidly produced upon withdrawl of doxycycline and three fold reduced when doxycycline was added back to the cells for 24 hours, while Western blot of protein from dnTcf4 expressing versus MG control cells demonstrated expression of the transgenic protein. Induction of the dnTcf4 protein actively inhibited Wnt signaling in luciferase promoter assays (TOPflash) resulting in an approximately 40-fold reduction in reporter activity.

To examine the role of Wnt signaling in tri-lineage differentiation at gastrulation, we employed an embryoid body differentiation paradigm. When mESC are grown in the absence of LIF in suspension culture or in hanging drops of medium without substrate contact, they form cell aggregates termed embryoid bodies (EBs) due to the loose organization of cells into three layered structures resembling the early postimplantation mouse embryo (Desbaillets *et al*., 2000). However, the EB is significantly different from the embryo since there are no organized axes, primitive streak, or signaling centers. Lacking organization, differentiation within the EB has been characterized as “chaotic” or random. Despite the disorganization and lack of organized signaling centers, EB differentiation has been widely employed to model early lineage differentiation; particularly, as a model of gastrulation and the role of Wnt signaling in this process (ten Berge *et al*., 2008; Nakanishi *et al*., 2008; Gaude *et al*., 2006). Using this model, expression of the dnTcf4 protein resulted in increased

expression of the pluripotency marker Oct3/4 and the epiblast marker FGF5, likely identifying cells that retain an epiblast phenotype and are unable to differentiate due to the requirement for Wnt signaling in formation of the primitive streak and mesendoderm differentiation. Consistent with this result, expression of markers of endoderm (Sox17 and Foxa2) and mesoderm (Brachyury) were significantly reduced. Only one lineage, primitive neural ectoderm marked by Sox3 expression, increased in the absence of Wnt signaling. These results are consistent with previous reports demonstrating that canonical Wnt signaling is crucial for formation of the primitive streak, gastrulation, and mesendoderm differentiation during embryogenesis. For example, deletion of core components of the Wnt pathway such as of β-catenin (Haegel *et al*., 1995; Huelsken *et al*., 2000), Wnt3a (Yoshiakai *et al*., 1997), Wnt3 (Liu *et al*., 1999), frizzled co-receptors Lrp5 and Lrp6 (Kelly *et al*., 2004), or Lef1 and Tcf1 (Galceran *et al*., 1999) produced embryos lacking the primitive streak or paraxial mesoderm, with expansion of neural ectoderm in surviving embryos. Conversely, mouse embryos in which canonical Wnt signaling was activated via deletion of inhibitory proteins including: Axin2 (Zeng *et al*., 1997), Tcf3 (Merill *et al*., 2003) or APC (Ishikawa *et al*., 2003), or transgenic mis-expression of Wnt8c (Popperl *et al*., 1997), were characterized by multiple axes (multiple primitive streaks), and increased differentiation of mesendoderm at the expense of neural ectoderm. It is clear both in mESC and the early embryo that Wnt signaling is required for formation of the primitive streak and gastrulation and in its absence more of the embryonic epiblast can be converted to neural ectoderm.

Decades of research using the amphibian embryo have demonstrated that neural induction occurs when BMP inhibitors secreted by the organizer bind BMP receptors and inhibit BMP signaling, activating neural gene expression in the ectoderm - identifying the “default” state of the primitive ectoderm as neural (Bachiller *et al*., 2000; Munoz-SanJuan and Brivanlou, 2002). The situation in the mammalian embryo is complicated by the presence of multiple signaling centers, and it is clear from gene deletion studies in the mouse embryo (Anderson *et al*., 2002) that additional signaling pathways are involved in neural induction. Thus, non-neural tissues form from the pluripotent mouse epiblast in response to BMP, Wnt, and Nodal signaling (Tam 2004), with the corollary that the expression of early neural markers requires inhibition of these signaling pathways. Consistent with these results, BMP and Nodal signaling are required in the epiblast prior to gastrulation to inhibit default neural differentiation. The situation is further complicated by the fact that gastrulation and neural induction occur concurrently in the mouse embryo with anterior signals acting to pattern the neural ectoderm and inhibit posterior differentiation, thereby positioning the primitive streak. Wnt signaling must be restricted to the posterior region of the embryo in order to prevent conversion of the entire epiblast to mesendoderm (Kemler *et al*., 2004). Both the node and the anterior visceral endoderm express Wnt inhibitors, including Dickkopf-1 (Glinka *et al*., 1998), Sfrp1 (Hoang *et al*., 1998), Sfrp2 (Leimeister *et al*., 1998), Frzb/Sfrp3 (Hoang *et al*., 1998), and Sfrp5 (Finley *et al*., 2003) to restrict Wnt signaling to the posterior of the embryo, and in combination with BMP and Nodal inhibitors to induce neural tissue.

Mouse ESC have been widely employed to model both neural induction and tri-lineage differentiation at gastrulation. Simply removing cells from a source of BMPs or adding BMP inhibitors to the culture medium of ESC that promotes neuronal differentiation has been cited as support for the default model. However, it is clear that additional factors including cell density and other unidentified factors in supplements and serum also play a role in controlling differentiation. Furthermore, since FGFs and Nodal are required to maintain BMP4 expression (Ben-Haim *et al*., 2006) it is likely that as in the intact embryo, the situation is more complex. Inhibition of Wnt signaling in mESC has previously been reported to be sufficient to promote neuronal differentiation (Aubert *et al*., 2002; Cajánek *et al*., 2009; Haegele *et al*., 2003; Nordin *et al*., 2008; Verani *et al*., 2007). However, absent Wnt signaling, the neural precursor cells formed in the EB assay failed to progress from Sox3 positive precursors to βIII tubulin positive neurons. To avoid the confounding random differentiation and to control cell density problems characteristic of the EB assay, we also employed a monolayer model of differentiation. Monolayer culture of the dnTCF4 cells in neural permissive media absent doxycycline (to induce transgene expression) promoted differentiation of neural precursors, but they once again failed to progress to primitive neurons, remaining at the precursor stage.

To investigate the role of Wnt signaling in the mouse embryo, shRNA plasmids directed against β-catenin were delivered to pregnant dams, thereby eliminating Wnt signaling at a nuclear level in embryos during early post-implantation development. Using knock-down in lieu of a knock-out, many

embryos were able to escape the lethality associated with early β-catenin loss. *In vivo* β-catenin shRNA phenocopied previous results with Wnt signaling null embryos including gastrulation abnormalities with resulting axis elongation defects. When embryos survived gastrulation lethality, it was possible to examine the requirement for active Wnt signaling in neuronal differentiation. Consistent with out observations in mESC, absent Wnt signaling, neural ectoderm precursors failed to differentiate to NeuN positive neurons.

In the developing nervous system, Wnt signaling is required for both proliferation of neural precursors (Chesnutt *et al*., 2004; Israsena *et al*., 2004; Kalani *et al*., 2008; Shimizu *et al*., 2008; Zechner *et al*., 2003) and later for neuronal differentiation (Hirabayashi *et al*., 2004: Muroyama *et al*., 2004). Subsequent patterning of the nervous system requires Wnt signaling (Ciani and Salinas, 2005; Nordstrom *et al*., 2002), and the key bHLH transcription factors that drive neuronal specification, including neurogenin1 and neuroD, are transcriptional targets of the canonical Wnt pathway (Hirabayashi *et al*., 2004; Israsena *et al*., 2004). While Wnt signal inhibition may be required to inhibit mesendodermal lineage pathway differentiation, active signaling appears to be required for precursor proliferation and neuronal differentiation. When dnTcf4 expression was abrogated midway through the culture period there was a dramatic conversion of Sox3 positive precursors to immature neurons. Previous conclusions that a simple block in Wnt signaling was sufficient to promote neuronal differentiation (Aubert *et al*., 2002; Cajánek *et al*., 2009; Kong and Zhang 2009; Verani *et al*., 2006) likely resulted from a reduction rather than a

complete block in signaling since a single Wnt gene or co-receptor (Wnt 1 or LRP6) was deleted. Similarly, over-expression of extracellular inhibitors (DKK1 or SFRP2) likely did not reach a sufficiently high concentration to eliminate Wnt signaling. However, the dnTcf4 employed in the current investigation should inhibit all canonical Wnt signaling at the promoter of target genes preventing all downstream signaling.

As in the CNS, lineage differentiation of most cells and tissues likely involves multiple rounds of Wnt signaling, signal inhibition, and resumption of signaling rather than acting as an on-off switch. The inducible dnTcf4 cell line should be extremely useful in uncovering previously unidentified requirements for Wnt signaling in many tissues that were unappreciated in transgenic experiments.

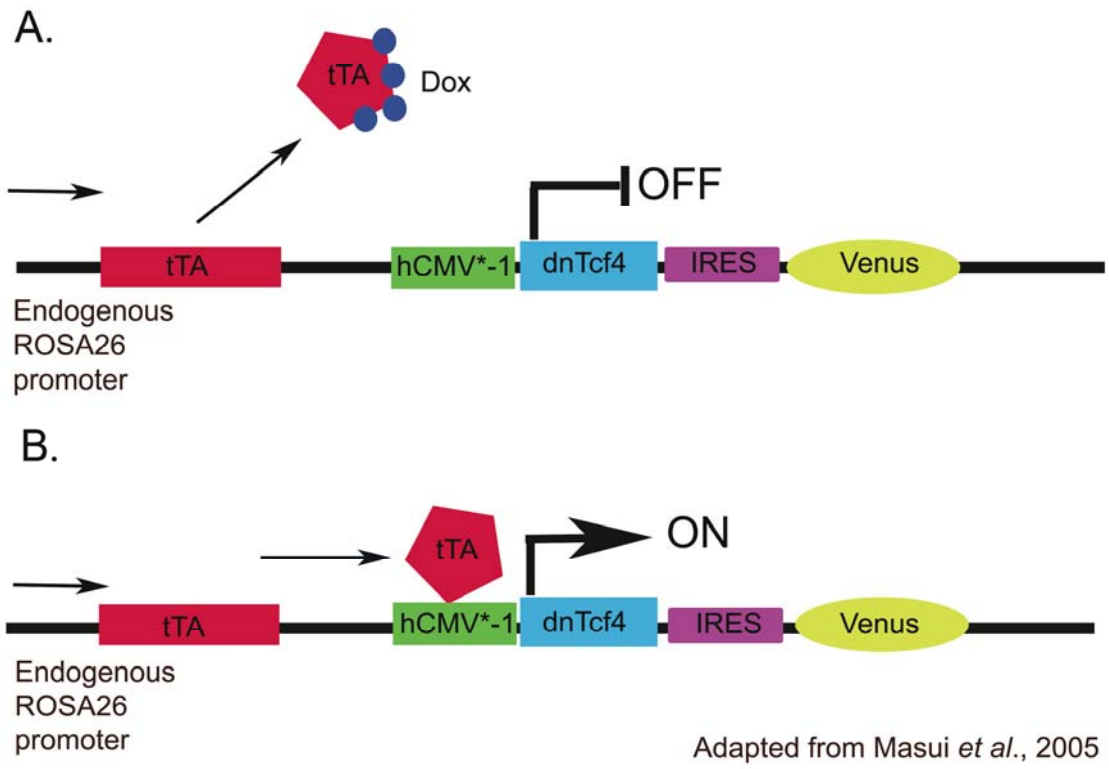
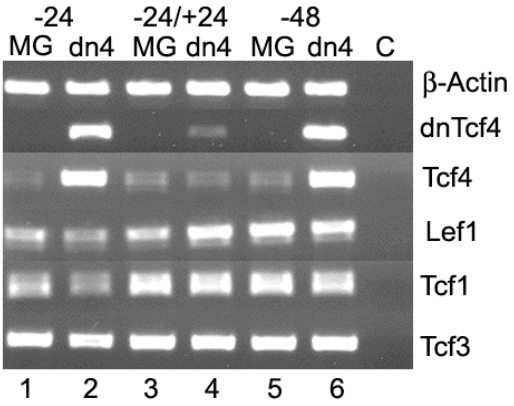


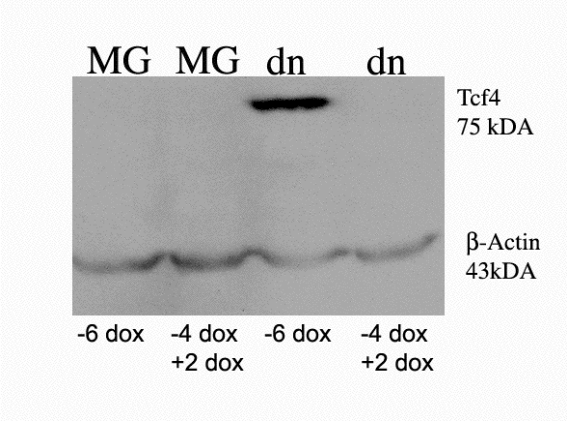
Figure 2.1
The TET-off system stably expressed at the ROSA26 locus in the dnTcf4 inducible line.
A. In the presence of Doxycycline (Dox), the Tetracycline-regulated transactivator (tTA) is unable to bind to hCMV*-1 promoter (Tetracycline response element followed by the minimal promoter of the human cytomegalovirus immediate early gene) and initiate downstream gene expression.
B. Once the drug is removed from the tissue culture medium, the tTA binds the promoter and the dnTcf4 and Venus protein are transcribed.

Figure 2.2
Inducible expression of the dnTcf4 protein blocks Wnt signaling in mESC.
A. Control MG) and dnTcf4 (dn4) mESC were grown for 24 (-24, lanes 1,2) or 48 (-48, lanes 5,6) hours without Dox or for 24 hours without Dox then 24 hours with Dox (-24/+24, lanes 3,4). Lane C is a no template negative control. PCR with a primer that anchors in the FLAG tag present in only the dnTcf4 mRNA shows that transgene expression is tightly controlled by Dox exposure and that reversible induction of the dnTcf4 had no effect on expression of endogenous Tcf4 or other Tcf/Lef transcription factors (Lef1, Tcf1, Tcf3).
B. Western blot demonstrating expression of the dnTcf4 protein 6 days after doxycycline withdrawl. Expression is eliminated when doxycycline is reintroduced into the culture media for 48 hours. Expression is not detected in control cells.
C. Wnt signaling is strikingly inhibited by dnTcf4 expression even in the presence of a GSKβ inhibitor (Chir99021) that strongly stimulates the Wnt pathway assessed using luciferase assays. The mean fold change (TOP Flash/FOP Flash) in the MG control line was significantly higher than the dnTcf4 (dn4) line without Dox for 4 days (-Dox, *p value $\leq$ 0.01), after Dox was withdrawn for 3 days and added back to cells for 1 day (+Dox, **p value $\leq$ 0.03), and without Dox for 4 days but with Chir99021 for 1 day (+Chiron, *p value $\leq$ 0.01) by the Student's t test (n=3). Error bars represent SEM.

A.


-24
MG dn4
-24/+24
MG dn4
-48
MG dn4
C
β-Actin
dnTcf4
Tcf4
Lef1
Tcf1
Tcf3
1 2 3 4 5 6

B.


MG MG dn dn
Tcf4
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β-Actin
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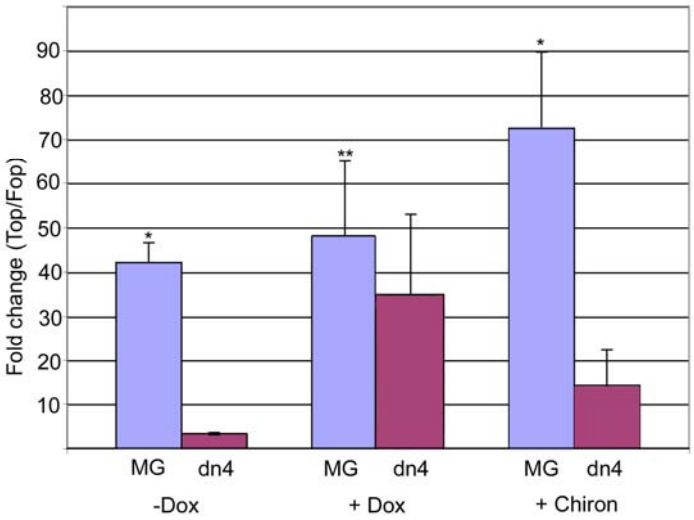
-6 dox
-4 dox
+2 dox
-6 dox
-4 dox
+2 dox

C.


Fold change (Top/Fop)
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MG dn4
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+ Dox
MG dn4
+ Chiron

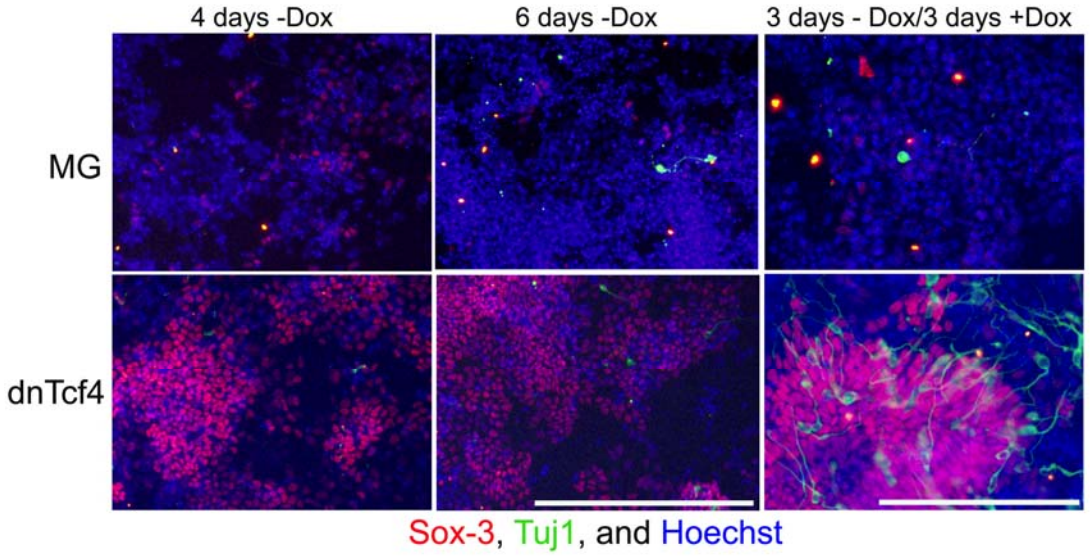
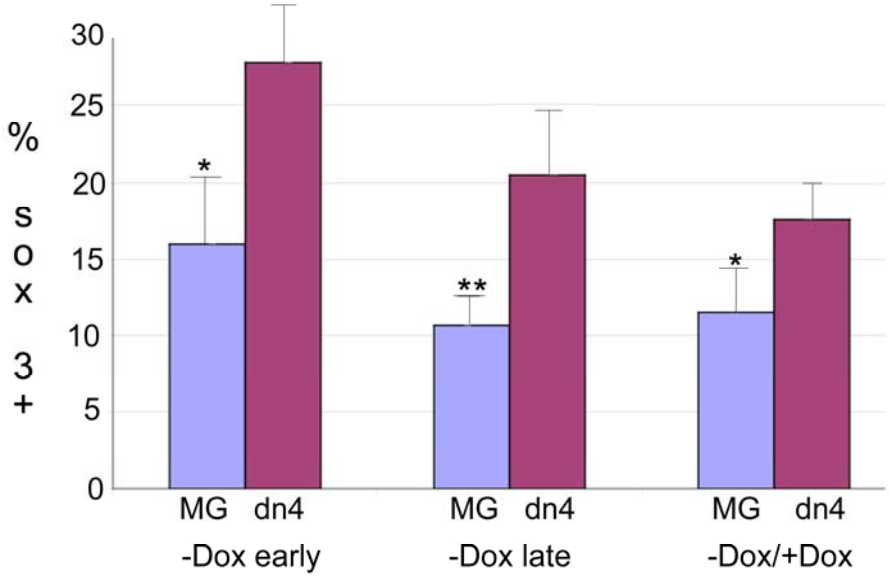


Figure 2.3
Regulated expression of the dnTcf4 protein promotes neural differentiation of mESC in a monolayer assay.
IHC of cell type restricted antigens in MG control and dnTcf4 mESC grown in monolayer. When the transgene was induced and Wnt signaling was abrogated throughout differentiation (4 or 6 days) there was widespread expression of the neural precursor marker Sox3 (red, Cy3 secondary). There was little expression of the neuronal marker βIII tubulin (Tuj1 antibody, green, FITC secondary). Reactivation of Wnt signaling after 3 days of differentiation (3 days -Dox/3 days +Dox) resulted in a significant increase in conversion of Sox3 positive precursors to immature neurons. Nuclei were stained with Hoechst (Blue). Scale bar = 200 μM.

Figure 2.4
Regulated expression of the dnTcf4 protein significantly increases neural differentiation of mESC in a monolayer assay.
A. FACS analysis of the number of neural precursors (Sox3 +cells) following induction of the dnTcf4 transgene. Cells were cultured for 3 days without Dox (- Dox early), 6 days without Dox (- Dox late), or 3 days without Dox then 3 days with Dox (-Dox / + Dox). The number of Sox3 positive cells was significantly increased in dnTcf4 (dn4) expressing compared to MG control cells at all time points. *$p \leq 0.001$, p**≤ 0.007, p***≤ 0.03, students t test. The graphs represent averages of 5 experiments with 3 replicates/experiment.
B. The Image J program (NIH) was used to quantify the number of neurons in MG control and dnTcf4 mESC grown in monolayer culture. IHC localization of βIII tubulin was carried out in cultures in which Wnt signaling was abrogated during the entire culture period (No Dox) or when signaling was abrogated only during the first half of the culture period (-Dox / +Dox). The average number of βIII tubulin pixels was divided by the average number of Hoechst pixels in 10 fields per well, 3 replicates/experiment, in 4 biological experiments. There was a significant increase in neuronal differentiation only when Sox3 positive precursors were allowed to complete differentiation in the presence of Wnt signaling during the last half of the culture period. *$P \leq 0.003$ Student's t test.

A.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20
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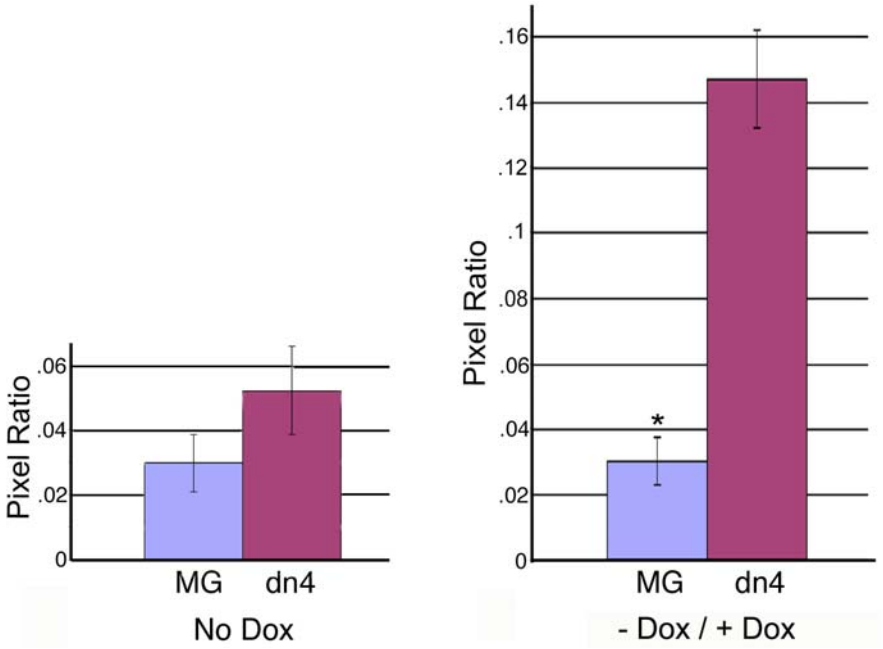
MG dn4
-Dox early
MG dn4
-Dox late
MG dn4
-Dox/+Dox

B.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MG dn4
No Dox
Pixel Ratio
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.14
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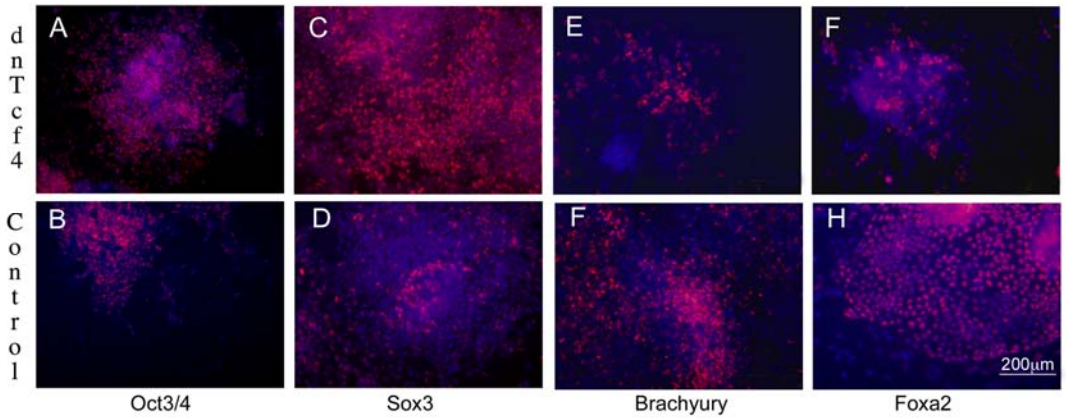
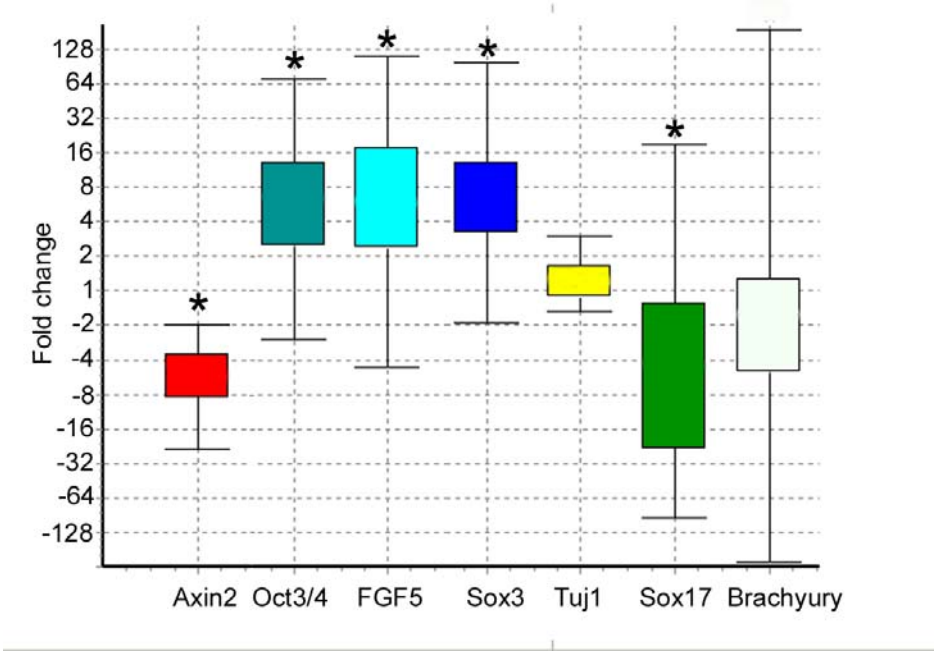
*
MG dn4
- Dox / + Dox

Figure 2.5
Wnt signaling blockade increases differentiation of neural precursors and decreases mesendoderm differentiation in an EB assay.
A. MG control and dnTCF4 (dn4) mESC were grown without Dox for 4 days as EBs then transferred to adherent culture for an additional 2 days before analysis. IHC localization of the pluripotency marker (Oct3/4), neural precursor marker (Sox3), mesodermal marker (Brachyury), or endodermal maker (Foxa2). When Wnt signaling is inhibited, cells continue to express high levels of Oct3/4 and Sox3 with a concomitant decrease in mesendoderm differentiation. Secondary antibodies were conjugated to Cy3 (red) and Hoechst (blue) identifies nuclei.
B. Quantitative PCR indicated that the Wnt target gene Axin2 was decreased 10 fold in dnTcf4 compared to MG control cells. Genes expressed in the epiblast, Oct3/4 and FGF5 were increased 6 fold suggesting that Wnt signaling is required for lineage differentiation. The neural precursor gene Sox3 was increased 6 fold, but Tuj1, a marker of immature neurons, remained unchanged. Sox17 was down-regulated 5 fold and there was a trend towards a reduction of Brachyury expression (mesoderm) when Wnt signaling was inhibited. Box and whisker plot produced with (REST software, Pfaffl *et al*., 2002). The top and bottom "whiskers" indicate the range while the box indicates the upper quartile, and lower quartile values. Gene expression was calculated relative to β-actin and gene expression of the MG control mESC line was set to 1. Asterisks indicate statistically significant differences ($P \leq 0.05$) calculated by the REST software ($n=3$).

A.


dnTcf4
Control
A
C
E
F
B
D
F
H
200μm
Oct3/4
Sox3
Brachyury
Foxa2

B.


Fold change
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32
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-2
-4
-8
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-128
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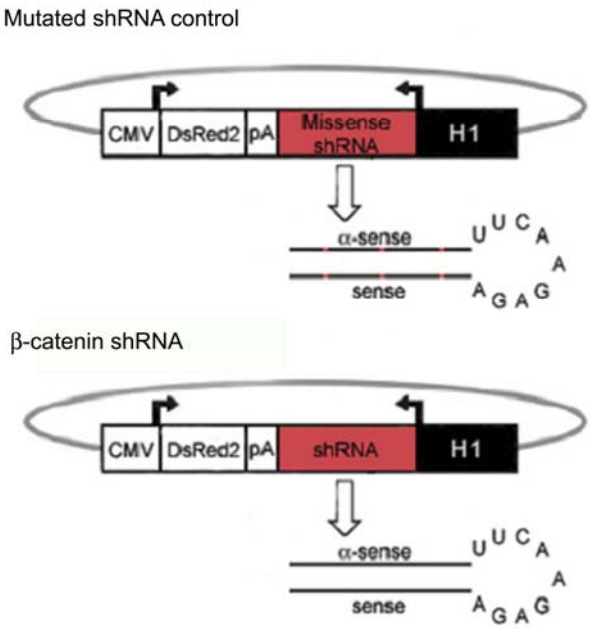
Axin2
Oct3/4
FGF5
Sox3
Tuj1
Sox17
Brachyury

A.



B.

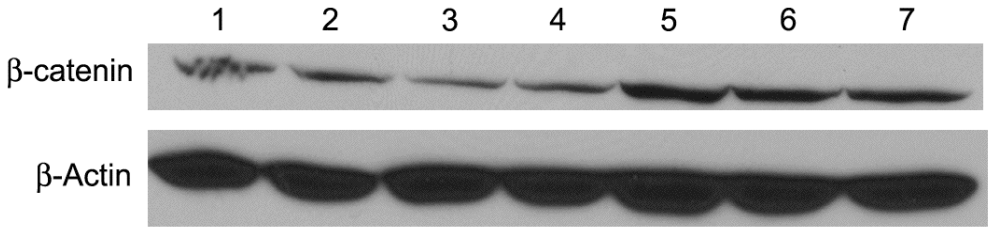
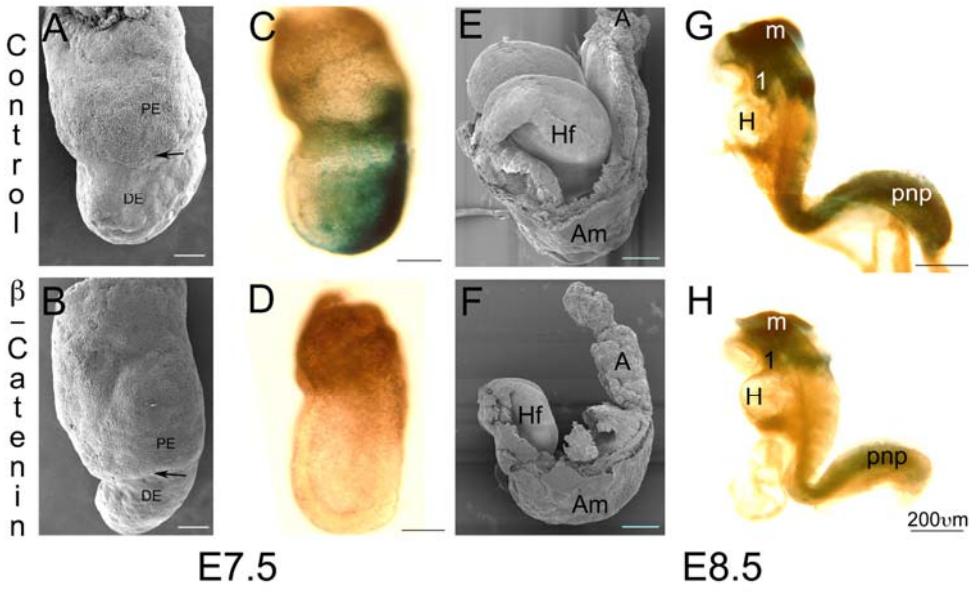


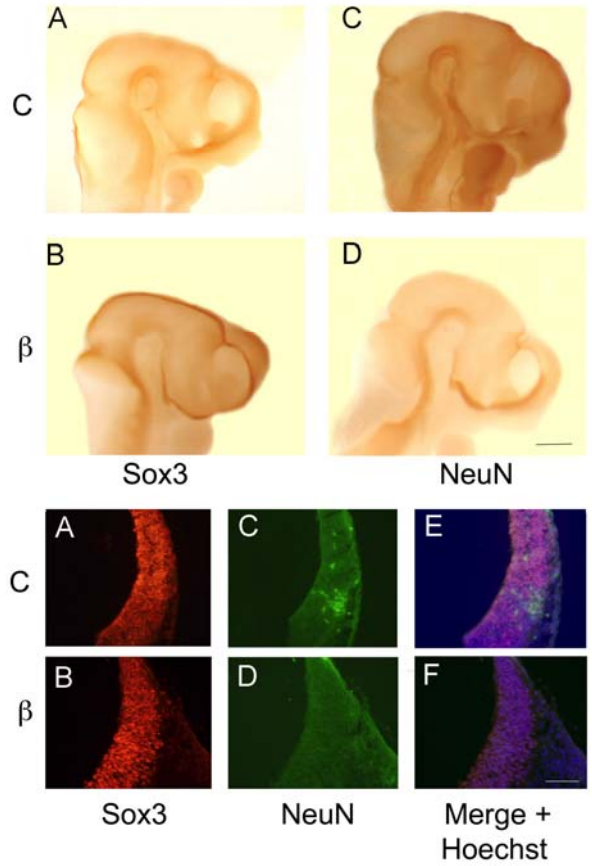
Figure 2.6
Transfection of mESC with a plasmid containing β-catenin shRNA decreases the level of β-catenin protein.
A. Diagrams of plasmids containing mutated shRNA control or β-catenin shRNA that were injected into mESC and pregnant dams.
B. Western blot of mESC transfected with shRNA constructs targeting β-catenin singly (lanes 2,3) or in combination (lane 4) versus mutated control shRNA constructs singly (lanes 5,6) or in combination (lane 7) demonstrating 2 fold knock-down. Top blot is β-catenin and bottom blot is a loading control blotted for β-actin. Lane 1 is untransfected mESC.

Figure 2.7
Wnt signaling is required in the embryo for conversion of Sox3 neural precursors to NeuN positive neurons.
A. Compared with control embryos (A,C,E,G), embryos exposed to shRNA targeting β-catenin (B,D,F,H) exhibit defects in gastrulation, axis elongation, and neural differentiation. Side views of E7.5 and 8.5 embryos examined using SEM (AB, EF) or stained with Xgal to identify sites of β-galactosidase expression and Wnt signaling (CD, GH). The arrows in A and B indicate the boundary between primitive endoderm (PE) and definitive endoderm (DE) that largely replaces the PE at gastrulation. The proximal-distal displacement of the boundary is an indication of the extent of gastrulation. The control embryo (A) has expanded in both the proximal-distal and anterior-posterior axes compared to the β-catenin shRNA exposed embryo (B). In control embryos (C) Wnt signaling is strong in the posterior primitive streak (PS) compared with shRNA exposed embryos (D) where signaling is nearly abrogated and strikingly reduced. By 8.0 in control embryos (E) the neural folds are elevating and later embryos (G) are beginning the process of adopting the fetal C shape. β-galactosidase is expressed at high levels in the midbrain (m), first branchial arch (1), and posterior neuropore (pnp) in control embryos (G), but is strikingly down-regulated in β-catenin shRNA treated embryos (H). Anterior is to the left in each figure, Am=amnion, A=Allantois, Hf=headfold, H=head. SB=200μm
B. Side views of control (A, C) and embryos exposed to shRNA against β-catenin (B, D). Embryos were dissected and whole mount IHC carried out to compare Sox3 positive neural precursors (A, B) and NeuN positive neurons (C, D). By E10.5 in control embryos there were more NeuN positive neurons and fewer Sox3 positive precursors compared with β-catenin shRNA exposed embryos (B, D). Embryos exposed to β-catenin shRNA also exhibited increased midbrain mesenchyme and abnormal positioning of midbrain flexure. When similar embryos were sectioned, there was widespread expression of Sox3 in both control and β-catenin shRNA exposed embryos (A, B) while NeuN was observed in differentiating neurons in control embryos (C) but not in embryos exposed to β-catenin shRNA (D). E and F are overlays of AC+BD with Hoechst staining of nuclei. SB=200μ

A.


Control
β-Catenin
A
B
C
D
E
F
G
H
PE
DE
Hf
Am
m
1
H
pnp
200υm
E7.5
E8.5

B.


C
β
A
C
B
D
Sox3
NeuN
E
F
Merge +
Hoechst

Chapter 3

Geminin promotes an epithelial to mesenchymal transition and mesendodermal differentiation in an embryonic stem cell model of gastrulation

Introduction

At gastrulation the pluripotent epiblast is allocated to three germ layers: endoderm, mesoderm, and ectoderm. This process involves an epithelial to mesenchymal transition (EMT), complex morphogenetic movements, and differential regulation of gene expression that culminates in lineage fate decisions. Despite the importance of understanding cell fate choice in early development, progress has been limited due to the early lethality of gene null models and general inaccessibility of the conceptus at this early stage. Mouse embryonic stem cells (mESC) are derived from the inner cell mass of the blastocyst and have the ability to generate all cell types found in the mouse embryo (Evans and Kaufman, 1981; Martin 1981). In addition, mESC and the epiblast have a similar gene expression pattern and share a uniquely abbreviated cell cycle. Because of these similarities with the epiblast, differentiation of mESC offers a simple model of cell fate choice at gastrulation and an experimentally tractable system to examine gene function in development. Because the loss of

one germ layer at gastrulation frequently leads to expansion of other germ layers (Sheng *et al*., 2003), one approach to obtaining enriched populations of differentiated ESC is to eliminate unwanted lineages and thereby expand the desired tissue progenitors. Therefore, the use of mESC as a model of gastrulation may not only increase our understanding of the process itself but may also lead to improved methods for the directed differentiation of specific cell types for replacement therapy.

In addition to being multipotent, mESC and the epiblast also share an attenuated cell cycle. Just prior to gastrulation, the mouse embryo expands from 20-25 cells to approximately 660 cells in roughly 1.5 days (Ciemerych and Scinski, 2005; Stead, *et al*., 2002). During this period of rapid cell division, it is critical that the epiblast remains undifferentiated. ESC and the epiblast spend the majority of their cell cycle in S phase with a very short G1 phase (Burdon *et al*., 2002 and White *et al*., 2005), unlike somatic cells where G1 predominates. The short cell cycle of the epiblast and mESCs likely underlies the rapid expansion capability of both and may also play an important role in prohibiting differentiation and maintaining multipotency.

One candidate gene that can modulate both cell cycle progression and differentiation is *Geminin*, which was originally identified in two independent functional screens in *Xenopus* embryos (Kroll *et al*., 1998; McGarry and Kirschner, 1998). The first screen was designed to identify genes that were degraded during mitosis and the second screen was designed to identify genes involved in neural induction. Geminin functions as a cell cycle licensing factor,

allowing replication of the genome once and only once during mitosis (Luo and Kessel, 2007; Wohlschlegel *et al*., 2000). Geminin inhibits DNA re-replication during late S phase of the cell cycle by binding to the Cdt1 protein and preventing its interaction with origins of replication. Geminin must be degraded during M phase of the cell cycle to allow Cdt1 to interact with DNA origins of replication. However, over-expression of Geminin in *Xenopus* embryos expanded the neural plate independent of its effect on cell division (Kroll *et al*., 1998). The mechanism was proposed to be via an inhibition in BMP signaling and a resulting increase in Neurogenin-1 expression concomitant with an expansion of the neural plate at the expense of the ectoderm.

During the G2 phase of the cell cycle, Geminin binds to Hox, Six, and AP4 transcription factors in the nucleus to antagonize their activity (Del Bene *et al*., 2004; Luo *et al*., 2004, Kim *et al*., 2006). Additionally, Geminin interacts with chromatin remodeling proteins Brg-1 and Brahma (Roukos *et al*., 2007; Seo *et al*., 2006) in neural precursor cells to block their interaction with neurogenic bHLH proteins, thereby inhibiting bHLH target gene expression to prevent premature differentiation. Geminin expression is restricted to the neural plate in *Xenopus* embryos by Tcf and Vent sites (Taylor *et al*., 2006), indicating that the BMP and Wnt pathway signaling cooperate to control Geminin expression during differentiation. Because *Geminin* null mouse embryos die around E3.5 due to endoreduplication errors (Gonzalez *et al*., 2006 and Hara *et al*., 2006), there is a lack of information about Geminin's function during postimplantation development.

To gain an understanding of how *Geminin* functions in lineage specification, we created a mESC line in which Geminin can be inducibly expressed, and employed short hairpin RNAs (shRNA) to target the native Geminin mRNA. Reduction of Geminin protein via targeted shRNA resulted in cell death due to DNA damage, demonstrating that as in the cleavage staged embryo, Geminin is required to prevent reduplication errors (Hara *et al*., 2006; Gonzalez *et al*., 2006). Over-expression of Geminin promoted EMT and mesendodermal differentiation in embryoid bodies. Initiation of mesendodermal differentiation appears to occur via Wnt pathway activation by binding of Geminin to Groucho/transducin-like Enhancer of split (TLE) proteins in the nucleus that function to block Tcf/Lef target gene expression in the absence of activated β-catenin (Brantjes *et al*., 2001).

Materials and methods

Mouse ES Cell Culture and differentiation

Undifferentiated mESCs were maintained in 0.1% gelatin coated tissue culture flasks in complete media composed of DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) with 5 ng/ml LIF (Chemicon). Neural permissive culture conditions were achieved by plating cells at low density ($1.6x10^4$ to $2.6x10^4$ cells per cm^2) in gelatin coated 12 well plates in 20% Neural basal medium (Invitrogen), 80% Ham's F12 medium (Invitrogen) with N2 and B27 salts

(Invitrogen), 1 □M retinoic acid (Sigma), and 0.5% Knock-out serum replacement (Invitrogen). To form embryoid bodies (EBs), cells were plated at $1.25x10^5$ cells per ml in non-adherent petri dishes in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) for 4 days then transferred to gelatin coated 12 well dishes for an additional 2 days of culture in the same medium. Cells were maintained at 37°C with 5% CO_2.

Plasmid Construction and Transfection

Geminin shRNA plasmids were described previously (O'Shea *et al*., 2006). The HI promoter was used to drive expression of the shRNA while a separate eGFP cassette driven by a CMV promoter was used to measure transfection efficiency and identify transfected cells. ESC were passaged 12 to 24 hours prior to transfection and plated in gelatin coated 6 well plates ($2.5x10^4$ cells per cm^2) in complete media with LIF. ES cells were transfected with Lipofectamine Plus (Invitrogen) as directed by the maufacturer and cultured overnight in serum free conditions (OptiMem, Invitrogen).

Inducible Geminin mESC

We obtained the MGZRTcH2 mESC cell line and corresponding exchange vector from Dr. Shinji Masui (Masui *et al*., 2005). The MGZRTcH2 cell line contains a tetracycline-regulated transactivator (tTA), a tetracycline response element followed by the minimal promoter of the human cytomegalovirous

(hCMV*-1) immediate early gene, a hygromycin resistance cassette, directional loxP sites, and an IRES-Venus (yellow fluorescent protein) cassette knocked-into the endogenous ROSA26 promoter. The corresponding exchange vector replaces the hygromycin resistance cassette with the desired cDNA in addition to adding a puromycin resistance cassette. When the exchange vector is correctly enzymatically recombined into the ROSA26 locus, the resulting clones are no longer hygromycin resistant but are puromycin resistant allowing for both positive and negative selection thereby ensuring the elimination of cells with random integration of the exchange vector.

A PCR cloned and sequence verified *Geminin* cDNA was inserted into one exchange vector. A control exchange vector with no cDNA was also generated for the development of control cell lines that are puromycin resistant and express the Venus protein upon doxycycline withdrawl. Each exchange vector was transfected individually into the MGZRTcH2 cell line with a Cre recombinase vector followed by selection with 2 μg/ml puromycin (Sigma). Clones were expanded in complete media with 1 μg/ml doxycycline (Sigma) to inhibit transgene expression and 2 μg/ml puromycin to maintain selection. Clones were checked for random integration with 100 μg/ml hygromycin (Sigma). Over-expression of Geminin was verified by Western blot and quantitative PCR. During all differentiation experiments, selection was maintained with 2 μg/ml puromycin and gene expression was induced by doxycycline withdrawl.

Cell Cycle Analysis

Cells were grown in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) without doxycycline for two days. At day four cells were plated at $2x10^5$ cells/well in 6 well plates in triplicate without doxycycline. After 48 and 96, hours cells were trypsinized and counted in triplicate in a Coulter Counter (BD). For cell cycle analysis, samples were fixed by adding absolute cold ethanol and keep at -20C until ready for analysis. Fixed cells were resuspended in PBS and RNase A-DNAse free (Sigma) was added at 20□g/ml final concentration. Samples were incubated at 37°C for 30min and DNA was labeled with propidium iodide (Sigma) at final concentration of 0.1mg/ml. Acquisition and analysis were done in a FACS Calibur using Cell Quest Pro MAC 9.0. Cell cycle analysis was done with Mod Fit LT Mac 3.1 SP3.

Immunohistochemistry

ES cells were fixed with 2% paraformaldehyde (PFA) for 15 minutes at room temperature and washed twice with PBS. Fixed cells were incubated with 10% donkey serum for 30 minutes followed by overnight incubation at 4°C with primary antibody (rabbit anti-Sox3, Mike Klymkowski, University of Colorado 1:1000; goat anti-Oct3/4, Santa Cruz 1:500; rabbit anti-Foxa2, Upstate 1:500, or goat anti-Brachyury, Santa Cruz 1:500). Cells were then washed with PBS and incubated with secondary antibodies (1:400) conjugated to Cy3 or FITC (Jackson Immunoresearch) for 2 hours at room temperature. Nuclei were visualized with

Hoechst 33258 (Sigma) staining and images were obtained using with a Leica DM inverted fluorescence microscope and Olympus DP70 camera with associated software.

Quantitative RT PCR

RNA was harvested with Trizol (Invitrogen) following the manufacturer's protocol and genomic DNA was removed by DNAse digestion (Sigma). Complete DNA digestion was confirmed by semi-quantitative PCR using primers for β-actin before reverse transcription. A 0.5 to 1.0 μg RNA sample was used for reverse transcription with Verso RT (Thermo Scientific) using random nonamers (Invitrogen) following the manufacturer's protocols. For quantitative PCR, cDNAs were diluted 1:3 and 1μl was used per reaction with Abgene SYBR green master mix. All primer pairs were rigorously screened to eliminate primer dimer and reaction conditions were optimized resulting in reaction efficiencies between 90% and 110%. Quantitative PCR results were calculated and statistical analysis was performed with REST2008 software (Pfaffl *et al*., 2002). Primer sequences and detailed reaction conditions are available upon request.

Western Blot

ESC were lysed in RIPA buffer (50mM Tris-HCL pH7.4, 1% NP-40, 0.25% Na-deoxcholate, 150mM $NaCl_2$) with Complete protease inhibitor (Roche), cell debris was pelleted by centrifugation, and the protein containing supernatant was removed and analyzed by the Pierce Protein Assay to determine total protein

concentration. A total of 10 to 30 μg of protein was loaded onto 10% or 12% polyacrylamide SDS PAGE gels then transferred to PVDF membranes. Membranes were blocked with 5% milk powder /TBST (Tris buffered saline and 0.2% Tween 20) and incubated with rabbit anti-Geminin (1:2000; Santa Cruz), anti-activated β-catenin (1:2000; Millipore), goat anti-Tcf3 (1:500; Santa Cruz), goat anti-panTLE (1:500; Santa Cruz), and mouse anti-β-actin (1:10,000; Sigma) primary antibodies in 5% milk powder/TBST overnight at 4^{o}C. Membranes were washed in TBST and incubated for 1 hour at room temperature in horseradish peroxidase conjugated secondary antibody (1:1000 - 1:10,000; Jackson Immunoresearch). Membranes were developed with Pierce Supersignal West Pico chemiluminescent substrate.

Top/Fopflash assay

MG control and Geminin mESC were grown in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) without doxycycline for 48 hours to induce transgene expression. Cells were transfected with TOPFlash or FOPFlash plasmids (Millipore) using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. A Renilla plasmid (pRL-CMV, Promega) was co-transfected as a transfection control. After 24 hours, cells were lysed with passive lysis buffer and subjected to the Dual-luciferase assay (Promega) according to the manufacturer's directions. Samples were read with a Veritas microplate

luminometer (Turner Biosystems). Data are expressed as the ratio of TOPFlash relative to Renilla over FOPFlash relative to Renilla.

Co-immunoprecitpitation

MG control and Geminin mESC were grown in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 mM β-mercaptoethanol (Sigma) without doxycycline for 4 days to induce transgene expression. Cells were scraped from the tissue culture dishes and lysed in Blenis lysis buffer (10mM KPO_4, 1mM EDTA pH7.05, 5mM EGTA pH7.2, 10mM $MgCl_2$, 50mM β-glycerolphosphate, 0.3% Chaps) with Complete protease inhibitor (Roche). Protein samples were divided in half and 2μl of goat anti-TLE (Santa Cruz) or rabbit anti-Geminin (Santa Cruz) antibodies were added to 1 aliquot from each sample set and incubated overnight at 4^{o}C with end over end agitation. Pre-blocked Protein A or Protein G sepharose beads (GE) were added to samples with antibody as well as to samples with input protein alone as a negative control and incubated for 1 hour at 4^{o}C with end over end agitation. Samples were centrifuged and the first supernatant was saved for a loading control. Sepharose beads were washed 3 times with lysis buffer at 4^{o}C. Loading buffer was added to the Sepharose beads and the samples were boiled for 5 minutes to release antibody/antigen complexes from the beads before analysis by Western blot as described above.

Results

Geminin can be inducibly expressed in mESC

To explore the role of Geminin protein in multil-ineage differentiation of mESC, we created a cell line that inducibly over-expresses *Geminin* (Figures 3.1 and 3.2). A total of 24 Geminin and 24 MG control (puromycin resistant cells that inducibly express only venus yellow fluorescent protein) lines were cloned and expanded for further study. Proper targeting of all lines was verified by testing for hygromycin sensitivity and puromycin resistance. Several lines were selected for additional study based doxycycline regulated Geminin protein over-expression and/or Venus protein expression. In the presence of doxcycline, the Geminin cell line grows similarly to the MG control cell line (data not shown). Geminin protein levels in Geminin and MG control mESC lines were roughly equal two days after doxycycline withdrawl, but by four days Geminin was seven fold over-expressed and by six days increased to 199 fold over-expression (Figure 3.2). These data indicate the Geminin protein is reliably over-expressed in response to doxycycline withdrawl.

Geminin promotes EMT and mesendodermal differentiation in an EB model of gastrulation

To examine the role of the Geminin protein in tri-lineage differentiation of mESC, we employed a three-dimensional differentiation assay. Cells were plated in non-adherent dishes (Petri dishes), in the absence of LIF and

doxycycline but with 10% serum, promoting the formation of embryoid bodies (EBs) and inducing differentiation. A large percentage of Geminin over-expressing EBs began to adhere to the Petri dish by 3 to 4 days after doxycycline withdrawl and cells left the EB itself and rapidly migrated away (Figure 3.2 B). However, the vast majority of MG control line EBs remained intact and floating for the entire six day assay. Therefore, cells were grown as floating EBs for four days then replated into adherent tissue culture dishes for an additional two days before fixation for immunhistochemistry (IHC) or harvesting of RNA to force the attachment of MG control cell EBs and promote differentiation. The behavior of the Geminin EBs on adherent tissue culture dishes was identical to that seen on the Petri dishes, once the EBs adhered to the tissue culture dish, cells rapidly migrated away. We examined E-cadherin expression in Geminin and MG control EBs by IHC. Geminin over-expressing EBs had strikingly less cytoplasmic E-cadherin compared with MG control cell EBs that expressed high levels of E-cadherin (Figure 3.2 A). There was also a trend toward down-regulation of E-cadherin in Geminin versus control cells at the level of transcription as determined by quantitative PCR (Figure 3.3 B).

When lineage restricted markers were examined by IHC, compared with controls, Geminin over-expression lead to a decrease in the expression of the pluriptotent stem cell marker Oct3/4 and of the neural precursor marker Sox3. However, the mesodermal marker Brachury and the endodermal marker Foxa2 were increased (Figure 3.2 C). We also examined expression of lineage restricted markers (Figure 3.3 A) and genes important in EMT (Figure 3.3 B) by

quantitative PCR. Oct3/4 expression decreased as did the expression of the epiblast marker FGF5, indicating that the Geminin over-expressing cells differentiated more rapidly than MG control cells. Markers of mesendodermal lineage, BMP4, Brachyury, and Sox17 were increased while the surface ectoderm marker Claudin6 and the neural precursor marker Sox3 were decreased. There was also a trend word increasing Snail1 expression along with increased Snail2, Twist1, and Twist2 expression in Geminin over-expressing cells. Taken together these data indicate that Geminin over-expression is inducing EMT in the mESC model of gastrulation.

Geminin over-expression increases Wnt signaling in mESC

Because Wnt signaling plays a critical role in formation of the primitive streak and mesendodermal differentiation in the mouse embryo, we examined the status of the Wnt pathway in Geminin over-expressing mESC. Induction of Geminin protein expression increased canonical Wnt signaling approximately 40 fold in the TopFlash luciferase assay (Figure 3.4 B), which was approximately equal to the increase in Wnt signaling produced by exposure of MG control cells to the Gsk3β inhibitor Chir99021 previously shown to promote Wnt signaling (Finaly *et al*., 2004). Geminin did not maximally stimulate Wnt signaling because addition of Chir99021 in combination with Geminin over-expression increased Wnt signaling approximately 190 fold. In addition, a well-characterized canonical Wnt target gene, *Axin2*, (Hughes and Brady 2006; Jho *et al*., 2002; Leung *et al*., 2002; Yan *et al*., 2001) was up-regulated following doxycycline withdrawl after

four days in monolayer culture (Figure 3.4 A) and six days in EB culture (Figure 3.5 D). These data indicate that in mESC, Geminin over-expression functions to stimulate Wnt signaling. Since BMP signaling is also required for mesendodermal differentiation of the mouse embryo, we examined *BMP4* and *Axin2* expression after only three days of EB differentiation by quantitative PCR. *Axin2* was increased 57 fold while BMP4 was only increased three fold, suggesting that Geminin is acting on Wnt signaling that may in turn up-regulate BMP4 expression.

Geminin induces Wnt signaling via binding TLE proteins

To identify the mechanism underlying increased Wnt signaling, we examined the expression of Wnt pathway members. Geminin did not increase the level of activated β-catenin (Figure 3.5 A), suggesting that Geminin does not likely regulate expression of Wnt ligands or inhibit GSK3β. Tcf3 expression was also unchanged with Geminin over-expression (Figure 3.5 B) as was the expression of TLE proteins (Figure 3.5 C), both of which typically inhibit the Wnt pathway.

Since Geminin has been shown to bind proteins in the nucleus and inhibit their function, we performed co-immunoprecipitation experiments with Tcf3 and Geminin antibodies, but were unable to detect an interaction (Data not shown). However, we were able to detect a weak but consistent interaction of Geminin with TLE proteins (Figure 3.4 D). Based on this interaction, we hypothesize that

Geminin may bind and inhibit TLE proteins from repressing Wnt signaling in the nucleus (Figure 3.6).

Geminin reduces proliferation and alters cell cycle in mESC

Since Geminin inhibits DNA replication to prevent endoreduplication, we assayed proliferation and cell cycle in monolayer culture following doxycycline withdrawl. Geminin over-expression significantly reduced proliferation, increased the length of time cells spent in the G2/M phase of the cell cycle and reduced the time spent in G1 phase (Figure 3.7). The decrease in proliferation may play a role in increased differentiation since exit from the cell cycle is required for many cell fate decisions. Additionally, cells may be unable to progress from G2/M because they are unable to initiate new rounds of DNA replication since Geminin is normally destroyed by the proteasome during M phase. Clearly Geminin plays an important role in controlling cell cycle and proliferation in mESC similar to the role demonstrated in the ICM.

Geminin shRNA results in DNA damage and death

To determine if Geminin protein was necessary for either Wnt signaling and/or EMT and mesendodermal differentiation, we employed shRNAs (Figure 3.8 A) to reduce Geminin protein levels. We were able to achieve six fold knock-down of Geminin protein 24 hours post-transfection (Figure 3.8 B); however, Geminin shRNA transfected cells failed to survive long enough to perform differentiation assays (data not shown). A phospho histone HA2X antibody was

employed to determine the extent of any DNA damage. As predicted, cells transfected with a Geminin shRNA exhibited increased DNA damage (Figure 3.9 C) precluding an analysis of differentiation and Wnt signaling.

Discussion

Our results demonstrate that expression of Geminin in a mESC model of gastrulation promotes EMT and mesendodermal differentiation. In the mouse, there are four TLEs that bind to Tcf/Lef transcription factors to inhibit Wnt signaling in the absence of nuclear β-catenin (Daniels and Weiss, 2005). Based on our results, we hypothesize that Geminin inhibits interactions between Tcf/Lefs and TLEs to de-repress Wnt signaling. However, as cell cycle regulation is critical for the transition from proliferating epiblast to differentiating mesedodermal cells, it is also possible that Geminin plays a role in blocking proliferation required for this transition.

The bi-functional protein Geminin has previously been characterized by its ability to promote neural differentiation at the expense of epidermis in both *Xenopus* (Kroll *et al*, 1998) and *Drosophila* embryos (Quinn *et al*, 2001). Despite repeated efforts, Geminin over-expressing mESC consistently generated fewer neural precursors and neurons than controls both in monolayer culture (data not shown) and following EB differentiation (Figure 3.2 C). It remains possible that the level of Geminin expression in our experiments was somehow inhibitory to neural differentiation. Alternatively, in the mESC model Geminin over-expression

increases rather than decreases BMP4 expression, as well as an increasing Wnt signaling which in combination would potently inhibit neural differentiation of mESC. Geminin's second function is to prevent endoreduplication by binding and sequestering the cell cycle licensing factor Cdt1 immediately following DNA replication in S phase (Luo and Kessel, 2007; Wohlschlegel *et al*., 2000). Once DNA replication is complete, Geminin is released from Cdt1 and during the G2 phase of the cell cycle is free to bind other factors within the nucleus. Geminin can bind and inhibit the function of several nuclear factors including: AP4 (Kim *et al*., 2006), the chromatin remodeling protein Brg-1 (Seo *et al*., 2006), Hox proteins (Luo *et al*., 2004), and Six3 (Del Bene *et al*., 2004). Geminin has been hypothesized to regulate the transition from proliferation to differentiation during development by binding Brg-1 and inhibiting its interaction with proneural bHLH transcription factors, allowing cells to remain undifferentiated and proliferative as precursors (Seo *et al*., 2005; Seo and Kroll, 2005). In addition to controlling chromatin, Geminin has the potential to act as a molecular switch between proliferation and differentiation by alterations in its binding to Cdt1 versus transcription factors (Pitulescu *et al*., 2005), in this case TLE proteins.

In many cell types, cell fate decisions are tightly coupled to cell cycle exit. In fact, the most crucial decision point in the cell cycle, i.e., proliferate, differentiate, quiesce, senesce, or apoptose, occurs at the G1 checkpoint (Blomen and Boonstra, 2007). Both ESC and the epiblast have a short cell cycle with a very attenuated G1 phase that may prevent differentiation by insulating cells from growth factor exposure (Burdon *et al*., 1999; Orford and Scadden,

2008). Over-expression of Geminin decreased proliferation and increased length of G2/M phase in mESC. This increase likely reflects the requirement for Geminin degradation, to free Cdt1 to initiate a new round of replication. Persistent Geminin protein is likely to maintain the sequestration of Cdt1 and block progression from M phase. In turn, lengthening G2/M would increase time Geminin might interact with other proteins in the nucleus and perhaps further promote differentiation. A decrease in proliferation will also promote differentiation since cell cycle exit is typically required for differentiation.

During gastrulation, cells undergo dramatic changes in morphology that require exit from the cell cycle due to drastically different requirements for cytoskeleton organization in each process (Grosshans and Wiechaus, 2000; Seher and Leptin, 2000). In the zebrafish embryo, cell cycle exit is required for delamination of mesendoderm from the epiblast (Liu *et al*., 2009).

The Wnt signaling pathway has been implicated in proliferation and/or differentiation of virtually every tissue in the embryo. The Wnt family of secreted ligands bind to frizzled receptors, inhibiting GSK-3β phosphorylation and the destruction of β-catenin, resulting in the accumulation of nuclear β-catenin that binds to Tcf/Lef transcription factors to activate and/or repress gene expression (Blauwkamp *et al*., 2008). All four Tcf/Lef factors bind similar promoter sequences and can act as repressors in the absence of Wnt ligand activity (by binding Groucho/TLE family of co-repressor proteins) but can also act as transcriptional activators when β-catenin enters the nucleus and displaces Groucho/TLE proteins (Brantjes *et al*., 2001; Daniels and Weiss, 2005).

Wnt signaling is critically involved in establishing the primitive streak and promoting the EMT required for mesendodermal differentiation (Kelley *et al*., 2004; Kemler *et al*., 2004; Liu *et al*., 1999; Mohamed *et al*., 2004; Maretto *et al*., 2003; Rivera-Perez and Magnuson, 2005; and Yamaguchi *et al*., 1999). In fact, β-catenin null mouse embryos die around E7.0 due to failure of gastrulation and mesodermal differentiation (Haegel *et al*., 1995 and Huelsken *et al*., 2000) while over-expression of β-catenin and expanded Wnt signaling induces a premature EMT of the epiblast (Kemler *et al*., 2004) and mis-expression of a Wnt ligand induces multiple primitive streaks (Popperl *et al*., 1997).

Wnt signaling has previously been shown to be required for the differentiation of mESC to mesendoderm (Gadue *et al*., 2006; Lindsley *et al*., 2006; Nakasnishi *et al*., 2008; ten Berge *et al*., 2008). This is not surprising since Brachyury, one of the key regulators of mesedodermal differentiation, is a direct target of Wnt signaling (Arnold *et al*., 2000; Yamaguchi *et al*., 1999). Wnt signaling may play an important role in bridging the switch from the pluripotent to the differentiated state via Tcf3 binding to a protein complex with Oct3/4, Sox2, and Nanog on gene promoters (Cole *et al*., 2008 and Pereira *et al*., 2006; Tam *et al*., 2008). Interestingly, Wnt signaling also appears to act as part of the switch from proliferation or self-renewal to differentiation in several other stem cell populations (Alonso and Fuchs, 2003; Chien *et al*., 2009; He *et al*., 2004; Kleber and Sommer, 2004; Zhang *et al*., 2010). Our results suggest that Geminin via its ability to de-repress Wnt signaling may be a key regulatory factor controlling cell cycle, EMT, and differentiation required for gastrulation.

In the embryo, EMT occurs as cells migrate into and through the primitive streak; a process the requires the loss of apical-basal polarity, changes in cell shape, loss of epithelial junctions via the down-regulation of E-cadherin and up-regulation of N-cadherin, expression of integrin receptors, and finally, migration from the epiblast (Hay, 1995). The transcriptional repressors Snail1 and Snail2 both down-regulate expression of E-cadherin and in the absence of *Snail1*, mesoendoderm is still specified, however gastrulation fails because cells are unable to leave the primitive streak (Carver *et al*., 2001; Nieto *et al*., 1994). It has been hypothesized that the down-regulation of E-cadherin releases β-catenin from cytoskeletal junction complexes, increasing Wnt signaling (Medici *et al*., 2008; Stemmer *et al*, 2008) that in turn feeds back to increase Snail1 expression (Bachelder *et al*., 2005; Murray *et al*., 2006; Sakai *et al*., 2005 *Yook et al*., 2006; Zhou *et al*., 2004). However, based upon the dramatic increase in Axin2 expression at three days of differentiation and no increase in Snail1 or Snail2 expression (data not shown) it seems that in the EB model of gastrulation, Geminin de-represses Wnt signaling to promote Snail1 and Snail2 expression. Geminin should now be included in the list of genes that initiates EMT and mesendodermal differentiation.

Geminin expression is consistently associated with proliferating tumors and high levels of Geminin expression have been correlated with poor patient prognosis (Nishihara, *et al*., 2009; Wholschlegel *et al*., 2002; Xouri *et al*, 2004). Therefore, based on our observations in mESC high levels of Geminin may promote not only high cell division rates, but may also increase EMT and

therefore metastasis. Geminin may be a potent therapeutic target. Knock-down of Geminin protein might inhibit cancer metastasis as well as initiate endoreduplication and apoptotic cell death in dividing cancer cells.

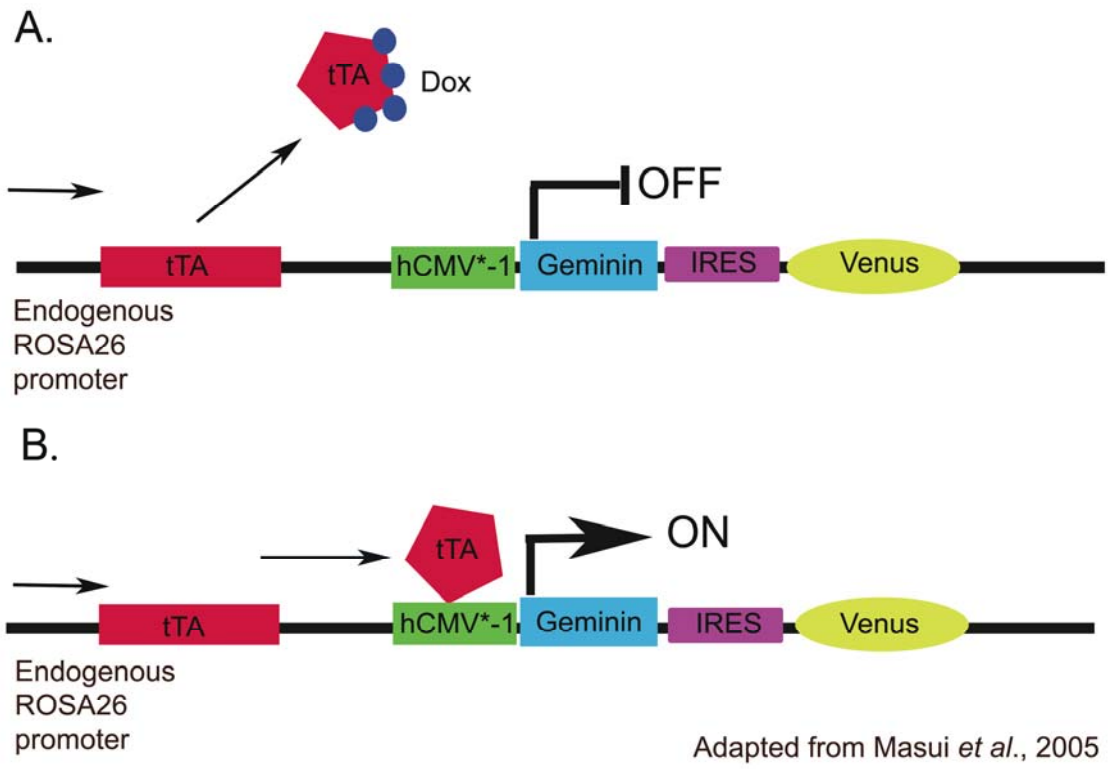
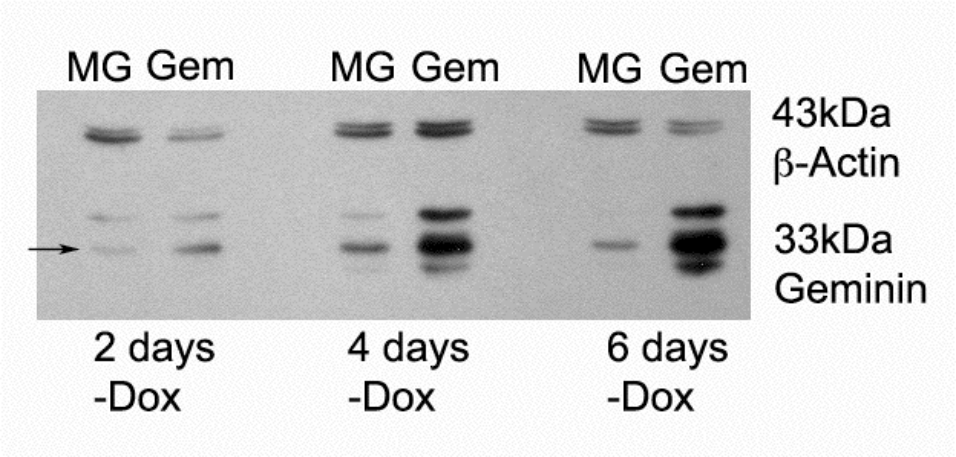


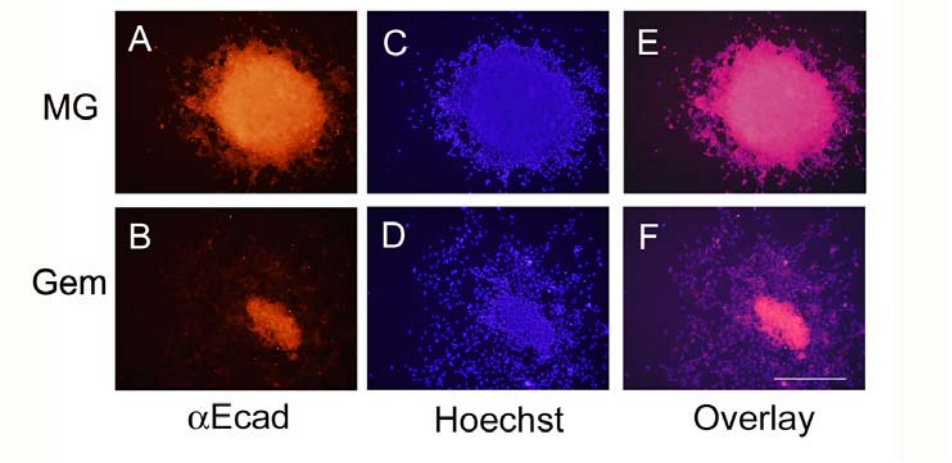
Figure 3.1
The TET-off system stably expressed at the ROSA26 locus in the Geminin inducible line.
A. In the presence of doxycycline (Dox), the Tetracycline-regulated transactivator (tTA) is unable to bind to hCMV*-1 promoter (Tetracycline response element followed by the minimal promoter of the human cytomegalovirus immediate early gene) and initiate downstream gene expression.
B. Once doxycycline is removed from the tissue culture medium, the tTA binds the promoter and the Geminin and Venus proteins are transcribed.

Figure 3.2
Geminin can be inducibly expressed in mESC and promotes EMT and mesendodermal differentiation.
A. Western blot of control (MG) and Geminin (Gem) mESC lines that were grown for two, four, and six days without doxycycline. Full length Geminin is the middle band (arrow) at 33 kDa. At two days Geminin expression was roughly equal in MG control cells and Geminin cells, but at four days Geminin was 7-fold over expressed compared to MG control cells and by six days had increased to 199 fold compared to control cells.
B. E-Cadherin IHC of control (C) and Geminin (G) mESC grown without doxycycline as EBs for 4 days followed by 2 days in adherent culture. Control EB cells have attached but cells have not yet migrated from them as illustrated by Hoechst staining (C). However, Geminin over-expressing EBs attach and cells migrate rapidly from the EB (D). Compared to controls that expressed high levels of E-cadherin (A), Geminin (B) over-expression dramatically down regulated expression of E-cadherin protein, a hallmark of cells that have undergone EMT. Scale bar = 200 μM.
C. IHC of cell type restricted antigens in MG control (A) and Geminin mESC grown as in B above. Geminin over-expression decreased the expression of the pluripotency marker (Oct3/4, B) and of a neural precursor marker (Sox3, C,D) but increased expression of a mesodermal marker (Brachyury, E,F) and an endodermal marker (Foxa2, F,H). Nuclei were stained with Hoechst (Blue). Scale bar = 200 μM.

A.


MG Gem
MG Gem
MG Gem
43kDa
β-Actin
33kDa
Geminin
2 days
-Dox
4 days
-Dox
6 days
-Dox

B.


MG
Gem
A
C
E
B
D
F
αEcad
Hoechst
Overlay

C.

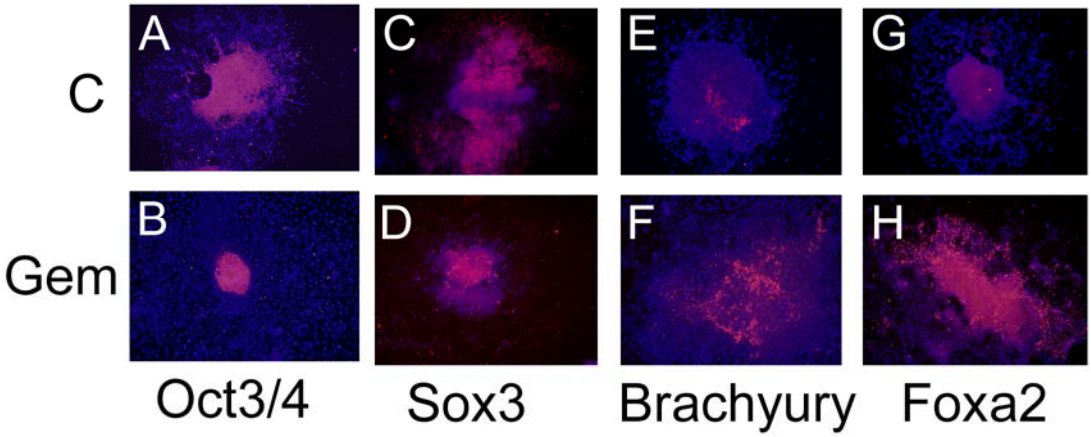
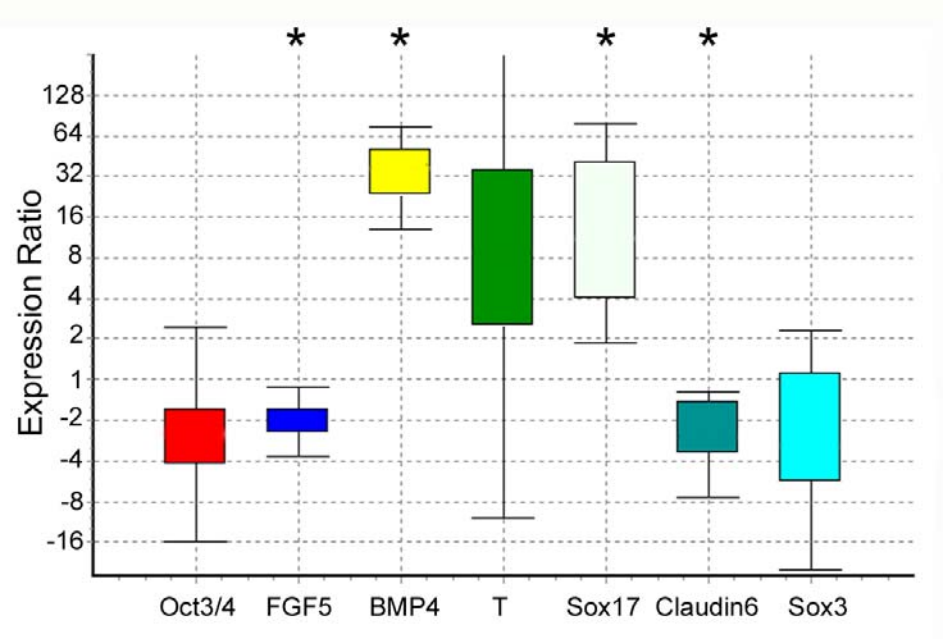

C
Gem
A
C
E
G
B
D
F
H
Oct3/4
Sox3
Brachyury
Foxa2

Figure 3.3
Analysis of gene expression in Geminin versus control EBs.
Quantitative PCR of mRNAs from EBs, grown as described in Figure 3.2. Box and whisker plots produced with REST software (Pfaffl *et al*., 2002). The top and bottom "whiskers" indicate the range while the box indicates the upper and lower quartile values. Gene expression was calculated relative to β-actin and set to 1 in MG control mESC line. Asterisks indicate statistically significant differences ($p \leq 0.05$) calculated by the REST software (n=3 replicates).
A. Geminin over-expression decreased expression of the pluripotency gene Oct3/4 (2.5 fold) and of the epiblast gene FGF5 (2.0 fold). There was increased expression of the mesendodermal markers BMP4 (35.2 fold) and Sox17 (12.8 fold) and there was a trend towards increasing Brachyury expression (10.3 fold) in Geminin EBs. Finally, there was reduced expression of the epidermal ectoderm marker Claudin6 (2.5 fold) and of the neural precursor marker Sox3 (2.5).
B. Geminin over-expression (21.9 fold) increased expression of the Wnt pathway target Axin2 (7.7 fold). There was a trend towards increased Snail1 (3.1 fold) expression, as well as several other genes involved in EMT in Geminin cells compared to MG control cells, including: Snail2 (8.8 fold), Twist1 (4.8 fold), and Twist 2 (73.4 fold). E-cadherin expression was decreased 3.3 fold in Geminin cells compared to MG control cells.

A.


Expression Ratio
128
64
32
16
8
4
2
1
-2
-4
-8
-16
*
*
*
*
Oct3/4
FGF5
BMP4
T
Sox17
Claudin6
Sox3

B.

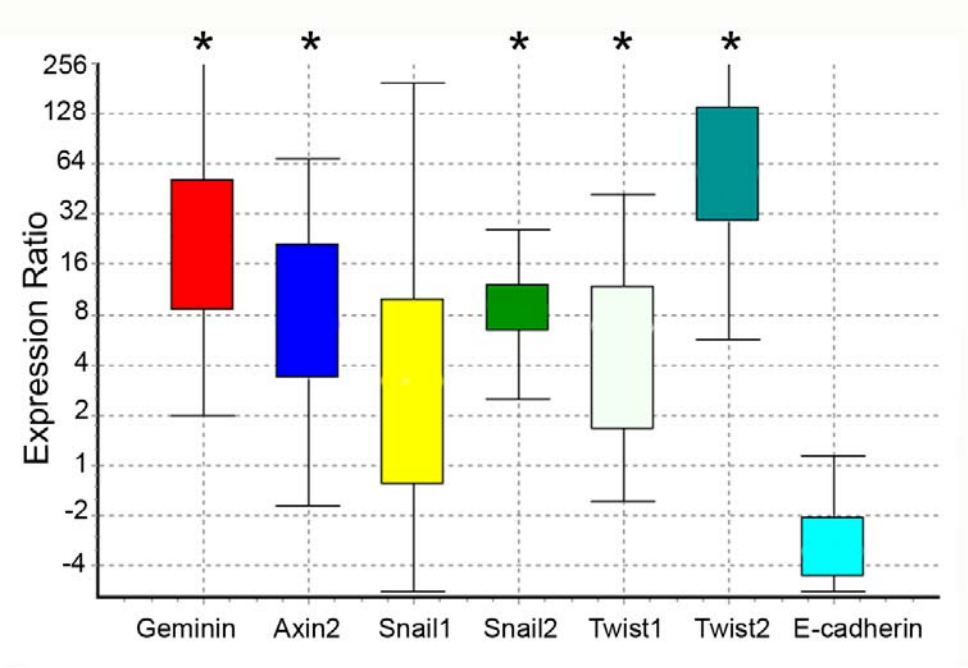
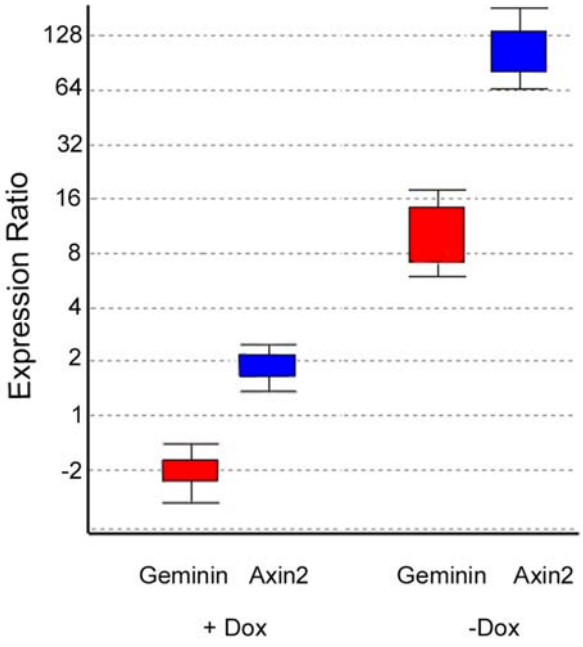

Expression Ratio
256
128
64
32
16
8
4
2
1
-2
-4
*
*
*
*
*
Geminin
Axin2
Snail1
Snail2
Twist1
Twist2
E-cadherin

Figure 3.4.

Geminin over-expression increases Wnt signaling in mESC.
A. Quantitative PCR of the Wnt pathway target gene Axin2 from samples of MG control cells versus Geminin cells grown with and without doxycycline (+/- Dox) for four days demonstrating that activation of the Wnt pathway was dependent on Geminin over-expression. Box and whisker plot produced with REST software (Pfaffl *et al*., 2002). The top and bottom "whiskers" indicate the range while the box indicates the upper quartile, and lower quartile values. Gene expression was calculated relative to β-actin and gene expression of the MG control mESC line was set to 1.
B. Geminin strikingly up-regulated Wnt signaling, nearly to the same level as the control cells treated with the GSK3β inhibitor Chir99021 (Chiron - a Wnt pathway agonist), as assessed by luciferase assays. The mean fold change (TOP Flash/FOP Flash) in the Geminin line was significantly higher than the MG control line without doxycycline (*$p \leq 0.003$) as well as without doxycycline +Chiron (**$p \leq 0.05$) Student's t test (n=5). Error bars represent SEM.

A.


Expression Ratio
128
64
32
16
8
4
2
1
-2
Geminin
Axin2
Geminin
Axin2
+ Dox
-Dox

B.

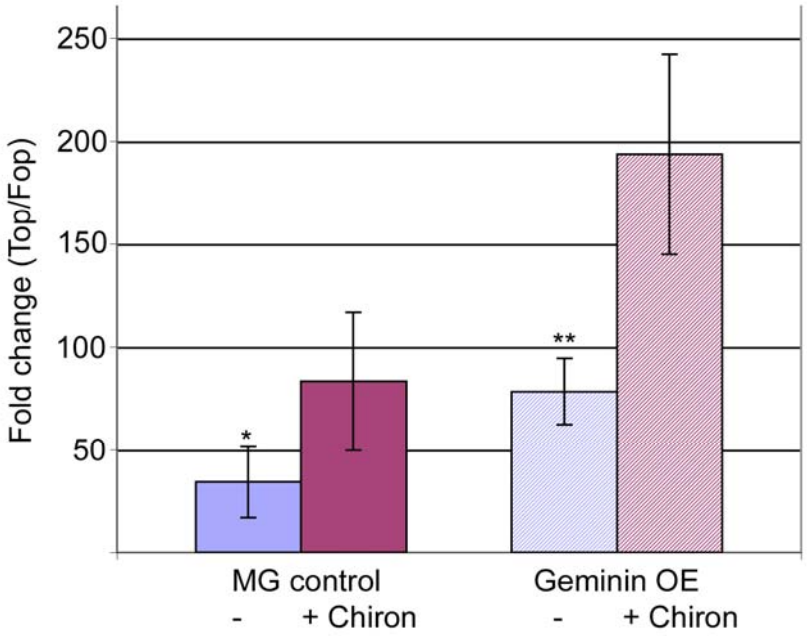
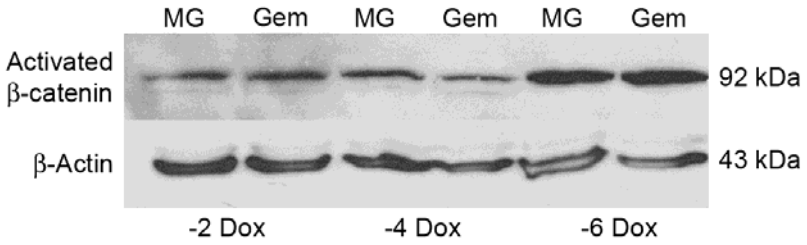
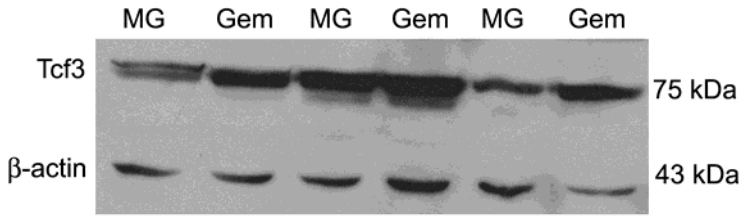

Fold change (Top/Fop)
250
200
150
100
50
*
**
MG control
- + Chiron
Geminin OE
- + Chiron

Figure 3.5
Geminin binds to TLE proteins in mESC.
Control (MG) and Geminin (Gem) mESC were grown two, four, and six days without doxycycline, then protein was harvested and used for Western blotting. There was no change in the expression of activated β-catenin (**A**), of Tcf3 protein, a frequently inhibitory Tcf/Lef factor (**B**), or of TLE proteins that function to inhibit Tcf/Lef function (**C)** with Geminin over-expression. However, there was a relatively weak but consistent interaction of Geminin with TLE proteins in samples grown 4 days without doxycycline as assayed by co-immunoprecipitation using anti-Geminin to pull down and anti-TLE to blot (**D**).

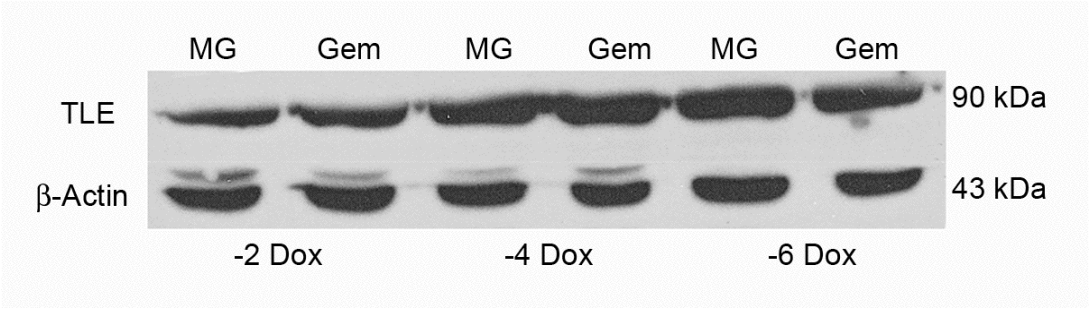
A.


MG
Gem
MG
Gem
MG
Gem
Activated
β-catenin
92 kDa
β-Actin
43 kDa
-2 Dox
-4 Dox
-6 Dox

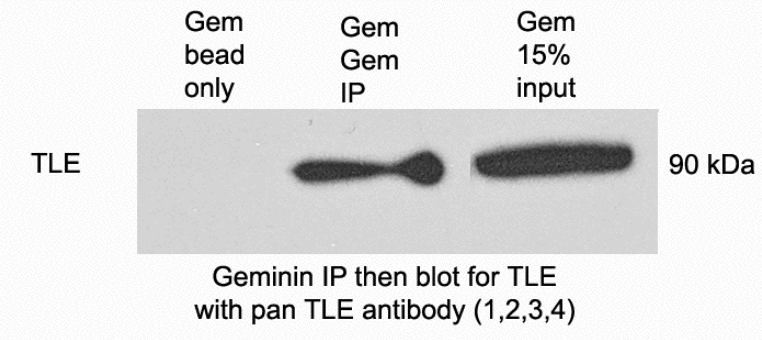
B.


MG
Gem
MG
Gem
MG
Gem
Tcf3
75 kDa
β-actin
43 kDa

C.


MG
Gem
MG
Gem
MG
Gem
TLE
90 kDa
β-Actin
43 kDa
-2 Dox
-4 Dox
-6 Dox

D.


Gem
bead
only
Gem
Gem
IP
Gem
15%
input
TLE
90 kDa
Geminin IP then blot for TLE
with pan TLE antibody (1,2,3,4)

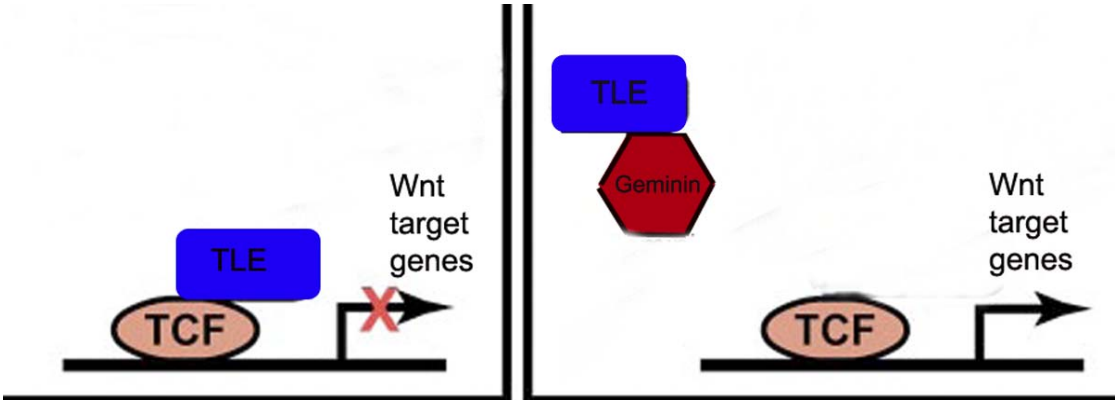
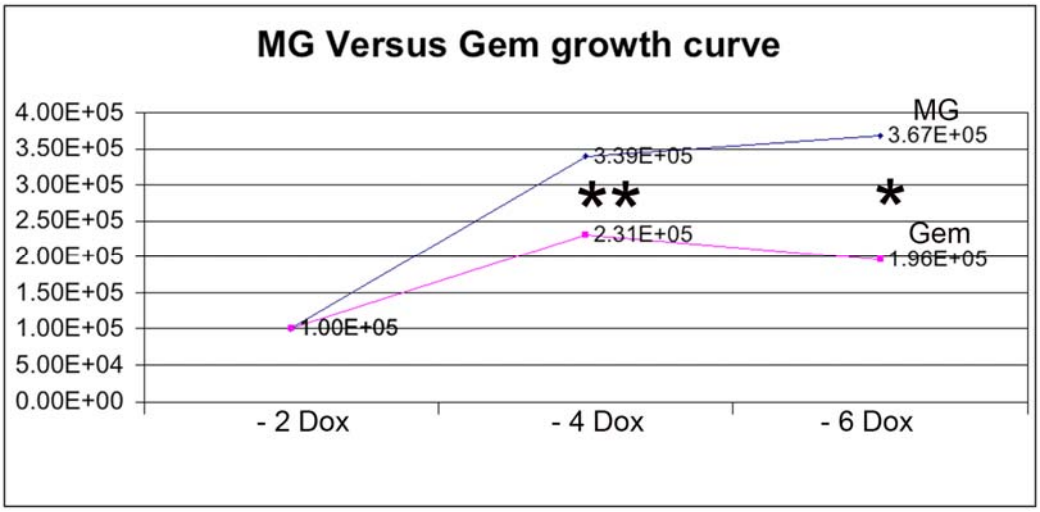


Figure 3.6
Proposed model for Geminin activation of Wnt signaling in the nucleus.
The hypothesis based on the Western blot data is that Geminin binding to TLE proteins in the nucleus prevents them from inhibiting Tcf transcription.

A.



B.

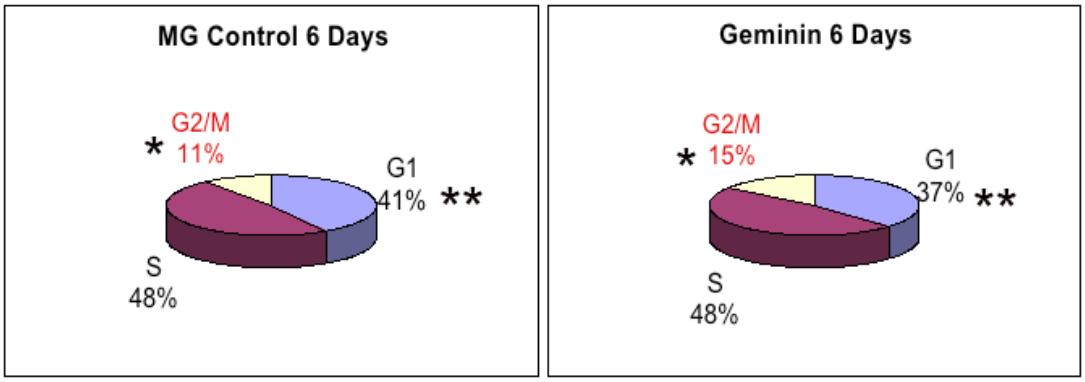
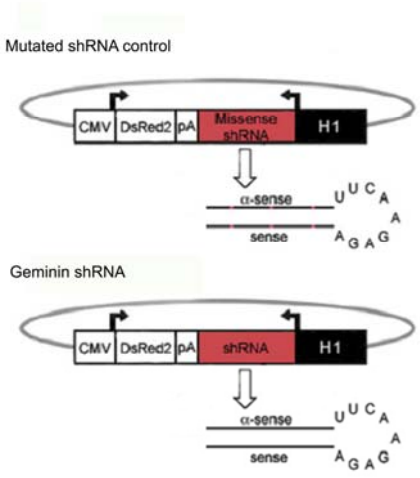


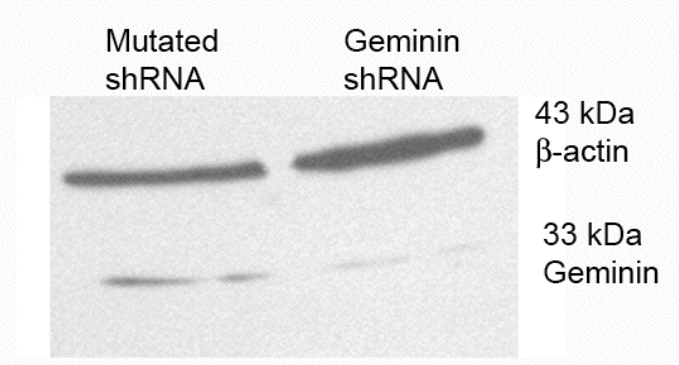
Figure 3.7
Geminin over-expression decreases proliferation and increases the G2/M phase of the cell cycle.
A. MG control (MG) and Geminin (Gem) mESC were grown for two days in the absence of LIF and doxycycline then plated to assay proliferation. Geminin over-expression reduced proliferation at four and six days after doxycycline withdrawl (*$p \leq 0.005$) and **$p \leq 0.0001$ Student's t test, n=3). Experiment performed with the assistance of Dr. Maria Morell.
B. Cell cycle analysis after six days doxycycline withdrawl. Geminin over-expression increased the length of G2/M with a decrease in the length of G1 (*$p \leq 0.05$) and **$p \leq 0.03$ Student's t test, n=3). Experiment performed with the assistance of Dr. Maria Morell.

Figure 3.8
Transfection of mESC with a plasmid containing Geminin shRNA causes cell death by DNA damage.
A. Diagrams of plasmids containing mutated shRNA or Geminin shRNA.
B. Western blot of protein from mESC transfected with mutated shRNA or Geminin shRNA for 24 hours, demonstrating a six fold reduction in Geminin protein.
C. IHC of mESC transfected with mutated or Geminin shRNA for the DNA damage marker phospho histone HA2X indicated that loss of Geminin protein induced DNA damage. Scale bars = 200 μM Experiment performed with the assistance of Dr. Maria Morell.

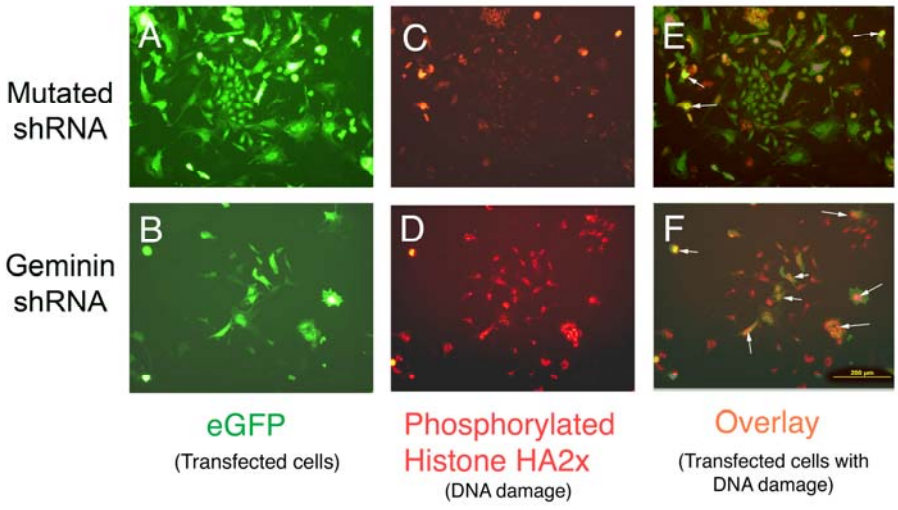
A.


Mutated shRNA control
CMV
DsRed2
pA
Missense shRNA
H1
α-sense
sense
Geminin shRNA
CMV
DsRed2
pA
shRNA
H1
α-sense
sense

B.


Mutated shRNA
Geminin shRNA
43 kDa β-actin
33 kDa Geminin

C.


Mutated shRNA
Geminin shRNA
A
B
C
D
E
F
eGFP
(Transfected cells)
Phosphorylated Histone HA2x
(DNA damage)
Overlay
(Transfected cells with DNA damage)

Chapter 4

Significance and future directions

The canonical Wnt signaling pathway is critically involved in a range of biological processes including: cell proliferation, differentiation, survival, and migration - that ultimately control cell fate during development as well as tissue and organ homeostasis in adult organisms. The main effector of the pathway, β-catenin, plays important roles in cell adhesion by binding to E-cadherin and in cell signaling by interacting with chromatin remodeling factors and members of other key signaling pathways (Barker *et al*., 2001; Heuberger and Birchmeir, 2010; Medici *et al*., 2006; Nawshad *et al*., 2007). Dysregulation of the Wnt pathway frequently leads to organ developmental defects and cancer (Moon *et al*., 2004). Therefore, it is of utmost importance to understand not only the processes in which Wnt signaling plays a role, but how signaling is regulated during development and in adult physiology.

Dynamic changes in Wnt signaling are required for neuronal differentiation of mouse embryonic stem cells

Significance

In the embryo, Wnt signaling is required to specify the mesendodermal lineage from the epiblast (Doble and Woodgett, 2007; Maretto *et al*., 2003; Mohamed *et al*., 2004; Sinner *et al*., 2004; Yamaguchi *et al*., 1999). Our working hypothesis was that inhibition of Wnt signaling during the differentiation of mESC would promote differentiation of the neural lineage. However, initial transient transfection experiments with a dominant negative Tcf4 protein (dnTcf4) demonstrated that this hypothesis was an over-simplification (data not shown). In the absence of Wnt signaling, a majority of mESC differentiated to neural precursors that were then unable to continue differentiation to form primitive neurons.

To examine dynamic changes in Wnt signaling, a mESC line that inducibly expresses a dnTCF4 protein was developed. Expression of the dnTcf4 promoted differentiation of neural precursors that differentiated to primitive neurons only when the transgene was down-regulated allowing Wnt signaling to resume. It is the first report of a sequential role for Wnt signaling in early neuronal differentiation. This is important for the field of regenerative medicine, since the most desirable cells for replacement therapy should be relatively immature and able to respond to the local microenvironment of the transplant site and differentiate into region specific neuronal subtypes. The inducible dnTcf4 cell line appears to be the first pluripotent cell line in which Wnt signaling can be

controlled at the level of target gene expression and should be a useful resource to study the role of Wnt signaling in the differentiation of additional lineages.

Puripotent mESC grown in complete medium (LIF and 10% fetal bovine serum) were unable to activate the Wnt pathway in luciferase assays (data not shown) even when stimulated with a small molecule antagonist of GSK3β (Chir99021) previously shown to increase Wnt signaling (Finaly *et al*., 2004). This implies that pluripotency either requires or enforces a block on canonical Wnt signaling, and further this suggests that one of the earliest steps in mESC differentiation may somehow prime the cells to respond to Wnt signaling. In support of this observation, blocking the Wnt pathway with the dnTcf4 protein during differentiation resulted in an increase of Oct3/4 and FGF5 expression implying that some cells were unable to progress beyond the epiblast stage and to initiate differentiation. Embryos null for *Wnt3*, the key Wnt for primitive streak formation, form mostly a proliferating primitive epithelium that is unable to differentiate (Liu *et al*., 1999). Taken together these data strongly suggest that pluripotency either requires or occurs in the absence of Wnt signaling.

Previous work has suggested that the transcriptionally inhibitory Tcf3/Transducin like enhancer of split (TLE) complex is required for the down-regulation of Nanog expression and differentiation of mESC, although the precise mechanisms were not fully defined (Cole *et al*., 2008; Pereira *et al*., 2006; Tam *et al*., 2008). A requirement for inhibitory Tcf/Lef complexes during the initial steps in differentiation implies a block of β-catenin activation or a restriction in the ability of activated β-catenin to enter the nucleus, and convert Tcf/Lef complexes

to a transcriptional activators. The protein or proteins performing these functions and how these mechanisms could be rapidly inhibited once differentiation has begun remain unidentified. However, these mechanisms could significantly increase our understanding of pluripotency of both the epiblast and mESC and perhaps improve the rate at which pluripotency can be induced from somatic cells.

Future directions

While these data indicate that Wnt signaling is required for the progression from neural precursor to primitive neurons, it is critical to identify the specific targets required for differentiation. Since the promoters of many neurogenic bHLH genes contain Tcf/Lef binding sites (Gao *et al*., 2009; Hirabayashi *et al*., 2004; Israsena *et al*., 2004 Kuwabara *et al*., 2009) neurogenic bHLH genes are very attractive Wnt signaling targets. There is strong evidence that some populations of neuronal precursors require Wnt signaling for proliferation while others require Wnt signaling for differentiation (Israsena *et al*., 2004; Kalani *et al*., 2008 Muroyama *et al*, 2003, Zechner *et al*., 2003). Controlled expansion versus differentiation of neuronal precursor cells will be required for the treatment of human disease such as stroke and spinal cord injury.

Alterations in Wnt signaling might eventually be useful *in vivo* to expand or activate neural stem cells within their niche. For example, since the subventricular zone (SVZ) of the adult brain contains Wnt responsive neural stem cells (Adachi *et al*., 2007), it would be advantageous to be able to expand,

mobilize, and hopefully differentiate functional neurons and oligodendrocytes from these neural stem cell populations after injury. One thus far unexplored possibility is that different Wnt ligands have different effects on neural stem cells. If verified, this would greatly simplify expansion versus differentiation of neural stem cells *in vivo* since one might be able to over-express one Wnt ligand to expand neural progenitors and then over-express another Wnt ligand to induce differentiation of the expanded neural progenitor population.

It would also be informative to inhibit Wnt signaling extracellularly with a protein such as Dikkopf-1 (Dkk-1) followed by intracellular stimulation of Wnt signaling with Chir99021. While Dkk-1 has been employed by other investigators to promote neuronal differentiation of mESC (Kong and Zhang, 2009; Verani *et al*., 2006); given our results it is likely that they did not obtain maximal neuronal differentiation or this paradigm promoted differentiation of a particular neuronal subtype. If different results with Dkk-1 compared with dnTcf4 are observed, additional Tcf/Lef inducible mESC lines could be developed to explore the likelihood that different Tcf/Lef proteins have different target genes. Alternatively, the dnTcf4 line could be differentiated with varying increments of Doxycycline in an attempt to replicate a partial Wnt signaling block as is potentially seen when Dkk-1 is over-expression.

When EBs were differentiated with Wnt signaling blocked via expression of the dnTcf4 protein, there was an increase in the differentiation of neural progenitors; however, in this case, relieving the Wnt signaling block did not result in differentiation of primitive neurons even in serum free medium lacking BMP

proteins. It is likely that the high cell density present in EB cultures inhibited terminal differentiation of these cells by lateral inhibition. When a neural progenitor differentiates into a neuron it induces Notch pathway signaling in neighboring cells, thereby inhibiting their differentiation (Kageyama *et al*., 2008). Inhibition of Notch signaling in conjunction with the de-repression of Wnt signaling in both monolayer culture and in EB cultures would be a useful approach to determine if neuronal differentiation was blocked by high cell density found within EBs. However, it also remains formally possible that a specific Wnt ligand may be required to promote neuronal differentiation and this ligand may not be produced by the mESC in the EB cultures.

Overall, research with the inducible dnTcf4 cell line has revealed a surprising and important requirement for dynamic changes in Wnt signaling that were not obvious from mouse Wnt pathway null animals or mESC.

Geminin promotes an epithelial to mesenchymal transition and mesendodermal differentiation in an embryonic stem cell model of gastrulation

Significance

Geminin has been characterized as a dual function protein that prevents endoreduplication during cell division and promotes neural differentiation by inhibiting BMP expression (Kroll *et al*., 1998). In addition, Geminin can also bind transcription factors and chromatin remodeling proteins in the nucleus to inhibit their function (Del Bene *et al*., 2004; Luo *et al*., 2004, Kim *et al*., 2006; Roukos *et*

al., 2007; Seo *et al*., 2006). It has also been suggested that Geminin may play a role in preventing the differentiation of neural precursors to neurons by promoting proliferation and blocking differentiation (Seo *et al*., 2006). Based on these observations, the original working hypothesis for this research was that over-expression of Geminin would promote the differentiation of mESC to neural precursors. However, when Geminin over-expressing cells were grown in a monolayer neuronal differentiation paradigm, we observed no obvious differences in neuronal differentiation compared to control cells.

To determine if Geminin played a role in lineage differentiation, an embryoid body differentiation paradigm was employed. Geminin EBs rapidly adhered to a Petri dish and individual cells immediately migrated out from adherent EBs. Lineage analysis determined that Geminin promoted mesendodermal differentiation at the expense of neural lineages. Geminin down-regulated E-cadherin expression - a hallmark of the epithelial to mesenchymal transition (EMT). This is the first report that Geminin is implicated in mesendodermal differentiation and EMT.

Luciferase assays demonstrated that Wnt signaling was significantly increased with Geminin over-expression. This is the first report of Geminin increasing Wnt signaling. Our current hypothesis, based on results of co-immunoprecipitation experiments is that Geminin increases Wnt signaling by binding and inhibiting the function of TLE proteins, which is a novel result.

Finally, Geminin expression is frequently up-regulated in cancer cells and increased Geminin expression often correspond with poor prognosis (Nishihara,

et al., 2009; Wohlschlegel *et al*., 2002; Xouri *et al*, 2004). It has been difficult to reconcile how a gene that functionally inhibits DNA replication and thereby inhibits proliferation could produce more aggressive cancers. Based on our results, we hypothesize that Geminin could promote metastasis by promoting EMT thereby resulting in aggressive and lethal tumors. In order to test this hypothesis, Geminin could be over-expressed in both normal epithelial cells and in non-metastatic cancer cells and those cells could be observed for down-regulation of E-cadherin and induction of EMT. Finally, down-regulation of Geminin could prove to be a very effective treatment for cancer. Loss of Geminin protein could both limit metastasis of tumors as well as inducing cell death of rapidly dividing cancer cells due to endoreduplication.

Overall, this work has identified an exciting new function for Geminin in promoting mesendodermal differentiation and EMT via the up-regulation of Wnt signaling.

Future Directions

While we propose that binding of Geminin to TLE proteins inhibits their ability to complex with Tcf/Lef genes and inhibit transcription, data fully supporting this hypothesis are in progress. It is now critical to determine if Geminin/TLE binding decreases Tcf/Lef/TLE binding. Geminin over-expression is predicted to eliminate or dramatically reduce the co-immunoprecipitation of Tcf3/TLE protein complexes. In addition, over-expression of TLE1 in Geminin

over-expressing cells (in progress) is predicted to block Wnt pathway stimulation, mesendodermal differentiation, and EMT.

Finally, it is important to determine if Geminin targets one specific TLE or all four TLEs. While Western blot demonstrates that there is no decrease in pan TLE protein following Geminin over-expression, it is possible that there is a decrease in one specific TLE that was not detected with the pan TLE antibody.

The dual functions of the Geminin protein have been attributed to two separate protein domains, a C-terminal "cell cycle domain" and an N-terminal "neuralizing domain" (Kroll *et al*., 1998). Currently we are unable to comment on which domain is required for EMT and mesendodermal differentiation of mESC since it is likely to require both changes in gene expression and cell cycle exit (Grosshans and Wiechaus, 2000; Liu *et al*., 2009; Seher and Leptin, 2000). Over-expression of the N-terminal domain in mESC is in progress to determine its role in cell cycle progression and mesendodermal differentiation or EMT. Previous results with the N-terminal domain of Geminin in *Xenopus* embryos suggested that neural differentiation was not dependent on changes in cell division (Kroll *et al*., 1998); therefore, the prediction would be that mesendodermal differentiation would not depend on changes in the cell cycle and will obtain similar results when the N-terminus is over-expressed as when the whole protein is over-expressed.

Currently we have observed that Geminin over-expression can increase Wnt signaling but have been unable to determine if Geminin is required for Wnt signaling due to the lethality of Geminin protein down-regulation. Over-

expression of the cell cycle domain might be used to prevent endoreduplication and subsequent cell death in cells exposed to the Geminin shRNA. Depending on the outcome of these experiments, it will also be important to determine which domain is required to promote Wnt signaling and further test if that domain interacts with TLE proteins.

Geminin null mouse embryos die due to endoreduplication during prior the formation of the inner cell mass (Hara *et al*., 2006; Gonzalez *et al*., 2006), precluding the analysis of Geminin function in gastrulation and neural differentiation, therefore, it would be beneficial to create tissue specific knock-outs with lineage restricted Cre drivers. A Sox3-Cre could be used to eliminate *Geminin* from the early neural ectoderm and a Wnt3-Cre could be used to eliminate *Geminin* in the primitive streak. These tissue specific knock-outs should bypass the early lethality in order to determine Geminin's role in neural differentiation and gastrulation *in vivo*.

Since Geminin is restricted to dividing cell populations in the adult, it will be critical to examine its role in various stem cell niches, such as the neural stem cell subventricular zone, the crypt of the small intestine, and bone marrow, where Geminin has been reported to be expressed. Elucidating Geminin's function in these stem cells - either controlling proliferation or differentiation, or more likely controlling both functions will further the understanding of the transition from proliferation to differentiation and perhaps contribute to the understanding of how tissue stem cells are maintained or activated

ESC as a model system to study differentiation

It is clear from these investigations that there is an optimal cell density that permits differentiation. Low cell densities promote apoptosis and high cell densities, likely due to the expression of inhibitors of differentiation (e.g. LIF) can inhibit differentiation. In this regard, it is also clear that Tet-off systems have significant advantages over Tet-on systems because at high density, not all cells within the culture will have be accessible to doxycycline, resulting in variations in the level of transgene expression as well as variation in the timing of transgene induction.

Another important observation made during the differentiation of mESC is that blocking differentiation of one lineage often seems to result in increased differentiation of the other lineages. Blocking Wnt signaling reduced mesendodermal differentiation and simultaneously increased neural precursor differentiation. Promoting Wnt signaling by Geminin over-expression increased mesendodermal differentiation with a concomitant reduction of neural precursor differentiation. Similar observations have been made during mouse embryo development. Reduction of Wnt signaling in *LRP* mutant embryos reduced mesendodermal differentiation but increased neural ectoderm fomation (Kelly *et al*., 2004) while increasing Wnt signaling increased conversion of epiblast to mesendoderm resulting in a loss of neural ectoderm (Popperl *et al*., 1997; Kemler *et al*., 2004). Therefore, blocking differentiation of unwanted lineages during ESC differentiation is a valid method to either enhance or simply induce differentiation of a target lineage.

The differentiation paradigm itself clearly influences the outcome of the ESC assay. For example, the effects of Geminin might have been overlooked if the EB assays had not been carried out. In assaying the role of a protein differentiation should be attempted in multiple assays, since each assay provides a slightly different environment to observe the role of the protein of interest. Monolayer assays at low density will maximize cell autonomous functions of a give protein while EB assays can be used to determine a protein's role during tri-lineage differentiation or in developmental events that are triggered by cell-cell contact or high cell density. Furthermore, mESC can be transplanted into the easily accessible chick embryo to determine if a mESC population is competent to integrate into a developing tissue.

ESC are an excellent model system to examine the role of gene expression in lineage differentiation. Correlating gene expression patterns and knock-out phenotypes in the mouse embryo informs the temporal and special requirements for lineage specific differentiation that can in turn be tested in the simplified model of ESC differentiation. In addition, downstream genes can be identified in controlled differentiation conditions.

Expression of factors suspected to regulate pluripotency in ESC and the epiblast can be tested for their ability to reprogram differentiated cells to a pluripotent state, adding to the understanding of how pluripotency is achieved and maintained. Core transcription factors in the embryo required for a given fate choice can be tested by direct reprogramming to determine if one differentiated cell type can be altered to another differentiated cell type,

establishing gene hierarchies in development. In combination, all three models should allow for rapid progress in understanding embryonic development and the conversion of pluripotent cells to specific lineages.

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