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

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ABSTRACT

Embryonic stem cells (ESC) and the epiblast share a similar gene expression profile and an attenuated cell cycle, making them an accessible and tractable model system to study lineage choice at gastrulation. Differentiation of the epiblast and ESC to the mesendodermal lineage has been shown to rely on Wnt/β-catenin signaling; which counterintuitively, is also required to inhibit differentiation and maintain pluripotency. To examine these seemingly contradictory roles, we developed a mouse ESC (ESC) 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 derepressed, demonstrating a sequential requirement for Wnt signaling in lineage differentiation. To determine if Wnt/β-catenin signaling is similarly required at sequential stages of neural differentiation in the intact embryo, we delivered shRNA targeting β-catenin to pregnant mice on E5.5 of development. Blocking canonical Wnt signaling during post-implantation development increased the number of neural precursors which failed to differentiate to mature neurons, and produced defects of embryonic axis elongation, neurulation and neural tube closure that phenocopy the β-catenin null embryo. These results demonstrate that lineage differentiation relies on sequential repression and derepression of critical signaling pathways involved in maintaining pluripotency versus differentiation.

Introduction

Mouse embryonic stem cells (ESC) 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. ESC can also be used as a model of lineage choice in development, providing a systematic simplification of this extraordinarily complex and otherwise inaccessible process. Pluripotency of ESC, in the absence of a feeder layer, can be maintained by leukemia inhibitory factor (LIF) signaling through Stat-3 (Smith et al., 1998; Williams et al., 1988). Blocking or stimulating other signaling pathways including: the BMP, PI3 kinase/AKT, MAP-ERK kinases, and Wnt pathways has also been suggested to be sufficient to maintain ESC pluripotency and conversely, manipulation of these pathways has also been reported to promote lineage specific differentiation of ESC (Lee et al., 2009; Paling et al., 2004; Qi et al., 2004; Watanabe et al., 2006: Ying et al., 2003). Untangling these opposing results requires rigorous and likely reversible control of each 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 or repress gene expression (Blauwkamp et al., 2008). Wnt signaling has been implicated in many diverse and seemingly opposing processes such as self-renewal and proliferation versus differentiation of both developing as well as adult tissues (Arce et al., 2006; Chien et al., 2009; van Amerongen and Nusse, 2009). 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 ESC (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., 2006; Ogawa et al., 2006). In other contexts, Wnt pathway activation promoted rather than inhibited differentiation of ESC (Gadue et al., 2006; Lindsley et al., 2006; Nakanishi et al., 2009; 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

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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 Amerongen and Nusse, 2009). During gastrulation, Wnt signaling is critically involved in establishing the primitive streak and promoting the epithelial to mesenchymal transformation (EMT) required for mesendodermal differentiation of 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. In addition, differentiation of neural ectoderm both in the embryo as well as during ESC differentiation has been reported to result from the inhibition of Wnt signaling (Aubert et al., 2002; Cajanek 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 ESC 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 (Zhang et al., 2010), 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; Kalani et al., 2008; Kuwabara et al., 2009; Lagutin et al., 2003; Muroyama et al., 2002; 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 ESC, we developed a tetracycline inducible (Masui et al., 2005) dominant negative Tcf4 (dnTcf4) expressing mouse embryonic stem cell line. Using these cells, it is possible to block and then relieve the repression on Wnt signaling during ESC 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) in which neural ectoderm differentiates at the expense of mesendoderm. There is then a requirement for active Wnt 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 examine the requirement for Wnt signaling at sequential stages of neuronal differentiation in the intact embryo, we delivered a shRNA targeting β-catenin to pregnant dams to reduce Wnt signaling and observed a significant increase in differentiation of Sox3 positive neural precursors but a decrease in their conversion to mature neurons, as well as defects of embryonic axis elongation, neurulation and neural tube closure, that phenocopy the null embryo.

Results

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

To explore the role of Wnt signaling in multi-lineage differentiation of ESC, a cell line was created that inducibly expresses a dominant negative Tcf4 (dnTcf4) protein (Tet-off) thereby inhibiting canonical Wnt signaling (Figs. 1, 2). A total of 24 dnTcf4 and 24 control lines (puromycin resistant cells that inducibly express only Venus yellow fluorescent protein) were cloned and expanded for further study. Proper targeting of all chosen lines was verified by loss of hygromycin resistance. Several lines of each were selected for further study based on doxycycline regulated dnTcf4 protein and/or Venus protein expression. In the presence of doxycycline, the dnTcf4 cell line divides and grows similarly to the control cell line (Supplemental Fig. 1). Control (C) and dnTcf4 (dn4) cells express all four Tcf/Lef transcription factors (Fig. 2A). The dnTcf4 mRNA is highly expressed within 24 h of doxycycline withdrawal and is three-fold down-regulated when doxycycline is added back to the culture medium for an additional 24 h. Neither doxycycline exposure nor expression of the dnTcf4 affected expression of either native Tcf4 or the three other Tcf/Lef transcription factors (Fig. 2A). The transgenic dnTcf4 protein is detectable at four and six days of doxycycline withdrawal and disappears completely following reintroduction of doxycycline for 48 h (Fig. 2B).

The dnTcf4 protein reduced canonical Wnt signaling approximately 40 fold in the TopFlash luciferase assay (Fig. 2C), and after reintroduction of doxycycline for 24 h, the levels of Wnt signaling were restored to nearly normal in dn4 cells. More importantly, the dnTcf4 inhibited Wnt signaling even in the presence of a Gsk3β inhibitor (Chir99021, Stemgent) previously shown to promote Wnt signaling (Finlay et al., 2004). These data indicate that expression of the dnTcf4 transgene is controlled by doxycycline, and functionally inhibits Wnt signaling.

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

To explore the role of Wnt signaling in the neuronal differentiation of ESC, control and dnTcf4 expressing cells were plated in a serum free monolayer differentiation assay in neural permissive media with or without doxycycline. 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 neural precursor cells after as little as 4 days (Figs. 3, 4), but there was little neuronal differentiation even if the cells were kept in culture as long as 8 days. These results are not attributable to alterations in cell density as proliferation of dn4 cells and control cells were similar as assessed by IHC localization of anti-phosphohistone H3 (Fig. 5A; Supplemental Fig. 1), and cell behavior. FACS analysis indicated that there was a statistically significant increase in the number of Sox3 positive cells in dnTcf4 versus control cells (Fig. 4A) that was particularly robust when the transgene was induced early in differentiation. When the dnTcf4 protein 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 βIII tubulin positive neurons in the cultures (Figs. 3, 4). Culture of the dn4 cells with doxycycline for 6 days resulted in differentiation that was very similar to control cells (Supplemental Fig. 1). Because FACS sorting of Tuj1 positive cells resulted in high levels of background signal that did not accurately reflect differentiation, we used ImageJ software to determine the mean number of green (βIII tubulin positive) pixels in each condition. Data are presented as mean number of Tuj1 pixels/mean number of Hoechst pixels (nuclei). Consistent with our IHC results, ImageJ analysis indicated that there was maximal differentiation of primitive neurons when Wnt signaling was initially inhibited, then released for the final differentiation of precursors to neurons (Fig. 4B).

Wnt signaling is required for mesendodermal differentiation in a model of gastrulation

To investigate the role of Wnt signaling in a more complex three-dimensional multi-lineage differentiation assay, we employed an