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

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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*., 1988; 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*., 2006; 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

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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*., 2005; Ogawa *et al*., 2006). In other contexts, Wnt pathway activation promoted rather than inhibited differentiation of ESC (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 (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; 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 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; Kuwbara *et al*., 2009; Lagutin *et al*., 2002; 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 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.

Materials and methods

ES cell culture and differentiation

Undifferentiated ESCs 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 μM β-mercaptoethanol (Sigma) with 5 ng/ml LIF (Chemicon). Neural-permissive culture conditions were achieved by plating cells at low density (2×10^4 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 μM retinoic acid (Sigma), and 0.05% Knock-out serum replacement (Invitrogen). To form embryoid bodies (EBs), cells were plated at 1×10^6 cells per 6cm dish in non-adherent (Petri) dishes in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 μM β-mercaptoethanol (Sigma) with or without doxycycline for 4 days, then transferred to gelatin coated 12 well plates for an additional 2 days of culture. Cells were maintained at 37°C with 5% CO_2.

Inducible dnTcf4 ESC line

We obtained the MGZRTcH2 ESC 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 locus. 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 both positive and negative selection and ensuring the identification of cell lines with random incorporation of the exchange vector (Figure 1).

The dnTcf4 cDNA was a 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. The dnTcf4 and control exchange vectors were co-transfected with pCAGGS-CRE into MGZRTcH2ES cells using Lipofectamine Plus, following the manufacturer's recommendations. Selection of inducible dnTcf4 and control cells was carried out using 2μg/ml puromycin with negative selection of improperly targeted colonies using 100μg/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.

Immunohistochemistry

For immunohistochemical (IHC) localization of cell type-restricted proteins, 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°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; rabbit anti-phosphohistone 3, Millipore, 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 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 (12 wells; 120 fields) were analyzed for each cell line. Data were averaged and analyzed using Student's t test.

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°C. Cells were permeabilized with 0.2% saponin in FACS buffer for 10 minutes at 4°C, incubated with rabbit antibodies against Sox3 (1:1000) for 30 minutes at 4°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 Becton Dickinson FACSCalibur.

Western blot

ES cells were lysed in Laemmli or RIPA buffer, cell debris pelleted by centrifugation, protein loaded onto 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), or mouse anti-β-catenin (1:1000, Millipore) and mouse anti-β-actin (1:10,000, Sigma) primary antibodies in 5% milk powder/TBST overnight at 4°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. Quantification of band intensity was carried out using ImageJ software (NIH).

Quantitative and semi-quantitative RT PCR

RNA was harvested with Trizol (Invitrogen) following the manufacturer's protocol and genomic DNA was removed by DNAse (Sigma) digestion. 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 1:2 and 1μl was used per reaction with Abgene SYBR green master mix (Thermo-Fisher). 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) and displayed as box and whisker plots. Semi-quantitative PCR was performed as described for the genomic DNA test above. Primer sequences and detailed reaction conditions are available upon request.

Top/Fopflash assay

Control and dnTcf4 cells were grown in DMEM (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), 50 mM HEPES (Sigma), and 1 μM 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 used as a transfection control. After 24 hours, cells were lysed with passive lysis buffer and subjected to the dual-luciferase assay (Promega) following the manufacturer's instructions. Samples were quantified with a Veritas microplate luminometer (Turner Biosystems). Data are expressed as the ratio of TOPFlash relative to Renilla over FOPFlash relative to Renilla.

Post-implantation mouse embryos

Wnt reporter mice (Mohamaed *et al*., 2004) were obtained (from Dr. Philip Gage, University of Michigan) and bred to generate time pregnant females. On day 5.5 post-coitum, pregnant females were injected via the tail vein (Gratsch *et al*., 2003) with 200 μl Ringer's saline containing 10 μg of two separate plasmids containing a short hairpin RNA against β-catenin or containing a mutated short hairpin RNA that does not target any sequence in the mouse genome (Figure 6A). Prior to use in pregnant dams, we verified that each construct efficiently decreased β-catenin protein in ESC by western blot (Figure 6B). Transfection with shRNAs produced a 40-64% reduction in β-catenin protein compared with the mutated control as quantified by ImageJ software. To harvest embryos, dams were killed by cervical dislocation and embryos dissected from uteri and decidua. Membranes were removed and embryos photographed using a Wild stereomicroscope, then allocated for analysis by

scanning electron microscopy, X-Gal staining, O-Nitrophenyl -β-D-Galctopyranosidase (ONPG) analysis (quantification of β-Galactosidase activity), flow cytometry, 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°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°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°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 μl 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 × g for 15 minutes at 4°C. Protein concentration was determined by the Bradford protein assay (BioRad). Total embryo protein was added to 400 μl of PM-2 buffer and samples were incubated at 37°C for 15 minutes. Next, 100ml of prewarmed ONPG solution (4mg/ml O-Nitrophenyl -β-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, 250μl Na_2CO_3 was added to stop the reaction, stop time recorded, and the absorbance was read at 420nm in a spectrophotometer (BioRad SmartSpec 3000). β-galactosidase activity was calculated with the equation: Units = (380 × 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 antibody (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. They 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°C in rabbit anti-Sox3 (1:1000) and mouse anti-NeuN (1:500). They 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 Olympus 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 processing to increase antibody penetration. Embryos were permeabilized further 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°C in rabbit anti-Sox3 (1:2000) and mouse anti-NeuN (Chemicon, 1:1000) then washed 2 hours at 4°C followed by 3 hours at room temperature in PBSMTX. Embryos were incubated overnight at 4°C in secondary antibodies (1:200) conjugated to horseradish peroxidase (Jackson Immunoresearch) then washed 2 hours at 4°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 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 (Figure 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 Figure 1). Control (C) and dnTcf4 (dn4) cells express all four Tcf/Lef transcription factors (Figure 2A). The dnTcf4 mRNA is highly expressed within 24 hours of doxycycline withdrawal 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 (Figure 2A). The transgenic dnTcf4 protein is detectable at four and six days of doxycycline withdrawal and disappears completely following reintroduction of doxycycline for 48 hours (Figure 2B).

The dnTcf4 protein reduced canonical Wnt signaling approximately 40 fold in the TopFlash luciferase assay (Figure 2C), and after reintroduction of doxycycline for 24 hours, 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 (Finaly *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 (Figures 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 (Figure 5A; Supplemental Figure 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 (Figure 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 (Figures 3,4). Culture of the dn4 cells with doxycycline for 6 days resulted in differentiation that was very similar to control cells (Supplemental Figure 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 tublin 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 (Figure 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 embryoid body (EB) differentiation paradigm. Cells were plated in non-adherent dishes (in the absence of LIF, with and without doxycycline but with serum) promoting the formation of EBs and thereby inducing trilineage differentiation that has been widely employed as a model of lineage differentiation at gastrulation. Cells were grown as spheres for four days then plated 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 5-fold after 6 days in culture (Figure 5B), confirming a functional Wnt signaling blockade. The block in canonical Wnt signaling caused a delay or stalling of differentiation with increased expression of the ESC and epiblast markers Fgf5 (6-fold) and Oct3/4 (6-fold; Figure 5 A,B). Decreased expression of the endoderm markers Foxa2 (Figure 5A) and Sox-17 (4.5-fold; Figure 5B), and a trend toward decreased expression of the mesoderm marker T (2-fold) also indicated a reduction in mesendodermal differentiation, while primitive neural ectoderm differentiation increased as identified by Sox3 expression (6-fold; Figure 5A,B), but Tuj1 did not change. Addition of doxycycline to control and dn4 cells during EB culture eliminated changes in differentiation seen when Wnt signaling was blocked (Supplemental Figure 1). As observed in dispersed cultures, the dnTcf4 expressing cells were unable to progress to βIII-tubulin positive neurons in the continued Wnt signaling blockade. When doxycycline was reintroduced midway through differentiation, there was no increase in the number of βIII-tubulin positive neurons, likely because of the crowded conditions within each EB (data not shown). These data indicate that in dense culture conditions, blocking Wnt signaling delays differentiation and promotes differentiation of neural precursors at the expense of mesendoderm.

β-catenin knock-down affects survival, gastrulation, and neuronal differentiation of post-implantation embryos

To examine the role of Wnt signaling in lineage differentiation *in vivo*, we delivered shRNAs targeting β-catenin via the tail vein of pregnant mice on E5.5 of gestation; n=26 litters exposed to scrambled control plasmid, and 27 litters to β-catenin shRNA. Knock-down avoids the peri-implantation lethality of conventional knock-out approaches and results in graded knock-down of gene expression allowing the correlation of phenotype and level of 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% in β-catenin shRNA embryos on E7.5. Since Wnt signaling is required first to position the primitive streak then for EMT at gastrulation, we examined the displacement of primitive endoderm (PE) by newly formed (embryonic) definitive endoderm (DE) in individual embryos and using scanning electron microscopy (SEM). On E7.5, 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 7A.A). Control embryos had typically expanded in the proximal-distal, but not anterior-posterior axis at this stage of development. In embryos exposed to β-catenin shRNA, gastrulation was slowed, DE typically progressing only 1/4 to 1/3 of the way along the proximal-distal axis (Figure 7A.B). 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 endoderm. We also measured the distance the DE had migrated in individual embryos. In control embryos (n=27) the distance the DE had migrated was 1.75 ± 0.4 vs 0.45 ± 0.1 in embryos exposed to the shRNAs (n=51 embryos), $p \leq 0.003$, Student's t-test.

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 7A.C). In β-catenin shRNA targeted embryos at this stage, there was a range in β—gal expression from slight to undetectable levels (Figure 7A.D), confirming the range of knock-down, and presumably inhibition of Wnt signaling.

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 7A.E). Gene targeted embryos were characterized by extremely shortened PD axes, often with an expanded AP axis and shortened posterior region giving them a “rocking horse” appearance (Figure 7A.F). 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 smooth. 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 characteristic eversion of neural tissue. However, in extremely shortened embryos, the entire neural tube had often failed to close.

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 in 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 7A.G). In targeted embryos, there was a consistent reduction in the expression of β-gal—particularly in the first branchial arch, midbrain and heart (Figure 7A.H).

To quantify Wnt signaling activity in control versus β-catenin shRNA embryos, we used the O-nitrophenyl-beta-D- galactopyranoside (ONPG) β-galactosidase assay in Wnt indicator mice. In control embryos (6 litters, 40 embryos) β-galactosidase activity was significantly higher 0.29 ± 0.22 versus 0.13 ± 0.04 ($p \leq 0.03$, Student's t test) than in β-Catenin shRNA treated embryos (8 litters, 59 embryos) demonstrating significant inhibition of Wnt signaling.

Immunohistochemisty and FACS analysis

Since Wnt signaling is required for neuronal differentiation of proliferating precursors, we examined the conversion of neural precursor cells to neurons using immunohistochemistry and FACS analysis in individual 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 7B,C) in β-catenin shRNA exposed embryos compared with control embryos exposed to a mutated shRNA. Immunohistochemical localization of NeuN demonstrated a clear decrease in differentiation of immature neurons that were beginning to stratify within the neural ectoderm in conditions of reduced Wnt signaling (Figure 7C). To quantify these changes, we dissected the CNS from E10.5 embryos, dissociated each to a single cell suspension, divided each embryo into two equal samples, and then carried out IHC for Sox3 or NeuN. Cell number from the central nervous system of individual embryos was quantified in FACS analysis; 40 scrambled and 43 β-catenin shRNA exposed embryos from 12 litters. There was a significant increase in Sox3 positive cells in embryos exposed to the β-catenin shRNA compared to control embryos (20.7 ± 6.3 versus 26.5 ± 7.2, $p \leq 0.004$) and a corresponding decrease in NeuN positive cells (7.8 ± 1.6 versus 6.2 ± 1.3, $p \leq 0.004$), 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

To probe the role of Wnt pathway activation in lineage differentiation, we derived a tetracycline regulated dnTcf4 expressing ES 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 highly similar promoter sequences (Arce *et al*., 2006; Kelly *et al*., 2011) 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). However, in ESC Tcf3 retains its repressor function even in the presence of nuclear β-catenin (Pereira *et al*., 2006) and acts to regulate levels of genes involved in pluripotency. Rather than using extracellular inhibitors to abrogate Wnt signaling, we chose to employ a dnTcf4 protein, which lacks the β-catenin binding domain, to block all transcription downstream of the Wnt pathway.

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 (Gaulden and Reiter, 2008). However, it is clear that additional variables including cell density and other unidentified factors in supplements and serum also play a role in controlling differentiation. Further, 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 ESC 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),

despite observations that constitutive over-expression of a dnTcf4 and dnLef1 abrogated neuronal differentiation in vitro as well as in teratomas (Kelly *et al*., 2011). However, monolayer culture of the dnTCF4 cells in neural permissive media absent doxycycline (to induce transgene expression) promoted differentiation of Sox3 positive neural precursors, but these cells were unable to differentiate further into Tuj1 positive neurons, unless Wnt signaing was restored.

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), where its source is likely astrocytes. 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 NeuroD1, are transcriptional targets of the canonical Wnt pathway (Hirabayashi *et al*., 2004; Israsena *et al*., 2004; Kuwabara *et al*., 2009). Therefore, while Wnt signal inhibition may initially be required to increase neuronal precursor differentiation by inhibiting mesendodermal lineage 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.

To examine the role of Wnt signaling in tri-lineage differentiation at gastrulation, we employed an embryoid body differentiation paradigm. When ESC 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 stratified aggregates with some resemblance to the early post-implantation 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, induction of the dnTcf4 protein resulted in sustained expression of the pluripotency gene Oct3/4 and the epiblast marker Fgf5, likely identifying cells that retain an epiblast phenotype and are unable to further differentiate due to a 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. Surprisingly, in this EB model these cells failed to differentiate into Tuj1 positive neurons when Wnt signaling was de-repressed. Since neuronal differentiation is exquisitely sensitive to cell density (Otero *et al*., 2004), the high-density microenvironment of the EB likely inhibited neuronal differentiation possibly via Notch signaling.

Our 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. Deletion of core components of the Wnt pathway such as 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 an 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), formed multiple axes (multiple primitive streaks), and were characterized by increased differentiation of mesendoderm at the expense of neural ectoderm. It is clear both in ESC and the early embryo that Wnt signaling is required for mesendodermal differentiation and in its absence more of the embryonic epiblast can be converted to neural ectoderm.

To examine the role of Wnt signaling neural differentiation in the intact embryo, shRNA plasmids directed against β-catenin were delivered to pregnant dams, thereby eliminating Wnt signaling at the nuclear level in embryos during early post-implantation development. Using knock-down in lieu of 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 knock-down embryos exhibiting gastrulation abnormalities and 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 our observations in ESC, when Wnt signaling was down-regulated, neural ectoderm precursors failed to differentiate to NeuN positive neurons. It is appears that β-catenin function is required both in the embryo and in ESC to maintain epithelial characteristics of the cells (e.g., Li et al., 2010).

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 sequential requirements for Wnt signaling in many additional tissues that were unappreciated in transgenic experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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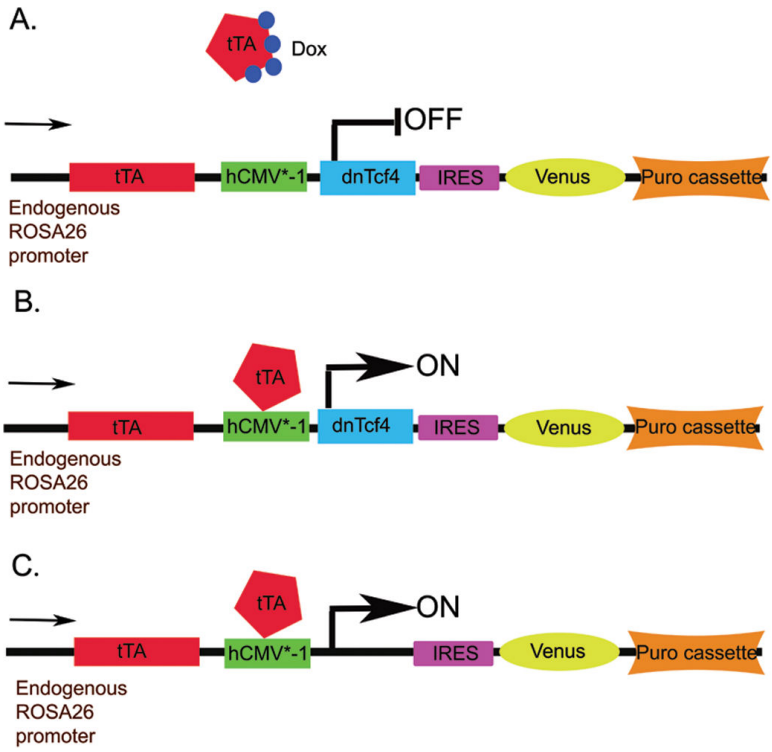

Figure 1. Expression of the dnTcf4 protein from the ROSA26 locus can be controlled by doxycycline

A. In the presence of Doxycycline (Dox), the tetracycline-regulated transactivator (tTA) is unable to bind to the 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 proteins are transcribed.

C. Control cells inducibly express Venus, but lack the dnTcf4 cDNA.

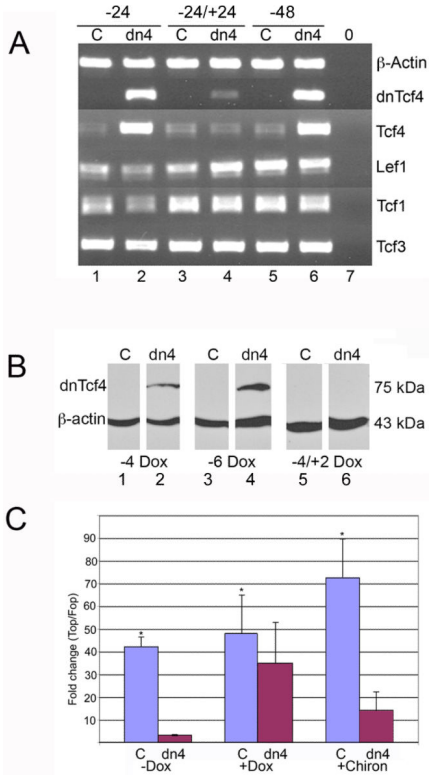

Figure 2. Inducible expression of the dnTcf4 protein blocks Wnt signaling in ESC

A. Control (C) and dnTcf4 (dn4) ESC were grown without Dox to induced transgene expression for 24h (-24, lanes 1,2) or 48h (-48, lanes 5,6) or for 24 hours without Dox followed by 24 hours with Dox (-24/+24, lanes 3,4). Lane 7 (0) 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 4 and 6 days after doxycycline withdrawal (Lanes 2,4). Expression is eliminated when doxycycline is reintroduced into the culture media for 48 hours (Lane 6). Expression is not detected in control cells (Lanes 1,3,5).

C. Wnt signaling is strikingly inhibited by dnTcf4 expression even in the presence of a GSKβ inhibitor (Chir99021) that strongly stimulates the Wnt pathway in luciferase assays. The mean fold change (TOP Flash/FOP Flash) in the control line (C) was significantly higher than the dnTcf4 (dn4) line without Dox for 4 days (-Dox), after Dox was withdrawn for 3 days and added back to cells for 1 day (+Dox), and without Dox for 4 days but with Chir99021 for 1 day (+Chiron,) * = $p \leq 0.05$, Student's t test (n=3). Error bars represent SEM.

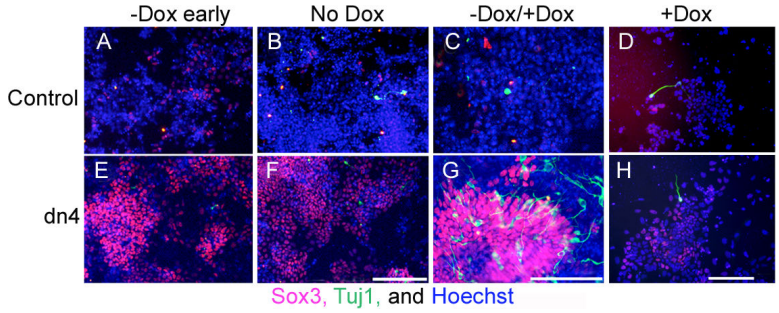

Figure 3. Regulated expression of the dnTcf4 protein promotes neural differentiation of ESC in a monolayer assay

Immunohistochemical localization of cell type restricted antigens in Control (A-D) and dnTcf4 (E-H) ESC grown in monolayer. When the transgene was induced and Wnt signaling was abrogated for 4 (-Dox early; E) or 6 days (No Dox; F) of differentiation there was widespread expression of the neural precursor marker Sox3 (red, Cy3 secondary) compared with controls (A,B), but was little expression of the neuronal marker βIII tubulin (Tuj1 antibody, green, FITC secondary; A,B, E,F). Reactivation of Wnt signaling during the last 3 days of differentiation (-Dox/+Dox; G) resulted in a significant increase in the conversion of Sox3 positive precursors to immature neurons, compared with Controls (C). There was little neuronal differentiation when Control or dn4 cells were grown continuously in doxycycline for 6 days (+Dox; D,H). Nuclei were stained with Hoechst (blue). Scale bar = 200 μM.

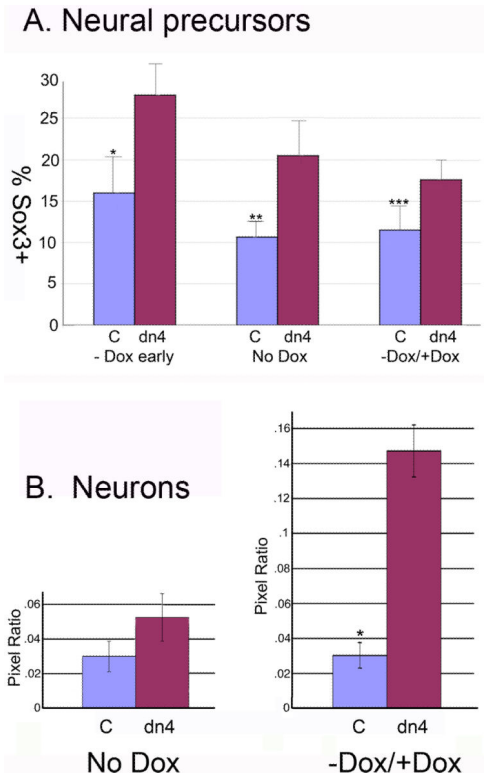

Figure 4. Regulated expression of the dnTcf4 protein significantly increases neural and neuronal differentiation of ESC in a monolayer assay

A. FACS analysis 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 (No Dox), or 3 days without Dox followed by 3 days with Dox (-Dox / +Dox). The number of Sox3 positive cells was significantly increased in dnTcf4 (dn4) expressing cells compared to control cells (C) at all time points. *p≤0.001, p**≤0.01, p***≤0.05, Student's t test. The graphs represent averages of 5 experiments with 3 replicates/experiment.

B. The number of neurons in control and dnTcf4 ESC grown in monolayer culture was quantified using NIH Image J. 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 (n=120 fields/group). 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 (-Dox / +Dox). *p≤0.003 Student's t test.

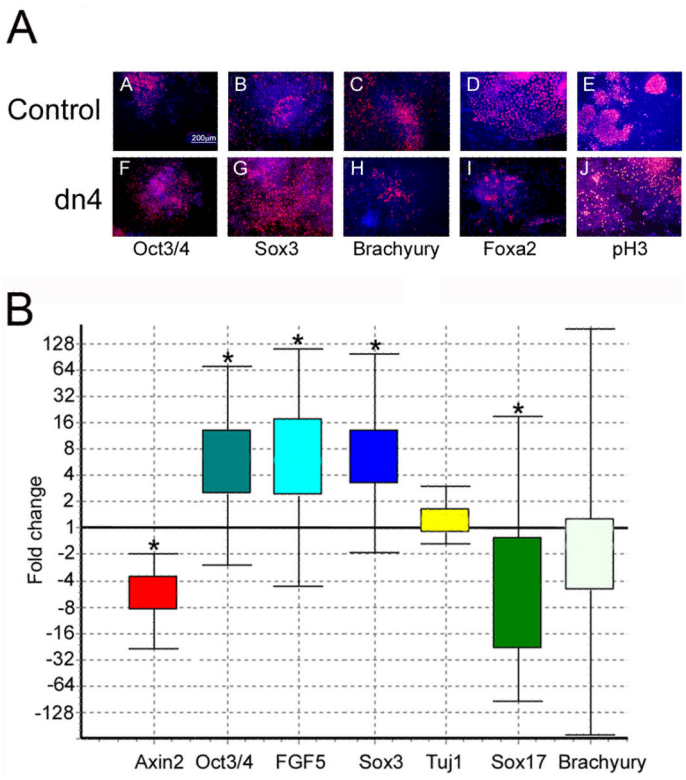

Figure 5. Wnt signaling blockade increases differentiation of neural precursors and decreases mesendoderm differentiation in an EB assay

A. Control and dnTCF4 ESC were grown as EB for 4 days then transferred to adherent culture for an additional 2 days. Cells were grown without Dox during the entire culture period to induce transgene expression. IHC localization of the pluripotency marker (Oct3/4; A,F), the neural precursor marker (Sox3; B,G), mesodermal marker (Brachyury; C,H), or the endodermal maker (Foxa2; D,I), or phosphohistone3 to identify dividing cells (pH3; E,J). When Wnt signaling is inhibited, cells continue to express high levels of Oct3/4 and Sox3 (F,G) with a concomitant decrease in mesendoderm differentiation (H,I), compared with controls (A-D). Panels E,J illustrate the very similar pattern of cell division identified using anti-phosphohistone3 antibody. Secondary antibodies were conjugated to Cy3 (red) and Hoechst (blue) identifies nuclei.

B. Quantitative PCR indicated that expression of the Wnt target gene *Axin2* was decreased 5-fold in dnTcf4 compared to control cells after 6 days of transgene induction. Genes expressed in the epiblast: *Oct3/4* and *Fgf5* were increased 6 fold suggesting that Wnt signaling is required for differentiation of the epiblast. The neural precursor gene *Sox3* was increased 6 fold, but *Tuj1*, a marker of immature neurons, was not changed. *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 and lower quartile values. Gene expression was calculated relative to β-actin and gene expression in the control ESC line was set to 1. Asterisks indicate statistically significant differences ($p \leq 0.05$) calculated by the REST software (n=3).

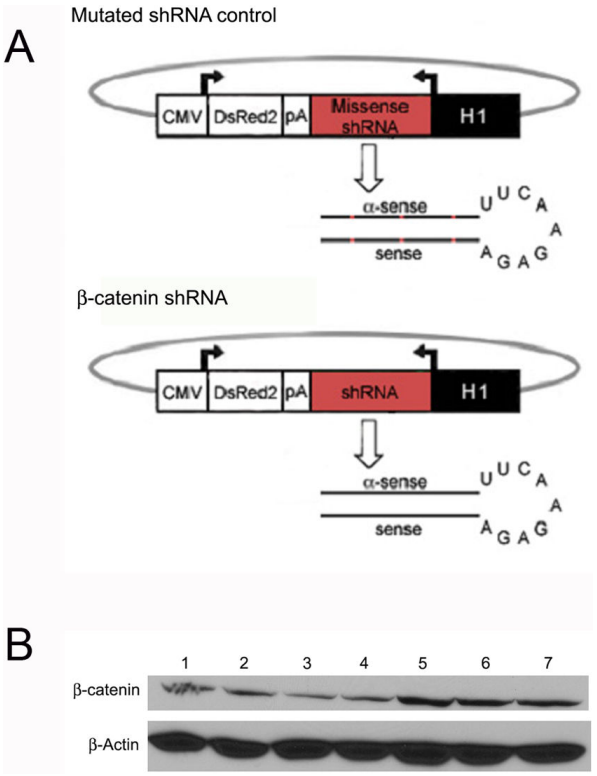

Figure 6. Transfection of ESC with a plasmid containing a β-catenin shRNA decreases the level of β-catenin protein

A. Diagrams of plasmids containing mutated shRNA control or β-catenin shRNA that were expressed in ESC and pregnant dams.

B. Western blot of protein from ESC transfected with shRNA constructs for 24 hours 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 40-64% knock-down. Top blot is β-catenin and bottom blot is a loading control blotted for β-actin. Lane 1 is protein from untransfected ESC.

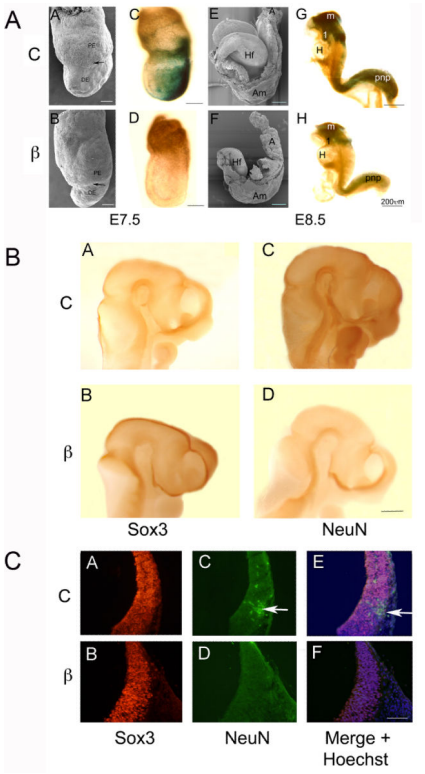

Figure 7. Wnt signaling is required in the embryo for the conversion of Sox3 neural precursors to NeuN positive neurons

A. Compared with control (C) embryos (A,C,E,G), embryos (β) 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 8.0-8.5 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 progress of gastrulation movements. The control embryo (A) has expanded in both the proximal-distal (P-D) and anterior-posterior (A-P) 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. By day 8 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=heart. SBs = 200μm

B. Side views of control (A, C) and embryos exposed to shRNA against β-catenin (B, D). Anterior is to the right in A-D. Whole mount IHC was 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 (C) and fewer Sox3 positive precursors (A) 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.

C. 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, arrow) 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