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Hedgehog signaling is required for the differentiation of ES cells into neurectoderm

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Abstract

Mouse embryonic stem cells can differentiate in vitro into cells of the nervous system, neurons and glia. This differentiation mimics stages observed in vivo, including the generation of primitive ectoderm and neurectoderm in embryoid body culture. We demonstrate here that embryonic stem cell lines mutant for components of the Hedgehog signaling cascade are deficient at generating neurectoderm-containing embryoid bodies. The embryoid bodies derived from mutant cells are also unable to respond to retinoic acid treatment by producing nestin-positive neural stem cells, a response observed in cultures of heterozygous cells, and contain cores apparently arrested at the primitive ectoderm stage. The mutant cultures are also deficient in their capacity to differentiate into mature neurons and glia. These data are consistent with a role for Hedgehog signaling in generating neurectoderm capable of producing the appropriate neuronal and glial progenitors in ES cell culture.

Keywords: ES cell; Hedgehog; Neurectoderm; Neuron

Introduction

The prospect of using human embryonic stem (ES) cells for transplantation therapies has heightened our interest in identifying the parameters that direct the differentiation of mouse ES cells into specific cell types. Mouse ES cells have been an active area of investigation for over 20 years (Evans and Kaufman, 1981; Martin, 1981). They derive from the inner cell mass of the blastocyst and demonstrate the classic properties of stem cells, the ability to both self-renew and differentiate. Since their origin is the early embryo, they have the capacity to differentiate into cell lineages representative of all three primary germ layers, and this ability is observed both in vitro and in vivo (Beddington and Robertson, 1989; Doetschman et al., 1985; Evans and Kaufman, 1981; Martin, 1981). Before protocols can be designed to provide a supply of specific progenitor cells for cell transplantation therapies, the in vitro conditions that generate these cell types must be defined.

ES cells grown in suspension culture aggregate to form embryoid bodies, consisting of an outer layer of visceral endoderm and an inner core of primitive ectoderm cells (Grabel, 1992). This primitive ectoderm begins as a solid core and subsequently forms a columnar epithelial layer surrounding a cavity, analogous to the proamniotic cavity in the embryo (Coucouvanis and Martin, 1995). Both BMP and Hedgehog signaling have been proposed to play a role in this cavitation process (Coucouvanis and Martin, 1999; Maye et al., 2000). The core ectoderm cells provide the source of the various differentiated embryonic cell types subsequently observed in culture, including neurectoderm. This transition can be optimized under certain embryoid body culture conditions, including the addition of conditioned medium from the HepG2 hepatocyte cell line (Rathjen et al., 2002) or culture in neuro-basal medium (Wichterle et al., 2002). Subsequent differentiation into the cells of the nervous system, neurons and glia, can be observed in continued suspension culture or after attachment culture under a variety of conditions. One protocol includes treatment of embryoid bodies with retinoic acid (Bain et al., 1995), which is key to

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promoting the appearance of neurons when the embryoid bodies are subsequently plated on adhesive substrates. A variety of neuronal subtypes appear in these cultures, including excitatory and inhibitory neurons (Bain et al., 1995). It remains unclear, however, how retinoic acid promotes the formation of embryoid bodies that are more committed to neuronal lineages. Recent observations using ES cells suggest that continued expression of the transcription factor Oct3/4, also key for maintaining undifferentiated ES stem cells, is involved in directing differentiation towards a neuronal fate (Shimozaki et al., 2003).

We provide evidence here that secreted Hedgehog signals promote the differentiation of ES cells into neurectoderm. Our previous studies demonstrated that mouse ES embryoid bodies provide an in vitro model of Hedgehog signaling: *Indian hedgehog (Ihh)*, one of the three hedgehog genes in the mouse, is expressed by the outer visceral endoderm layer, and the inner ectoderm core responds to the secreted protein by up-regulating the expression of conventional downstream target genes in this pathway, such as *Patched 1 (Ptch1)* and *Gli1* (Becker et al., 1997; Maye et al., 2000). Hedgehog signaling is mediated by the action of two membrane proteins, Patched and Smoothened (Smo) (McMahon, 2000). Hedgehog peptide binds to Patched, relieving its inhibitory action on Smoothened, thereby allowing Smoothened to mediate downstream action (Murone et al., 1999). A great deal of evidence supports a role for Hedgehog signaling in the patterning and differentiation of cells and tissues of the nervous system. The notochord and floor plate are ventral sources of Sonic hedgehog, (Shh), producing a ventral-to-dorsal gradient (Briscoe and Ericson, 1999). This gradient influences the fates of different cell types in the developing spinal cord (Briscoe et al., 2000). For example, the differentiation of motor neurons, which arise from the ventral neural tube, requires higher concentrations of Shh than the differentiation of interneurons, which arise dorsal to the motor neurons (Briscoe and Ericson, 1999). Treatment of ES embryoid bodies with Shh promotes the differentiation of interneurons and motor neurons (Wichterle et al., 2002). Recent analysis of *Shh, Smo*, and *Gli3* single and double mutants, as well as chimeric embryos including $Smo^{-/-}$ cells (Rallu et al., 2002; Wijgerde et al., 2002) supports a role for Hedgehog signaling in establishing ventral identity in the spinal cord and telencephalon, though additional signals may be involved as well.

While most studies have focused on the role of Shh in cell fate choice and patterning in the CNS, Shh can also function as a mitogen, stimulating proliferation of precursors for neurons and glia. $Shh^{-/-}$ mice have a substantial growth defect in the forebrain (Chiang et al., 1996). Treatment of telencephalic explants with Shh promotes expansion of nestin-positive ventral neuronal precursor cells (Kohtz et al., 1998). Ectopic expression of Shh leads to over-proliferation of CNS precursor cells (Rowitch et al., 1999). In the cerebellum, Shh is produced in the Purkinje cells, and acts as a mitogen for granule neuron progenitors (Wallace, 1999; Wechsler-Reya and Scott, 1999). This observation provides an explanation for the occurrence of medulloblastomas, cerebellar tumors, in mice heterozygous for a mutation in the *Ptch1* gene, since this mutation leads to an increase in Hedgehog signaling (Goodrich et al., 1997). Mutation of the human *PTCH1* gene has also been implicated in sporadic medulloblastomas (Goodrich and Scott, 1998). Localized expression of an activating mutation of the mouse *Smo* gene in the dorsal mid- and hindbrain results in extensive overgrowth as well as conversion to ventral fates in these regions (Hynes et al., 2000). In addition, Shh peptide promotes the proliferation of hippocampal-derived neural progenitors both in vitro and in vivo (Lai et al., 2003). These studies suggest that Hedgehog signaling promotes the proliferation of either neural stem cells or a more specialized progenitor cell type.

To define the roles of Hedgehog signaling in ES cell neurectoderm differentiation, we have taken a loss-of-function approach, using pharmacological reagents, as well as genetic mutation analysis. Our previous studies using reagents that promote Protein Kinase A (PKA) activity and thereby inhibit Hedgehog signaling, suggested a role for this pathway in generating the single-cell layer epithelial ectoderm core (Maye et al., 2000). We demonstrate here that embryoid bodies differentiating in the presence of the Hedgehog signaling inhibitor cyclopamine (Beachy et al., 1997; Taipale et al., 2000) express substantially lower levels of neurectoderm markers. *Ihh*-deficient and *Smo*-deficient ES cells form embryoid bodies that display little cavitation and express lower levels of neurectoderm markers, in comparison with embryoid bodies derived from heterozygous mutant ES cells. Interpretation of experiments examining the expression of early pluripotent lineage markers, including Oct3/4 and FGF-5, is consistent with the conclusion that cores of embryoid bodies formed from either the *Ihh* or *Smo* mutant ES cells are arrested at the primitive ectoderm stage and cannot differentiate into neurectoderm. Retinoic acid treatment promotes the transition from primitive ectoderm to neurectoderm in heterozygous, but not homozygous mutant cultures, inducing expression of the neural stem cell marker nestin. Consistent with the absence of neurectoderm in mutant embryoid bodies, neuron outgrowth cultures derived from the *Ihh* or *Smo* heterozygous ES cells demonstrate ample differentiation of nestin-positive neural stem cells, as well as mature neurons and glia, whereas homozygous mutant cultures are severely lacking in these cell types.

Materials and methods

ES cell culture

All ES cell lines were maintained on neo-resistant STO fibroblast feeder layers in DMEM (Gibco) and 15% fetal calf serum (Sigma or Atlanta Biologicals) in the presence of recombinant LIF. *Ihh* null and heterozygous cell lines were

generated as previously described (Byrd et al., 2002). *Smo* null and heterozygous lines were the generous gift of Andy McMahon (Wijgerde et al., 2002). Embryoid bodies were formed by removing the stem cells from the feeder layer and culturing them in suspension without LIF, in DMEM containing 15% FCS (Atlanta Biologicals) (Doetschman et al., 1985). For experiments involving treatment with cyclopamine and veratramine, 2 μM of these reagents was added to the culture medium after 24 h in suspension, and thereafter upon the addition of fresh medium. For these experiments, D3, R1, and R1-*Ptch1/lacZ* ES cell lines were used.

β-galactosidase and H&E staining

β-galactosidase staining was carried out on intact ES R1 *Ptch1*$^{+/-}$ −*lacZ* embryoid bodies as previously described (Mendelsohn et al., 1991). Embryoid bodies were then postfixed in 3.7% formaldehyde for 2 h at room temperature, dehydrated and paraffin embedded. H&E staining was carried out on dewaxed paraffin sections according to standard protocols.

In situ hybridization, immunocytochemistry, and RT-PCR

Whole mount in situ hybridization on embryoid bodies was performed as described in Maye et al. (2000). RNA extraction and conditions for RT-PCR and primers for *Ptch1*, *Ihh*, *HPRT* and *Shh* were also as described in Maye et al. (2000). Primers for *Sox1*, *Sox2*, *Otx2*, *Rax*, *Six3*, and *Foxd4* were as follows (those not referenced were designed by us):

Gene	Sense	Antisense	Reference
Sox1	aatccc-ctctca-gacggtg	ttgatg-cattttg-ggggtat	MGI (Freeman et al., 1998)
Sox2	caacgg-cagctac-agcatga	gggcca-tgtgcag-tctactg	
Otx2	aggagc-tgagtcg-ccacctc	gtagccc-agggag-ggatgca	(Belaoussoff et al., 1998)
Rax	gagttgc-tgcgagc-cctgtgt	ccgatg-ataggcg-ctgatgct	
Six3	ccagca-tcaggcc-atcggcga	ggccttg-gctatca-tacatcc	
Foxd4	ctccac-cgcccag-tcctctaa	gtgctcg-gaacttg-cggagga	

Immunocytochemistry for nestin was performed on paraffin sections as follows: After dewaxing and incubation in 100% EtOH, slides were incubated in 1% H_2O_2 in MeOH for 10 min. After rinsing in water and then PBS, slides were blocked at least 30 min in a buffer containing 5% normal goat serum in PBS plus 0.1% Triton X-100. They were then incubated overnight at 4 °C in anti-nestin monoclonal antibody (DSHB, rat 401, see reference below) at a dilution of 1/4 in blocking buffer. The next day, after several rinses in PBS, they were incubated for 1.5 to 2 h in a peroxidase-conjugated anti-mouse secondary, (Transduction Laboratories, 1/1000), and visualized with DAB.

Chimeric embryoid bodies

Embryoid bodies were grown as usual, but dishes were seeded with both *Smo*$^{-/-}$ ES cells, which express *lacZ*, and wild type ES cells, which do not. In each experiment, cells were mixed in proportions ranging from 25% to 75% *Smo*$^{-/-}$ cells. However, since the *Smo* null cells grow more slowly as embryoid bodies than wild type or heterozygous cells, the final proportions of null cells in each dish were lower. There was no apparent tendency for the null and wild type cells to segregate into separate embryoid bodies. At the end of the culture period, embryoid bodies were briefly fixed and stained for beta galactosidase to identify the *Smo*$^{-/-}$ cells, and processed as described above.

Retinoic acid induction of neuronal differentiation, and immunofluorescence

Neuronal differentiation was induced as described in Bain et al. (1995). Briefly, embryoid bodies were grown for 4 days without and then 4 days with 5×10^{-7} M retinoic acid. They were then plated in the growth medium (without retinoic acid) onto permanox chamber slides coated with 10 μg/ml laminin (Sigma) and allowed to differentiate. After approximately 5 to 7 days, morphological differentiation toward neuronal phenotypes was apparent in the heterozygous wells, at which point the slides were fixed and immunocytochemistry was performed according to standard protocols. Antibodies were against nestin (Monoclonal antibody developed by Susan Hockfield and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Dept. of Biological Sciences, Iowa City, IA 52242, 1/4 dilution of supernatant), TuJ1 (Research Diagnostics, 1/500), MAP2 (Sigma, 1/200), and RC2 (undiluted, gift of J. Crandall). Staining was visualized using anti-mouse IgG or anti-mouse IgM secondary antibodies, conjugated to Alexa 568 for red and Alexa 488 for green fluorescence. Antibodies directed against SSEA-1, developed by Davor Solter and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Dept of Biological Sciences, Iowa City, IA 52242, was used at 1/50 and visualized using anti-mouse IgM Alexa (Molecular Probes). Antibodies directed against Oct3/4 and FGF5 were obtained from Santa Cruz Biotechnology, Inc, used at dilutions of 1/40 and 1/20, respectively and visualized using Alexa conjugated anti-rabbit IgG from Molecular Probes.

Scoring of stained embryoid bodies

After immunostaining, the outgrowth region around the embryoid bodies was scored on a scale of − to +++, where − indicates the absence of nestin-positive cells, + indicates a few nestin-positive cells, generally at the periphery of the outgrowth, ++ indicates modest nestin staining and +++

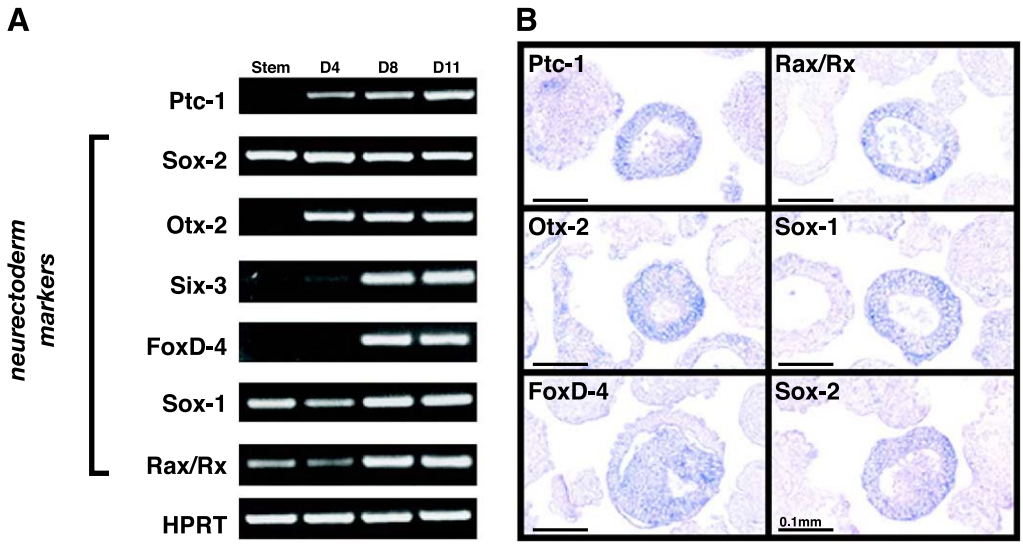


Fig. 1. RT-PCR and in situ hybridization analysis of neurectoderm markers in ES embryoid bodies. (A) RT-PCR demonstrates that *Ptch1* is up-regulated as embryoid bodies mature. *Sox-2* is expressed in stem cells and all embryoid body stages. *Otx-2* is not expressed by stem cells but is up-regulated in Day 4. *Six-3* and *FoxD-4* are not expressed in stem cells and are up-regulated in Day 8. *Sox-1* and *Rax/Rx* are expressed in stem cells and slightly up-regulated in Day 8. *HPRT* is shown as a control for equal loading of RNA. (B) In situ hybridization for most of these genes shows that they are expressed in the columnar ectoderm of Day 11 embryoid bodies. Scale bar equals 100 μm.

indicates extensive nestin staining originating at the edge of the embryoid body and extending throughout the outgrowth domain.

Photography and images

Slides were photographed using a Nikon E400 light microscope equipped with a Spot camera (Diagnostic Instruments), and processed using Adobe Photoshop.

Results

Embryoid body core expresses ectoderm and neurectoderm markers

When ES cells are placed into suspension culture, they differentiate into embryoid bodies that contain an outer layer of visceral endoderm surrounding an inner core of pluripotential cells. This inner core subsequently differentiates into primitive ectoderm and undergoes cavitation to form a columnar epithelial layer (Coucouvanis and Martin, 1995), which then gives rise to the multiple cell types, representative of all three primary germ layers, observed subsequently in culture (Doetschman et al., 1985). We have previously demonstrated that by Days 5–7 the outer visceral endoderm layer is a source of Ihh, while the core cells up-regulate *Ptch1* expression, which indicates a Hedgehog response (Becker et al., 1997; Maye et al., 2000). To characterize the cell type present in the inner epithelial layer of ES embryoid bodies, we used RT-PCR and in situ hybridization to examine the spatial and temporal expression of a variety of ectoderm and neurectoderm markers. Given the difficulty of obtaining quantitative information from RT-PCR data, experiments shown reflect observations made in at least three separate experiments. The up-regulation of *Ptch1* demonstrates the timing of the Hh response (Fig. 1A). Expression of *Sox-2*, which in the embryo is first expressed in the morula and inner cell mass (Avilion et al., 2003), then throughout the ectoderm before becoming restricted to the anterior neural ectoderm, is present both in undifferentiated stem cells and throughout the time course of differentiation (Fig. 1A). *Otx-2*, a marker of ectoderm and later anterior neurectoderm, (Ang et al., 1994) is not detected in stem cells, but up-regulated by Day 4 (Fig. 1A). This gene is also expressed in the anterior visceral endoderm (AVE) in the embryo, but in situ hybridization analysis, shown below in Fig. 1B, indicates expression in embryoid body cores. *Sox-1* is expressed throughout the anterior–posterior axis of the neural plate and neural tube in dividing cells (Pevny et al., 1998; Uwanogho et al., 1995). It is therefore somewhat surprising to observe expression of *Sox-1* in stem cells (Fig. 1A). This expression is likely not due to high levels of spontaneous differentiation in the stem cell sample, since it was observed in several experiments, but rather reflects anomalous gene expression sometimes observed in ES stem cells. Despite the apparent expression of *Sox-1* in stem cells, it is down-regulated by Day 4 and subsequently up-regulated between Days 4 and 8 of embryoid body culture. The anterior neurectoderm markers *Six-3, FoxD-4,* and *Rax/Rx* (Kaestner et al., 1995; Oliver et al., 1995; Furukawa et al., 1997) are all up-regulated between Days 4 and 8 (Fig. 1A). In situ hybridization analysis for a subset of these genes demonstrates that they are expressed specifically in the core (Fig. 1B), although just 20–30% of the embryoid bodies show expression of any of these markers, suggesting that under these conditions only a subset

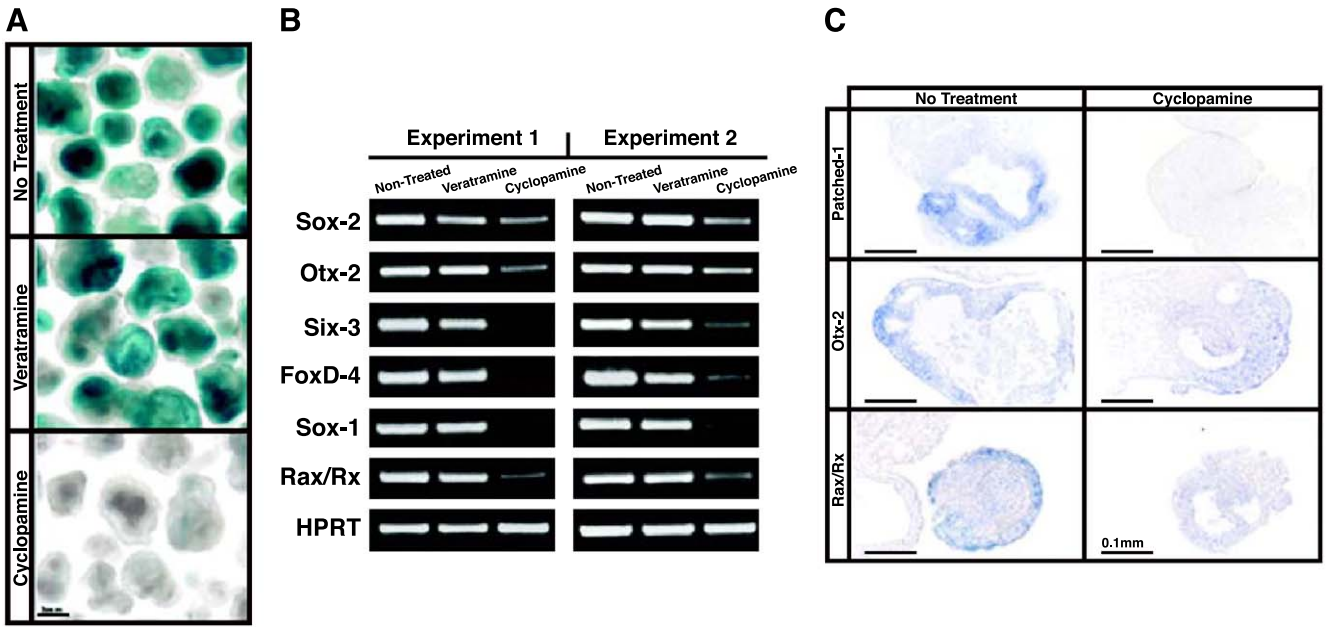


Fig. 2. Analysis of cyclopamine-treated Day 10 embryoid bodies. (A) Treatment of embryoid bodies with cyclopamine, but not with the control compound veratramine, inhibits the expression of *Ptch1-LacZ*. Two micromolar cyclopamine or veratramine were added to embryoid body cultures after 24 h in suspension and thereafter whenever the medium was changed. (B) RT-PCR analysis of RNA isolated from Day 10 embryoid bodies shows that cyclopamine, but not veratramine, inhibits the expression of molecular markers for ectoderm. Two separate experiments are shown. (C) In situ hybridization for *Ptch1*, *Otx-2* and *Rax/Rx* shows that expression of these genes is inhibited in embryoid bodies treated with cyclopamine, when compared to untreated controls. Scale bar equals 100 μm.

undergo maturation to neurectoderm. These data are consistent with observations made by others (Rathjen et al., 1999), and demonstrate that at least a subpopulation of the embryoid body cores can form neurectoderm-like cells from primitive ectoderm precursors.

Cyclopamine inhibits Hedgehog signaling and the expression of ectoderm and neurectoderm markers

Steroid alkaloids, such as cyclopamine, can be effective inhibitors of Hedgehog signaling at relatively low concentrations (Cooper et al., 1998; Incardona et al., 1998). These inhibitors appear to act at the level of *Smo*, the positive membrane effector of Hedgehog signaling (Taipale et al., 2000). Treatment of ES embryoid bodies with cyclopamine, but not the control compound veratramine (Beachy et al., 1997), decreases *Ptch1* expression in embryoid bodies, as assayed by decreased *lacZ* staining in an ES cell line that contains one copy of an insertion that includes a *lacZ* gene, expressed under the control of the *Ptch1* promotor. (Fig. 2A). RT-PCR analysis of RNA isolated from Day 10 embryoid bodies confirms this observation, and indicates that cyclopamine treatment inhibits accumulation of *Ptch1* and *gli1* (data not shown). These observations demonstrate that cyclopamine treatment inhibits the responses to Hedgehog signals, since target genes are no longer up-regulated. Under these conditions, we observe that the extent of ectoderm and neurectoderm differentiation is also inhibited. Fig. 2B shows RT-PCR analysis of RNA isolated from Day 10 embryoid bodies, which indicates that the expression of molecular markers for ectoderm and neurectoderm lineages, *Sox2*, *Otx-2*, *Six-3*, *FoxD-4*, *Sox1*, and *Rax/Rx* is inhibited by cyclopamine, but not veratramine treatment. Note that the more definitive neurectoderm markers, such as *FoxD-4*, are more severely affected. Fig. 2C shows in situ hybridization analysis for *Otx-2* and *Rax/Rx* expression that also suggests these genes are down regulated with cyclopamine treatment. Fig. 2C also shows that cyclopamine inhibits *Ptch1* expression, further documenting the successful inhibition of Hedgehog signaling. These data suggest that Hedgehog signaling is required for neurectoderm differentiation in the embryoid body core.

ES cells mutant for components of the Hedgehog cascade show decreased neurectoderm differentiation, but comparative rates of proliferation and cell death are difficult to obtain

To more definitively examine the role of Hedgehog signaling in neurectoderm differentiation, we examined the ability of $Ihh^{-/-}$ and $Smo^{-/-}$ ES cells to differentiate into embryoid bodies. Fig. 3A shows that following 7 or 10 days in suspension culture, while embryoid bodies formed from an *Ihh* heterozygous mutant cell line were able to form a cavity surrounded by a columnar epithelial ectoderm cell layer, homozygous mutant cells rarely formed cavities or contained an organized columnar

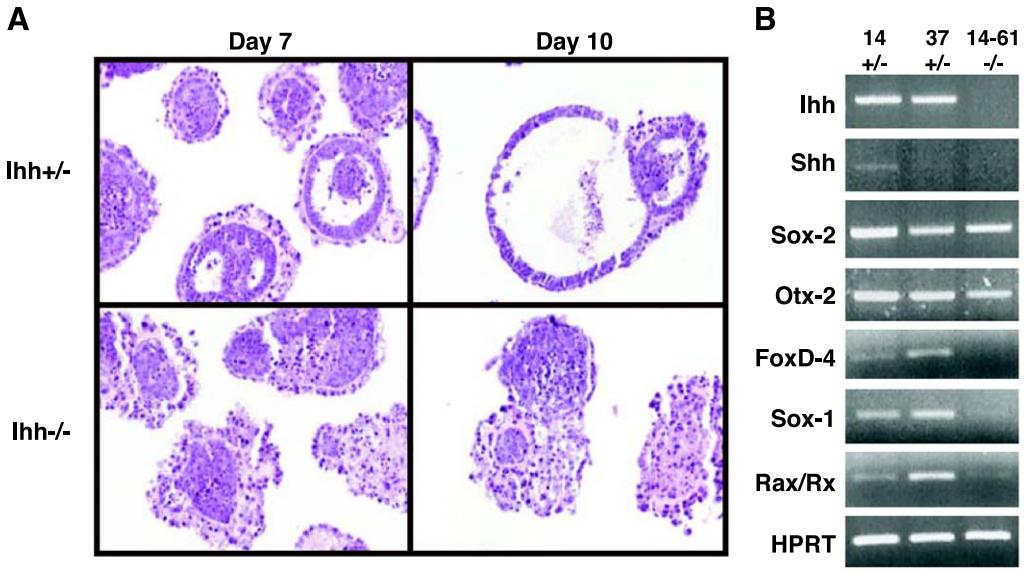


Fig. 3. Analysis of ES embryoid bodies mutant for *Ihh*. (A) Embryoid bodies formed from $Ihh^{+/-}$ ES cells cavitate and form a columnar layer of ectoderm, while those from $Ihh^{-/-}$ ES cell lines do not. H&E stained paraffin sections of embryoid bodies at Days 7 and 10 are shown. (B) RT-PCR analysis of RNA isolated from Day 10 embryoid bodies derived from two heterozygous and one homozygous mutant ES cell lines shows that, as expected, the $Ihh^{-/-}$ embryoid bodies do not express *Ihh*. Expression of *Otx-2* and *Sox-2* is slightly reduced in the $Ihh^{-/-}$ cell line, and expression of *Sox-1*, *FoxD4*, and *Rax/Rx* is almost absent.

epithelial layer. Fig. 3B demonstrates by RT-PCR analysis of RNA isolated from Day 10 embryoid bodies, that, as expected, the mutant cell line does not express *Ihh* mRNA. RT-PCR data are shown for two heterozygous (14 and 37) and one homozygous cell line (14–61). Previous time course analysis shows that *Shh* mRNA is expressed in all of these cell lines beginning at Day 10, a few days after the up-regulation of *Ihh* expression (Byrd et al., 2002). Fig. 3B confirms this observation and also demonstrates that the expression levels of *Sox1*, *FoxD4*, *Rax/Rx*, and to a lesser extent *Otx2* and *Sox2*, are decreased in homozygous mutant cultures in comparison to the heterozygous mutant cultures. The decrease in the level of expression of these markers in the $Ihh^{-/-}$ cultures is not as marked as observed with cyclopamine treatment. This discrepancy may be due to the presence of residual levels of *Shh* in the mutant cultures (Byrd et al., 2002) that could support neurectoderm differentiation.

We therefore examined ectoderm and neurectoderm differentiation in embryoid bodies derived from $Smo^{-/-}$ ES cells. Fig. 4A shows that, like the *Ihh* homozygous mutant cultures, the *Smo*-deficient cells form embryoid bodies that fail to cavitate or form an organized columnar epithelial layer. Fig. 4B shows that *Sox2* and *Otx2*, expressed in primitive ectoderm as well as neurectoderm, are slightly decreased, while the neurectoderm markers *Six3*, *FoxD4*, *Sox1*, and *Rax/Rx* are barely detectable in the homozygous mutant cultures relative to the heterozygous mutant cultures. Data are presented for two heterozygous lines, 37 and 41, and two homozygous mutant lines, 24 and 44. These RT-PCR data show more striking deficits in the expression of ectoderm and neurectoderm markers in the $Smo^{-/-}$ mutant embryoid bodies compared to $Ihh^{-/-}$ mutant embryoid bodies, most likely due to the absence of all Hedgehog responses in these cells. In situ hybridization analysis for *Ptch1*, *Otx2*, and *Rax/Rx* supports the conclusion that the *Smo* homozygous mutant cells are unable to generate neurectoderm (Fig. 4C). Note that although data are frequently shown for only one cell type for each genotype, an additional cell line for each genotype behaved similarly in all experiments.

The observation that there is decreased neurectoderm differentiation under conditions that compromise Hedgehog signaling would be consistent with reduced levels of proliferation of appropriate progenitor cells in embryoid bodies cores. This conclusion is supported by the observation that embryoid bodies derived from either *Ihh* or *Smo* mutant ES cells appear smaller than those derived from heterozygous or wild type cells. To determine if the decrease or absence of Hedgehog signaling results in decreased proliferation of the stem cell or ectoderm core cells, we attempted to measure rates of proliferation by a variety of methods, including BrdU labeling as well as expression of phospho-histone H3, a marker for mitotic cells. We first established that the stem cells proliferated at comparable rates regardless of their genotype (data not shown). We were consistently unable to see a statistically significant difference in proliferation levels between heterozygous and mutant embryoid body cultures at Days 4, 7, or 9. We were also unable to see a statistically significant difference in the level of programmed cell death, as assayed by TUNEL staining. This analysis, however, is confounded by cavitation occurring in the cultures of heterozygous cells, but not homozygous mutant cells, beginning as early

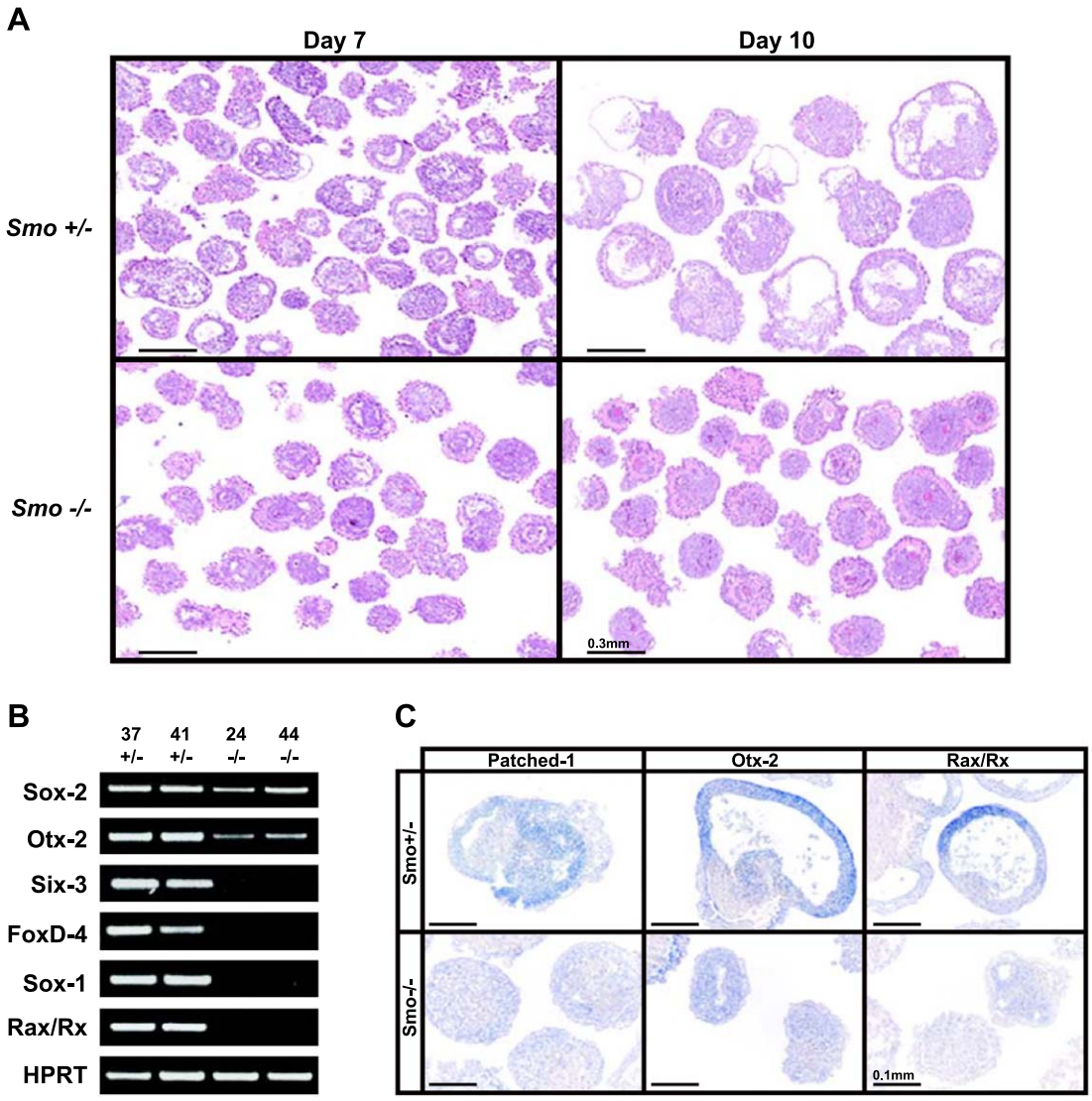


Fig. 4. Analysis of ES embryoid bodies mutant for *Smo*. (A) Embryoid bodies formed from $Smo^{+/-}$ cells cavitate and form a columnar layer of ectoderm, while those from $Smo^{-/-}$ ES cell lines do not. H&E stained paraffin sections of embryoid bodies at Days 7 and 10 are shown. Scale bar equals 300 μm. (B) RT-PCR analysis of RNA isolated from Day 10 embryoid bodies derived from two heterozygous and two homozygous mutant ES cell lines shows that *Sox-2* and *Otx-2* are slightly down-regulated in the $Smo^{-/-}$ embryoid bodies, and that *Six-3*, *FoxD4*, *Sox-1*, and *Rax/Rx* are severely down-regulated. *HPRT* is shown as a control for equal loading of RNA. (C) In situ hybridization analysis shows that *Ptch1*, *Otx-2* and *Rax/Rx* are significantly down-regulated in $Smo^{-/-}$ embryoid bodies, compared to heterozygous controls. Six-micrometer paraffin sections of Day 10 embryoid bodies are shown. Scale bar equals 100 μm.

as Day 4. Decreased levels of cell proliferation or increased levels of cell death in the mutant cultures may therefore be obscured by the apoptotic process that produces the cavities in the heterozygous cultures (Coucouvanis and Martin, 1995). We were therefore unable to determine if the absence of neurectoderm in mutant cultures was due to a lack of differentiation, or could, at least in part, be attributed to a defect in progenitor proliferation.

Chimeric embryoid body differentiation supports a role for Hedgehog signaling in ectoderm layer maturation

Experiments analyzing the behavior of ES cells carrying mutations for Hedgehog cascade genes suggest that embryoid body ectoderm differentiates only after receiving an Ihh signal from the outer visceral endoderm layer. To test this we next asked whether *Smo*-deficient ES cells could contribute to a cavitated inner ectoderm layer in embryoid bodies formed from a mixture of wild type and mutant cells. We mixed *Smo* mutant ES and wild type or heterozygous cell lines. The $Smo^{-/-}$ cells were derived from a ROSA mouse strain that constitutively expresses *lacZ* in all cells, whereas other cell lines used were not *lacZ*-expressing. Cells were mixed at various ratios of mutant/wild type, ranging from 25 to 75% mutant cells. Since the homozygous *Smo* mutant cells appear to grow more slowly than the heterozygous or wild type cell lines in the mixture, despite our inability to definitively estab-

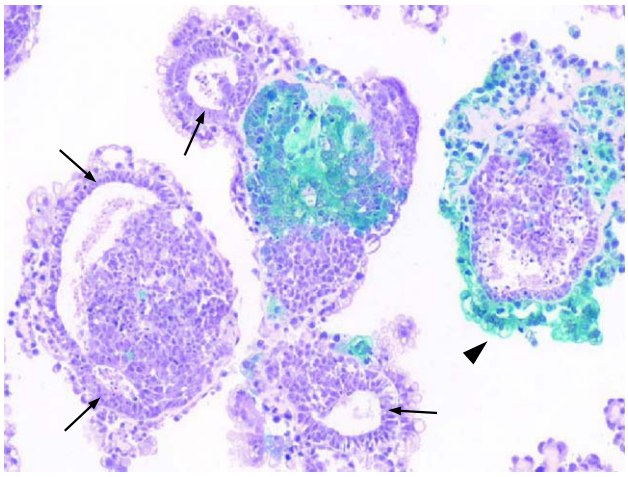


Fig. 5. Chimeric embryoid bodies. Embryoid bodies were formed by mixing *Smo*$^{-/-}$ and *Smo*$^{+/-}$ ES cells at a ratio of 1:1. After 9 days in culture, embryoid bodies were fixed and *Smo*$^{-/-}$ cells identified by β-galactosidase staining, followed by embedding, sectioning, and H&E staining. Heterozygous cells have formed a layer of columnar ectoderm (arrows), but *Smo*$^{-/-}$ cells (blue) were never observed to do this. *Smo*$^{-/-}$ cells did, however, readily contribute to the outer layer of endoderm and display the characteristic columnar morphology of visceral endoderm cells (arrowhead).

lish this, the percentage of mutant cells at the end of the experiment was always lower than at the start of the experiment. Fig. 5 demonstrates our observation that the *Smo*$^{-/-}$ mutant cells, even when present in abundance, did not integrate into a cavitated mature ectoderm layer (see arrows in Fig. 5). When localized to the core, these cells were part of a disorganized solid mass of cells. In contrast, mutant cells were readily observed in the outer endoderm layer and appear to be capable of differentiating into visceral endoderm, based upon their characteristic morphology (see arrowhead in Fig. 5). These observations support the importance of a Hedgehog response in the embryoid body core for ectoderm differentiation and maturation.

Characterization of the core of embryoid bodies deficient in Hedgehog signaling

The ES embryoid body in vitro system nicely mimics the in vivo progression of inner cell mass through primitive ectoderm, to neurectoderm. We have already demonstrated that the cores of embryoid bodies derived from *Ihh* or *Smo* mutant cells do not express neurectoderm markers. The continued expression of *Otx2* and *Sox2*, expressed in primitive ectoderm as well as neurectoderm but not in stem cells, suggests that embryoid bodies derived from mutant ES cells are able to differentiate into primitive ectoderm. To definitively determine if these cores are arrested at the equivalent of the inner cell mass stage, or are able to progress to the primitive ectoderm stage, we examined the expression of three markers of early embryonic cell types using immunohistochemistry. SSEA-1 antigen is an epitope expressed by inner cell mass and ES stem cells as well as by primitive ectoderm, and down-regulated in neurectoderm (Fox et al., 1981). Oct 3/4 is a POU-family transcription factor critical for early pluripotent cell lineages, expressed by inner cell mass and ES cells as well as by primitive ectoderm, and also down-regulated upon differentiation into neurectoderm (Rosner et al., 1990; Scholer et al., 1990; Minucci et al., 1996). FGF5 is not expressed by inner cell mass or ES cells, but is up-regulated in primitive ectoderm and then down-regulated once neurectoderm forms (Hebert et al., 1991; Pelton et al., 2002).

Fig. 6A (minus RA panels on the left) shows that at Day 9, embryoid bodies derived from either *Ihh*$^{+/-}$ or *Smo*$^{+/-}$ ES cells express SSEA-1 in columnar epithelial cores. In these cultures around 50% of the embryoid bodies exhibit strong staining in a cavitated columnar epithelial core layer. The rest of the embryoid bodies show staining in a solid core that has not cavitated. In contrast, virtually all of the core staining seen in embryoid bodies derived from mutant ES cells shows SSEA-1 cell expression in a solid core. Similarly, Oct 3/4 expression is predominantly seen in cavitated core cells, or alternatively in a solid core in heterozygous cultures, whereas the mutant cultures demonstrate expression exclusively in solid cores (Fig. 6B). Since the expression of these markers does not allow us to distinguish between a stem cell or primitive ectoderm identity for the core, we examined FGF5 expression, observed in primitive ectoderm but not in inner cell mass or stem cells (Pelton et al., 2002). Fig. 6C shows that embryoid bodies derived from mutant as well as heterozygous cell lines express FGF5, both in the matrix-rich region between the core and extraembryonic endoderm layer, and between core cells. Concentration of FGF5 in extracellular matrices has been previously observed (Kitaoka et al., 1997). These data suggest that the *Ihh* and *Smo* mutant cell lines can form primitive ectoderm, but cannot progress to cavitate or differentiate into neurectoderm.

The role of retinoic acid in promoting the primitive ectoderm to neurectoderm transition

To verify that the absence of Hedgehog signaling results in embryoid body cores stalled at the primitive ectoderm stage, we examined the ability of the mutant cultures to produce neurectoderm derivatives, neurons and glia. To promote neuronal differentiation, we turned to a previously published protocol that grows embryoid bodies for 4 days suspension culture without, followed by 4 days suspension culture with retinoic acid (Bain et al., 1995). Aggregates are then plated on adhesive substrates to promote the differentiation of neuronal derivatives (see Materials and methods for details). Before examining the extent of neuronal differentiation, we compared the extent of differentiation in untreated and retinoic acid-treated embryoid bodies at the end of 8 days in suspen-

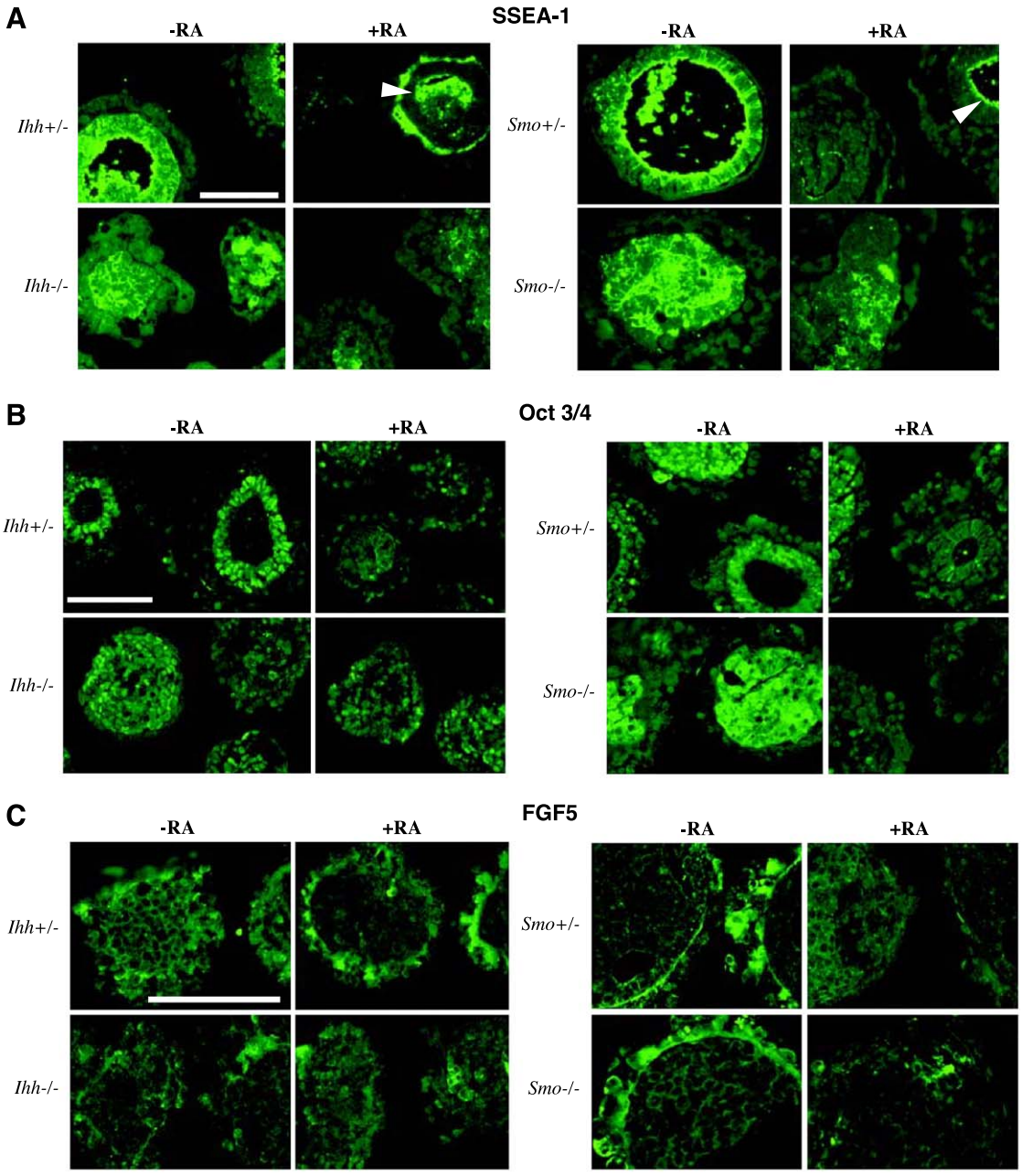


Fig. 6. SSEA-1, Oct 3/4, and FGF5 expression in untreated and retinoic acid-treated embryoid bodies. Embryoid bodies derived from $Ihh^{+/-}$, $Ihh^{-/-}$, $Smo^{+/-}$, or $Smo^{-/-}$ ES cells were grown in absence or presence of retinoic acid (RA) as described in Materials and methods. After 9 days embryoid bodies were fixed and paraffin sections analyzed by immunocytochemistry for the expression of (A) SSEA-1; (B) Oct 3/4; or (C) FGF5.

sion. Figs. 7A and B demonstrate that embryoid bodies formed from heterozygous *Ihh* or *Smo* cell lines do not express nestin, an intermediate filament protein expressed in neural stem cells and a few other cell types (Lendahl et al., 1990; Mokry and Nemecek, 1998). However, when these embryoid bodies are treated with retinoic acid to promote the subsequent generation of neurons following culture on adherent substrates (Bain et al., 1995), nestin expression is dramatically up-regulated in the ectoderm layer. The ability of the treated embryoid bodies to produce nestin-positive cells suggests that retinoic acid has promoted a primitive ectoderm-to-neurectoderm transition. This conclusion is supported by the observation that retinoic acid treatment of these cell lines also increases the level of expression of *Sox1*, an early neuroepithelial marker present throughout the anterior–posterior axis of the neural tube based upon RT-PCR analysis (Figs. 7C and D). Additional support comes from the observation that treatment of heterozygous cultures with retinoic acid decreases the level of expression of SSEA-1 and Oct 3/4, two markers that are expressed in ectoderm and down-regulated upon formation of the neural tube (Fig. 6). Low levels of SSEA-1 expression remain detectible in the treated

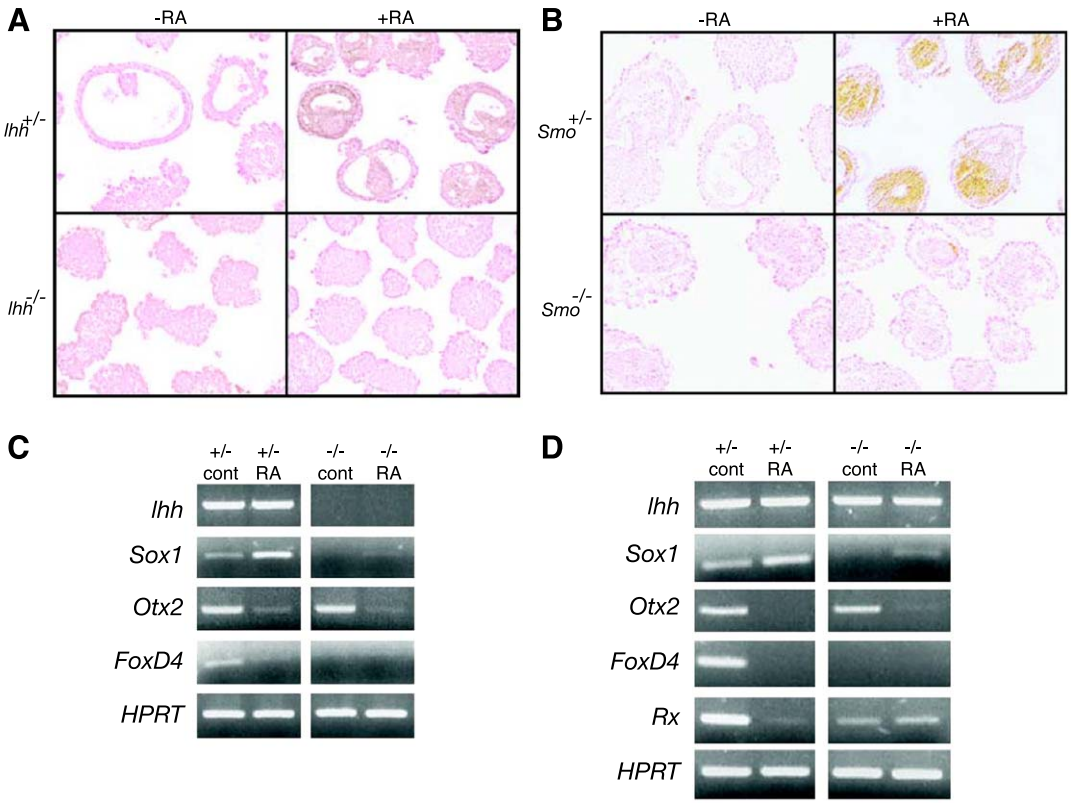


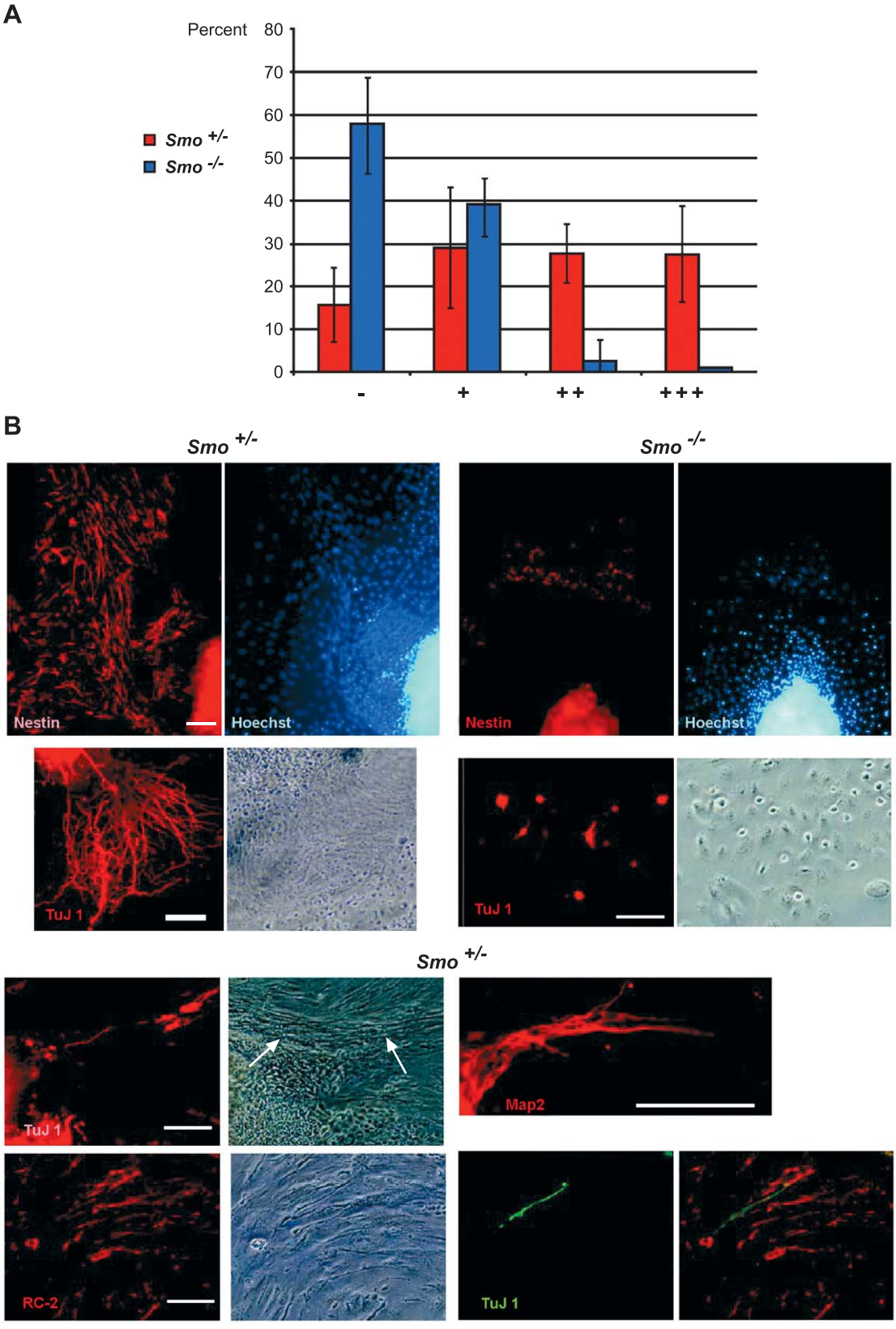
Fig. 7. Nestin staining and neurectoderm marker expression in retinoic acid-treated Day 9 embryoid bodies. (A) Embryoid bodies formed from $Ihh^{+/-}$ and $Ihh^{-/-}$ ES cells were grown with and without retinoic acid. Paraffin sections were stained with an anti-nestin antibody and counterstained with eosin. $Ihh^{+/-}$ embryoid bodies express nestin in response to retinoic acid treatment, while $Ihh^{-/-}$ embryoid bodies do not. (B) Embryoid bodies formed from $Smo^{+/-}$ and $Smo^{-/-}$ ES cells were grown with and without retinoic acid. Paraffin sections were stained with an anti-nestin antibody and counterstained with eosin. $Smo^{+/-}$ embryoid bodies express nestin in response to retinoic acid treatment, while $Smo^{-/-}$ embryoid bodies do not. RT-PCR analysis of RNA isolated from Day 9 $Ihh^{+/-}$ and $Ihh^{-/-}$ embryoid bodies (C), or $Smo^{+/-}$ and $Smo^{-/-}$ embryoid bodies (D), either untreated or treated with retinoic acid was performed for genes shown. The results of two separate experiments are shown. For both heterozygous cell lines, expression of *Sox1* is increased by retinoic acid treatment, whereas *Otx2* and *FoxD4* expression was inhibited by this treatment. *HPRT* is shown as a control for equal loading of RNA. Homozygous mutant cell lines display decreased levels of these markers relative to heterozygous cell lines, and retinoic acid still appears to modestly increase levels of *Sox1*.

embryoid bodies, lining the cavity of the core (see arrows in Fig. 6), reminiscent of the observation in the embryo of retained luminal surface staining of the neural tube (Fox et al., 1981). Consistent with the demonstrated caudalizing effect of retinoic acid on the developing CNS (Liu et al., 2001; Simeone et al., 1995), the expression of the rostral-specific markers *Otx2*, *FoxD4*, and *Rax* is inhibited by retinoic acid treatment (Figs. 7C and D).

In contrast, embryoid bodies derived from the homozygous mutant *Ihh* or *Smo* cell lines fail to express nestin upon retinoic acid treatment (Figs. 7A and B). RT-PCR analysis demonstrates that residual levels of expression of the anterior-specific marker *Otx2* are inhibited by retinoic acid (Figs. 7C and D). Retinoic acid treatment also decreases the level of expression of both SSEA-1 and Oct 3/4, and to a lesser extent FGF5, in embryoid bodies derived from mutant cells (Fig. 6), suggesting that down-regulation of these markers is not sufficient to promote neurectoderm differentiation. These data suggest that in the absence of Hedgehog signaling, retinoic acid is unable to induce a transition from primitive ectoderm to neurectoderm, despite the down-regulation of certain primitive ectoderm markers.

ES cells carrying homozygous mutations in Hedgehog signaling components show impaired capacity to differentiate into neurons

We next tested the ability of these mutant ES cell lines to form neuronal cell types. Embryoid bodies after 8 days of suspension culture using the retinoic acid protocol were plated on laminin-coated substrates and analyzed 5–8 days later for the expression of neuronal markers. Data are shown for only one cell line of both of the *Smo* genotypes, but similar results were obtained with an additional cell line of each of the *Smo* genotypes as well as with the $Ihh^{+/-}$ and $Ihh^{-/-}$ cell lines. In addition, three wild type cell lines behaved similarly to the heterozygous cultures. Fig. 8A quantifies the relative expression of nestin positive cells in *Smo* heterozygous versus homozygous mutant cultures. The outgrowth region around the embryoid bodies was scored on a relative scale of − to +++, where − indicates the


A
Percent
80
70
60
50
40
30
20
10
0
Smo +/-
Smo -/-
-
+
++
+++
B
Smo +/-
Smo -/-
Nestin
Hoechst
Nestin
Hoechst
TuJ 1
TuJ 1
Smo +/-
TuJ 1
Map2
RC-2
TuJ 1

absence of nestin positive cells, + a few nestin positive cells, frequently found at the periphery of the outgrowth, ++ modest nestin staining and +++ extensive nestin positive staining originating at the edge of the embryoid body and extending throughout the outgrowth domain. The cultures of heterozygous mutant cells display extensive nestin-staining, with approximately 85% of the outgrowths containing nestin positive cells, and over 25% displaying extensive staining (Fig. 8A). In contrast, over 55% of the homozygous mutant outgrowths have no nestin positive cells, and no outgrowths display extensive nestin staining (Fig. 8A). The predominant nestin-positive cell in the homozygous mutant cultures is a large flattened cell found at the periphery of the outgrowth. Previous reports have established that nestin is expressed in muscle precursors and epithelial cells, as well as in neural stem cells (Lendahl et al., 1990; Mokry and Nemecek, 1998). Our observation that the peripheral nestin-positive cells express α-smooth muscle actin (data not shown) is consistent with their identification as muscle precursor cells and not neural stem cells. The conclusion that these cells may not be neural stem cells, but another nestin-positive cell type, is supported by the observation described further below that the homozygous mutant cultures never generate cells with a neuronal phenotype that express either a β-tubulin isoform recognized by the TuJ1 antibody and expressed in neuronal precursors and immature post-mitotic neurons (Menezes and Luskin, 1994), or MAP2, a microtubule-associating protein expressed in differentiating and mature neurons (Caceres et al., 1984).

TuJ1-positive cells with neuronal morphology are observed only in the heterozygous mutant cell line, whereas rare TuJ1-positive cells in the *Smo*$^{-/-}$ cultures do not have this morphology. The heterozygous but not homozygous cultures express MAP2 and exhibit long processes, suggestive of neurons (Fig. 8B). In addition, we observed elongated cells that are closely aligned (see arrows) with a morphology reminiscent of radial glia. These cells stained positively using an antibody directed against the radial glial marker RC2 (Misson et al., 1988) indicating that they may correspond to a glial cell type. Often these RC2-positive cells were associated with TuJ1-positive cells (Fig. 8B). Taken together, these data suggest that the Hedgehog signaling pathway promotes the differentiation of multiple neural cell types from ES cells.

Discussion

In the embryo, the nervous system is established by the progressive commitment of pluripotential cell lineages. A portion of the inner cell mass differentiates into primitive ectoderm, the founding tissue for all three primary germ layers. A subpopulation of the primitive ectoderm becomes committed to definitive ectoderm, a subpopulation of which becomes neurectoderm. A group of transcription factors including Oct 3/4 (Rosner et al., 1990; Scholer et al., 1990) and the recently identified Nanog (Chambers et al., 2003; Mitsui et al., 2003) are involved in maintaining the pluripotency and self-renewal properties of the inner cell mass, primitive ectoderm, and ES cells. Less is understood about how neurectoderm is established.

Our data and studies by others (Rathjen and Rathjen, 2001; Rathjen et al., 2002; Wichterle et al., 2002) suggest that it is possible to mimic the steps from inner cell mass, to ectoderm, to neurectoderm, in vitro, using ES cell embryoid body culture. We show here that ES embryoid bodies express ectoderm and neurectoderm markers as their cores cavitate to produce a columnar epithelial cell layer. As noted by others (Rathjen et al., 2001), the core cells do not exclusively differentiate into neurectoderm under standard culture conditions, but into a variety of ectodermal, mesodermal, and definitive endodermal derivatives. We also demonstrate that when Hedgehog signaling is absent or decreased, the extent of neurectoderm differentiation is compromised. Careful examination of the expression of a variety of markers is consistent with arrest at the primitive ectoderm stage for embryoid bodies derived from either *Ihh*$^{-/-}$ or *Smo*$^{-/-}$ ES cells. Mutant cultures express the primitive ectoderm marker FGF5, as well as modest levels of *Otx2* and *Sox2* mRNA, two markers expressed in primitive ectoderm as well as in neurectoderm. In contrast, levels of definitive neurectoderm markers are dramatically down-regulated or absent in cyclopamine-treated or mutant cultures. What exactly does the Hedgehog signal promote in ES embryoid body cultures? The block at the primitive ectoderm stage may be due to the inability of the primitive ectoderm in these cultures to cavitate, perhaps an essential step in the progression to neurectoderm. Alternatively the block in neurectoderm may be attributed to a Hedgehog requirement for either establishing the neuronal lineages or promoting the proliferation of neural progenitors, a role previously described for

Fig. 8. Expression of neuronal markers in *Smo*$^{+/-}$ and *Smo*$^{-/-}$ cultures. (A) Embryoid bodies formed from *Smo*$^{+/-}$ and *Smo*$^{-/-}$ ES cells were scored for nestin staining (as described in Materials and methods). Over half the *Smo*$^{+/-}$ embryoid bodies displayed a substantial amount (++ or +++) of nestin staining, whereas almost none of the *Smo*$^{-/-}$ embryoid bodies did. (B) Retinoic-acid-treated embryoid bodies formed from *Smo*$^{+/-}$ and *Smo*$^{-/-}$ ES cells were plated onto laminin-coated chamber slides, allowed to grow for several days, and then fixed and stained for nestin, TuJ1, MAP2, and RC2. Top panel shows typical extensive nestin staining of *Smo*$^{+/-}$ cultures on the left, and on the right typical staining of *Smo*$^{-/-}$ cultures, restricted to large, flat cells at the periphery. Scale bar equals 100 μm; top panel is shown at lower magnification than lower panels. Second panel shows neuronal morphology of TuJ1-stained outgrowth cells in *Smo*$^{+/-}$ cultures on the left, and lack of neuronal morphology of the few TuJ1-stained *Smo*$^{-/-}$ cells on the right. Matching phase contrast panels are shown. Third panel on the left shows another example of TuJ1 staining of *Smo*$^{+/-}$ cells, with the matching phase contrast panel showing the radial glia-like morphology often displayed by TuJ1-positive fields (arrows). Third panel on the right, at higher magnification, shows MAP2 staining of *Smo*$^{+/-}$ cultures. No MAP2 staining was seen in *Smo*$^{-/-}$ cultures. Bottom panel shows double staining of RC2 and TuJ1 in *Smo*$^{+/-}$ cultures. From left to right, RC2, phase contrast showing the radial glia-like morphology, TuJ1, RC2 and TuJ1 images overlain. There was no RC2 staining seen in *Smo*$^{-/-}$ cultures.

Shh and hippocampal progenitors (Lai et al., 2002). We are now examining these alternatives using the protocols for generating neuronal cell types from ES cells that are described below.

Our data suggest a role for Hedgehog signaling in establishing neurectoderm. In contrast, a variety of in vivo studies suggest that neurectoderm and a variety of neuronal lineages are established normally in the absence of Hedgehog signaling. In zebrafish (Lewis and Eisen, 2001; Varga et al., 2001) or mouse embryos (Zhang et al. 2001) that completely lack Hedgehog signaling, there are defects in CNS patterning and a loss of specific cell types, including motor neurons, but other neuronal cell types are clearly present. Recent analysis of the behavior of $Smo^{-/-}$ cells in a wild type background in chimeric embryos indicates that mutant cells can participate in forming derivatives of the dorsal but not the ventral spinal cord (Wijgerde et al., 2002). The apparent absence of neurectoderm and neuronal differentiation in cultures of ES cells deficient for Hedgehog signaling is therefore a more severe phenotype than observed in the embryo. This discrepancy could be attributable to the presence of embryonic and/or maternal factors in utero that are capable of promoting neurectoderm differentiation, and are absent in vitro. The use of embryoid bodies derived from ES cells carrying mutations in Hedgehog cascade components should allow identification of such factors based upon their ability to rescue, at least in part, the neurectoderm phenotype described here.

Consistent with the inability to generate neurectoderm, embryoid bodies derived from mutant ES cells are also unable to produce neural derivatives. Several protocols have been identified that promote or restrict differentiation of ES cells into neural lineages. Most of these protocols rely upon an embryoid body stage and therefore the production of neurectoderm. The embryoid body-based protocol we have employed uses retinoic acid to promote the generation of neurons (Bain et al., 1995). Cultures derived from ES cells heterozygous for either *Ihh* or *Smo* contain ample nestin-positive cells as well as TuJ1 and MAP2-positive neurons. In contrast, homozygous *Ihh* or *Smo* mutant cell lines produce decreased numbers of nestin-positive neural progenitors in comparison with heterozygous cell lines. The nestin-positive cells present in homozygous mutant cultures may not be neural stem cells. Nestin is expressed in multiple cell types (Lendahl et al., 1990; Mokry and Nemecek, 1998), and our preliminary data indicating that the nestin-positive cells in homozygous mutant cultures also express α-smooth muscle actin, suggests they may be smooth muscle cells. TuJ1 staining is also greatly reduced and never found in cells with a neuronal morphology, and MAP2 staining is absent in mutant cultures. The heterozygous cultures also contain radial glia, as defined by the marker RC2, whereas homozygous mutant cultures do not. RC2-positive cells are frequently found in regions of closely aligned and elongated cells. In most instances, TuJ1-positive neurons also localized to these domains or are found elsewhere within the outgrowth of embryoid bodies that contained these RC2-staining regions.

In addition to promoting subsequent neural differentiation and increasing the levels of nestin and *Sox1* expression, we show here, as others have shown (Wichterle et al., 2002), that retinoic acid treatment caudalizes neurectoderm derived from ES cells, based upon the observed down-regulation of anterior neurectoderm markers.

Protocols that selectively promote neuronal differentiation from ES cells at the expense of other cell types, resulting in relatively pure populations of neuronal derivatives, have also been described. For example, it has been demonstrated that ES cell embryoid body cores can differentiate exclusively into a neurectoderm layer, at the expense of mesoderm or endoderm derivatives in the presence of conditioned medium derived from the HepG2 liver cell line (Rathjen et al., 2002). HepG2-conditioned medium likely contains a subset of the signals secreted by the visceral endoderm, a cell type similar to liver cells in terms of the profile of protein expression (Dziadek and Andrews, 1983). This subset of signals is sufficient to induce differentiation into ectoderm and neurectoderm derivatives, but not into derivatives of the other germ layers. Our data suggest that a Hedgehog peptide present in the HepG2-conditioned medium is responsible for promoting neurectoderm differentiation.

The culture of embryoid bodies in a defined medium that is 50% neurobasal medium (Gibco) also favors apparent neurectoderm differentiation (Wichterle et al., 2002). Under these conditions, Shh can induce the production of neural cell types derived from the ventral neural tube, motor neurons and interneurons, within ES embryoid bodies. In this study, there is also no apparent endoderm layer on the outer embryoid body surface, although this is not directly examined, and therefore perhaps no source of Ihh. Thus, the addition of an exogenous source of Hedgehog is required, in addition to retinoic acid, to promote significant levels of motor neuron differentiation. In addition to stimulating the production of motor and interneurons, retinoic acid and Hedgehog treatment also increases the number of cells expressing the pan-neural markers NeuN and Sox1, suggesting that a neural stem cell and not just a motor neuron progenitor may be a target of treatment (Wichterle et al., 2002).

An alternative protocol for promoting the differentiation of ES cells into neurons also begins with embryoid body formation, followed by culture in a serum-free defined medium to select for neural stem cells, and then the addition of growth factors to support their proliferation (Lee et al., 2000; Okabe et al., 1996). The advantage of this protocol is that it yields greatly enriched populations of neural progenitors by selecting for these cells in defined medium. The weakness of this approach is that it results in high levels of cell death upon transfer of the cells to the defined medium. Studies using this protocol suggest that FGF2 and/or EGF are essential for neural stem cell

proliferation; FGF8 and ascorbic acid increase the yield of dopaminergic neurons; and BMPs inhibit neural differentiation (Lee et al., 2000). Interestingly, Shh addition also promotes production of dopaminergic neurons in this protocol (Lee et al., 2000), while PDGF treatment appears to increase the yield of glial cells (Brustle et al., 1999). Our preliminary studies using this protocol suggest that ES cells deficient in Hedgehog signaling are able to produce few, if any, neural derivatives.

A modification of the protocols used for generating neurons from mouse ES cells has been used to generate enriched populations of neural progenitors derived from human ES cells (Carpenter et al., 2001). Studies such as ours, investigating the differentiation into neurons of mouse ES cells carrying mutations in genes encoding signaling molecules, will likely identify additional factors that can promote, or inhibit, the generation of desired cell types in these cultures. This information can be incorporated into the human ES cell protocols and provide the groundwork for defining conditions that will generate human ES cell derivatives for transplantation therapies that can potentially treat a variety of human neurological conditions.

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