Direct Neural Fate Specification from Embryonic Stem Cells: A Primitive Mammalian Neural Stem Cell Stage Acquired through a Default Mechanism

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Summary

Little is known about how neural stem cells are formed initially during development. We investigated whether a default mechanism of neural specification could regulate acquisition of neural stem cell identity directly from embryonic stem (ES) cells. ES cells cultured in defined, low-density conditions readily acquire a neural identity. We characterize a novel primitive neural stem cell as a component of neural lineage specification that is negatively regulated by TGFβ-related signaling. Primitive neural stem cells have distinct growth factor requirements, express neural precursor markers, generate neurons and glia in vitro, and have neural and non-neural lineage potential in vivo. These results are consistent with a default mechanism for neural fate specification and support a model whereby definitive neural stem cell formation is preceded by a primitive neural stem cell stage during neural lineage commitment.

Introduction

During vertebrate gastrulation, cells derived from the ectoderm are segregated into neural or epidermal primordia through a process of neural induction (Spemann and Mangold, 1924; Waddington and Schmidt, 1933; Oppenheimer, 1936; Beddington, 1994). According to the classical model, nascent embryonic ectoderm receives a positive inducing signal from organizer (mesendoderm) tissue during gastrulation, which enables ectodermal cells to adopt a neural fate. In the absence of this signal, ectodermal cells differentiate into epidermis, independent of organizer induction.

Results from subsequent in vitro experiments of isolated ectodermal (animal cap) cells derived from amphibian gastrula supported a different model for neural fate specification. Prolonged low-density dissociation of ectodermal cells, in the absence of organizer tissue, resulted in most of the cells expressing neural markers or forming neural structures upon reaggregation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). Furthermore, ectodermal explants (undissociated cells) expressing a dominant-negative receptor for activin (a member of the TGFβ superfamily of growth factors) were shown to become neural when cultured in vitro (Hemmati-Brivanlou and Melton, 1994). Molecules isolated from mesendodermal tissue, such as Noggin and Chordin, were found to be sufficient for inducing a second neural axis in analogous ectopic experiments performed in Xenopus (Smith et al., 1993; Sasai et al., 1995). However, the mechanism by which organizer signals promoted neural differentiation of ectodermal cells was not entirely consistent with a positive induction model. Noggin and Chordin act by binding extracellularly to bone morphogenetic proteins (BMPs), members of the TGFβ superfamily of molecules that strongly inhibit neural differentiation (Hemmati-Brivanlou and Melton, 1994). Thus, in a restricted manner, Noggin and Chordin prevent the binding of BMPs to their cognate receptors expressed on the surface of ectodermal cells (Zimmerman et al., 1996; Piccolo et al., 1999). In fact, BMP4 was shown to act as a positive signal for epidermal fate determination in the Xenopus ectoderm (Wilson and Hemmati-Brivanlou, 1995). These findings were consistent with the notion that the establishment of neural identity from uncommitted ectoderm occurs by default (i.e., a state achieved autonomously after the removal of the inhibitory signals).

We sought to determine whether a default-like mechanism underlies neural specification in uncommitted mammalian ES cells—the precursors to all embryonic lineages. ES cells are derived from the inner cell mass (ICM) of the preimplantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981) and can be sustained in an undifferentiated state in vitro while maintaining ICM characteristics. We report that chemically defined serum-free, feeder layer-free, low-density culture conditions are sufficient for neural differentiation of ES cells. We identify a novel colony-forming primitive neural stem cell population displaying properties that are intermediate to ES cells and forebrain neural stem cells. Furthermore, the transition from ES cell to primitive neural stem cell can be enhanced by the inhibition of TGFβ-related signaling, in a manner that is consistent with a default model of neural fate specification.

Note:

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Figure 1. LIF-Dependent Neural Colonies Are Clonally Derived
(A) ES cells cultured at 20 cells/µl form sphere colonies in the presence of LIF (1000 U/ml). Photo inset shows an ES-derived sphere colony after 7 days in culture (scale bar: 100 µm). Addition of FGF2 and heparin causes a slight, but nonsignificant increase in the numbers of primary sphere colonies compared to LIF alone (t = 1.1, p > 0.05) or LIF + B27 (t = 1.2, p > 0.05). The presence of FGF2 + heparin alone or B27 supplement alone (diluted to one-tenth the stock concentration: 1 x) is not sufficient for colony formation. Data represent 6–12 cultures per group from 4–11 separate experiments.
(B) Cells plated at limiting dilution in the presence of LIF. The frequency in which at least one neural stem cell will proliferate to form a sphere colony (37% mark on the ordinal scale) was ~0.2% (dashed line). Each data point represents the average of six cultures from two separate experiments.
(C) Sphere colonies are composed of Nestin-expressing cells. After 3 days in vitro or 7 days in vitro individual sphere colonies (n = 6 from each of 2 separate experiments) were transferred to a polyornithine substrate and allowed to adhere for 24 hr. Scale bar: 100 µm.
(D) Colony-forming ES cells displayed neural stem cell self-renewal characteristics. Single primary colonies generated in the presence of LIF alone (1a) were subcloned in LIF + FGF2, FGF2, or LIF to generate secondary colonies. Single primary colonies generated in the presence of LIF + FGF2 + B27 (1b) were subcloned in LIF + FGF2 + B27, FGF2 + B27, or LIF + B27 to generate secondary colonies. Single secondary colonies generated in LIF + FGF2 + B27 (2) were subcloned in LIF + FGF2 + B27 to generate tertiary colonies. Single tertiary colonies generated in LIF + FGF2 + B27 (3) were subcloned in LIF + FGF2 + B27 to generate quaternary colonies. Single sphere colonies (n = 6–24 isolated colonies per condition from at least 2 separate experiments) were dissociated into a single cell suspension after 7 days in vitro and recultured.

Results

Single ES Cells Differentiate into Colony-Forming Cells in the Absence of Serum, Feeder Layers, or the Formation of Embryoid Bodies
To determine directly the capacity for ES cells to generate clonal colonies of cells in the absence of serum-derived or feeder layer–derived factors and in the absence of cell–cell contact found in embryoid bodies (EB), we cultured ES cells at relatively low cell densities in a chemically defined, serum-free media. Under similar conditions, single neural stem cells isolated from the embryonic germinal zone of the neural tube can proliferate in response to exogenous EGF or FGF2 to give rise to clonal colonies of undifferentiated neural precursor cells that form floating spheres (Reynolds and Weiss, 1996; Tropepe et al., 1999). The colony-forming neural stem cells have the classical stem cell properties of self-renewal and multipotentiality (Morrison et al., 1997). That is, a small percentage of cells isolated from single dissociated colonies can generate new clonal colonies (self-renewal), while the majority of cells within the colonies will differentiate into neurons and glia.

ES cells cultured at relatively low cell densities in the presence of either EGF or FGF2 or in the absence of exogenous growth factors did not result in colony formation (Figure 1A). In contrast, in the presence of exogenous leukemia inhibitory factor (LIF), which is normally used to maintain ES cells in an undifferentiated state (Williams et al., 1988), floating sphere-like colonies were generated. There was no significant difference in the numbers of neural stem cell colonies generated when either EGF or FGF2 were combined with LIF compared to LIF alone (Figure 1A). Thus, exogenous EGF and FGF2 were neither necessary nor sufficient for colony formation in primary cell cultures. CNTF, a member of the cytokine family of signaling molecules to which LIF belongs (Kishimoto et al., 1994), was unable to substitute as a colony-promoting factor (data not shown), suggesting that the effects of LIF are specific.

To determine the frequency of cell colony formation, we cultured ES cells at various cell densities (from 1 cell/well to 20 cells/µl) in a limiting dilution assay (Tropepe et al., 1999). The estimated frequency of sphere colony-forming cells in the presence of LIF was ~0.2% (Figure 1B). No sphere colonies were observed at cell densities of less than 500 cells per well (0.5 ml of media), suggesting that a threshold number of cells may be required in order to facilitate the clonal proliferation of a single ES cell. To test this possibility further, ES cells were cultured at ~15 cells per microwell randomly distributed in Greiner hybridoma culture dishes subdivided into 700 microwells (0.04 cm² each). Even though the majority of microwells contained cells, an average of 35 colonies
were generated (two separate cultures) over the entire dish. Hence, a similar frequency of colony formation was observed over the entire culture dish (i.e., 15 × 700 – 10,500 cells; an average of 35 colonies/10,500 = 0.3%). In one additional experiment, single ES cells were cultured in 96-well plates (0.2 ml) and 1 sphere colony was generated in 600–700 wells scored. Thus, these results demonstrate that a threshold number of ES cells is not required for clonal colony formation, but rather that a very small percentage of single ES cells generate sphere colonies under these conditions as predicted by the limiting dilution analysis.

**Colony-Forming ES Cells Show Neural Stem Cell Characteristics**

Sphere colonies generated in the presence of LIF were composed of cells expressing the intermediate filament protein Nestin (Figure 1C), which is a marker of neural precursor cells in embryonic and adult CNS tissues. Nestin is also expressed transiently in muscle progenitors (Lendahl et al., 1990) and in some epithelial derivatives (Mokry and Nemecek, 1998). An analysis of smaller sized colonies identifiable at 3 days in culture (composed of 20–30 cells) demonstrated that all of the cells within these colonies (determined by counting Hoechst stained nuclei) appeared to express Nestin, suggesting that Nestin expression is correlated with the initial formation of the sphere colony. This early expression pattern coincides with Nestin expression in single ES cells at the onset of the cell culture period prior to colony formation (see below). Thus, individual ES cells acquire a neural precursor cell identity before they proliferate to generate colonies.

Individual colonies were dissociated and subcloned as previously reported (Reynolds and Weiss, 1996) in the presence of exogenous LIF, FGF2, or EGF alone, or in combinations. Regardless of the primary culture conditions, the formation of secondary neural stem cell colonies was dependent upon exogenous FGF2. LIF alone was not sufficient for secondary colony formation (Figure 1D). Colony-forming ability in tertiary and quaternary subcloned cell cultures could be sustained with combined FGF2 and LIF. However, substituting for LIF with B27 supplement (thought to prevent excessive cell death by inhibiting free radical-induced damage) in the FGF2 cultures was sufficient for repeated passaging (Figure 1D). The ability to generate sphere colonies in the presence of exogenous EGF alone, EGF + LIF, or EGF + LIF + B27 was not observed, and the effect of EGF + FGF2 was similar to the effects of FGF2 alone. The relatively small expansion of ES sphere colonies (2–16 new clonal colonies from a single primary colony; Figure 1D) is similar to the primary subcloning of FGF-responsive neural stem cells isolated from the E8.5 anterior neural plate (Tropepe et al., 1999). Under our conditions, however, new ES-derived colonies maintain their FGF2 and LIF (or B27) dependence upon repeated subcloning, whereas the E8.5-derived neural stem cell colonies require only FGF2. Furthermore, a separate EGF-responsive population of colony forming cells, which occurs during the development of the neural stem cell lineage between E10.5 and E14.5 in vivo (Tropepe et al., 1999; Martens et al., 2000), was not established from the ES-derived colonies.

![Figure 2. Cells from ES-Derived Sphere Colonies Express Neural-Specific Genes and Differentiate into Neurons and Glia](image-url)

(A) Differentiated ES sphere colonies contain neurons (MAP2⁺, arrowheads), astrocytes (GFAP⁺), and oligodendrocytes (O4⁺, arrowhead). Data are representative of 18 cultures from 2–3 separate experiments. (B) Gene expression analysis using RT–PCR. RNA was isolated from sphere colonies after 7 days in vitro and analyzed for the expression of markers for neural differentiation [Emx2 (151 bp), HoxB1 (325 bp), Six3 (571 bp), and Otx1 (128 bp)], endoderm differentiation [GATA4 (809 bp), HNF4 (629 bp)], mesoderm differentiation [Brachyury (857 bp)], and epidermis differentiation [Cytokeratin-17 (CK-17) (833 bp)]. To normalize for the amount of cDNA present in the sample, the cDNA for GADPH (401 bp) was amplified. R1 refers to primary ES cells; SC refers to ES-derived sphere colony; the plus sign refers to positive tissue control (forebrain, hindbrain, somitic mesoderm, liver, skin). Data are representative of at least three separate experiments. Scale bar: 20 μm.

To determine if the individual cells giving rise to the neural colonies had neural multilineage potential, individual colonies were encouraged to fully differentiate (placed on a MATRIGEL substrate and in the presence of 1% FBS) for a period of 7 days. Under these conditions, each of the differentiated colonies contained neurons (MAP2⁺ or βIII-tubulin⁺), astrocytes (GFAP⁺), and oligodendrocytes (O4⁺) (Figure 2A). The neural cells identified in these differentiated cultures (including undifferentiated, Nestin⁺ cells) appeared to account for all of the cell types present in the colonies. At least one non-neural marker, the muscle determination gene product MyoD, was not detectable by immunocytochemistry in these colonies (data not shown). Interestingly, ES cells (not from ES colonies) cultured for 7 days in the same differentiation conditions at high cell densities do not express the neuronal markers MAP2 or βIII-tubulin. Thus, at relatively high cell densities, ES cells must be specified to a neural identity (neural stem cell colonies) in order to differentiate into neurons and glia.

To further examine lineage commitment of the ES-derived sphere colonies, we analyzed the expression of genes restricted to neural and non-neural lineages using RT–PCR (Figure 2B). Colonies did not express the meso-
demarker brachyury (Beddington et al., 1992), which is abundant in EB (Elefanty et al., 1997). Colonies expressed the early endodermal marker GATA4, a zinc finger transcription factor that binds to a core GATA motif in the cis regulatory elements of many genes (Arcoci et al., 1993). However, the gene HNF4, which is a later endodermal marker (Li et al., 2000), was not expressed in ES-derived neural colonies, suggesting only partial endodermal potential within the colonies, unlike full endodermal potential documented for EB differentiation. Consistent with this observation, the absence of Otx1, expressed during the formation of the anterior visceral endoderm and later in the forebrain (Acampora et al., 1998), suggests that colonies do not engage in full visceral endoderm differentiation. Finally, the epidermal marker Cytokeratin-17 (McGowan and Coulombe, 1998) was not expressed in colonies.

Specific neural mRNAs were expressed in isolated ES-derived colonies. The forebrain marker Emx2 (Siemeone et al., 1992) and the hindbrain and spinal cord-specific transcription factor HoxB1 (Wilkinson et al., 1989) were expressed in the ES-derived neural colonies (Figure 2B). However, the anterior neural gene Six3 (Oliver et al., 1995), like Otx1, was not expressed. As a control, neural colonies derived from E14.5 forebrain germline zone were assayed for the expression of lineage-specific genes. Although neural-specific gene expression was confirmed in these samples (Figure 2B), expression of non-neural genes (brachyury, GATA4, and HNF4) was not observed. In addition, ES cells freshly trypsinized from their feeder layers were also used as controls. With the exception of GATA4, Otx1, and HNF4, the nonmanipulated ES cells express all of the genes tested and, indeed, are known to nonspecifically express a variety of genes (Elefanty et al., 1997). Thus, neural-specific gene expression persisted in the colonies, whereas mesodermal and epidermal markers were downregulated in the transition from ES cells to neural colonies. These findings suggest that colonies generated through the proliferation of a single neural cell are specified to primarily a neural identity and are composed of both neuronal and glial lineages. The fact that some non-neural genes (e.g., GATA4) are expressed in sphere colonies may suggest that these specified neural stem cell-derived colonies are not completely committed to a neural fate. The absence of Otx1, which is expressed in the anterior neural tube and anterior visceral endoderm, may indicate that early anterior–posterior polarity is not intrinsic to sphere colonies.

**LIF Functions as a Permissive Factor for Neural Stem Cell Differentiation of ES Cells**

The ability of LIF to promote neural colony formation may indicate that LIF induces uncommitted ES cells to a neural fate in primary cultures. However, LIF is necessary to maintain ES cells in an undifferentiated state, while LIF withdrawal is coincident with differentiation (reviewed in O’Shea, 1999). Two observations in the present study suggest that LIF may instead act in a permissive manner to enable ES cells to adopt a neural stem cell fate.

First, since neural stem cells isolated from early developmental stages are dependent upon FGF (Tropepe et al., 1999), we tested whether endogenous FGF signaling mediates neural colony formation in primary ES cell cultures in the presence of LIF. Neural colony formation in FGF-receptor-1-deficient (FGFR1-/-) ES cells (compared to a FGFR1+/+ control cell line; Ciruna et al., 1997) was diminished by 82% in the presence of LIF (Figure 3A), suggesting that ES cells may be responding to endogenous FGF that is released by the ES cells. Consistent with this notion, the addition of an anti-FGF2 antibody to a primary ES cell culture in the presence of
ES-Derived Neural Stem Cell Colonies Contribute Extensively to All Embryonic Tissues in Chimeric Mice

To determine if neural stem cell colonies have a broader potential to generate neural and non-neural lineages, we performed mouse chimera experiments. ES cells introduced into a blastocyst or aggregated with a morula predominantly contribute to the epiblast of the developing embryo, whereas extraembryonic tissues are primarily of host origin (Beddington and Robertson, 1989). We first determined that within 14 μm cryosections of whole ES sphere colonies after 7 days in culture all of the cells appeared to express Nestin, which is similar to Nestin expression in sectioned forebrain-derived neural stem cell colonies. However, we did not observe any nuclear Oct4-expressing cells in ES sphere colony sections or forebrain colony sections (data not shown), suggesting that no cells within ES derived neural colonies maintained an undifferentiated ES cell phenotype.

We used blastocyst stage or morula stage embryos as hosts and neural stem cell colonies derived from yellow or cyan fluorescent protein (YFP, CFP) expressing ES cells, β-gal expressing embryonic and adult forebrain cells (ROSA; Friedrich and Soriano, 1991), or green fluorescent protein (GFP) expressing embryonic forebrain cells (Hadjantonakis et al., 1998). Approximately 92% (22/24) of the single YFP or CFP ES-derived colonies aggregated with morulae after 24 hr in vitro contributing to the ICM in normally developed blastocysts (Figure 4A, inset) and had substantial contribution to all embryonic tissues in embryos recovered at E9.5 (Figure 4A). However, blastocyst injections of cells derived from E14.5 or adult ROSA neural colonies did not integrate into the ICM of the host embryos after 24 hr and in many cases tended to adhere to the host mural trophoderm. Embryos recovered between E7.5 and E8.5 from these chimeras did not contain any β-gal+ cells (0/19). Furthermore, E14.5 ROSA or E9.5 GFP neural stem cells were unable to adhere with morulae over a 24 hr period. Consequently, host morulae developed normally over the 24 hr culture period into healthy blastocysts while the sphere colonies remained outside of the embryo (Figures 4B and 4C). To test whether ES-derived sphere colonies (that readily adhere to morula cells) could facilitate the integration of colonies derived from the E9.5 forebrain, we cultured CFP ES colonies with GFP E9.5 forebrain colonies together with the host morula. In all cases, no E9.5 GFP colonies were observed to integrate (0/18), even though in many cases the CFP ES colonies did. These data suggest that ES derived neural stem cell colonies are competent to colonize many different tissues when exposed to an appropriate environment. However, this ability is only transient since neural stem cell colonies isolated from embryos in the earliest stages of neural development do not appear to have this same capacity.

Inhibition of TGFβ-Related Signaling Enhances Neural Stem Cell Differentiation of ES Cells

Given that very few of the cultured ES cells generated sphere colonies (0.2%), we sought to determine if the release of endogenous BMP from the ES cells inhibited neural sphere colony formation, as would be predicted
It is evident that although Noggin can enhance the numbers of ES cells that differentiate into neural colony-forming stem cells, the effect is moderate. Noggin is known to be less effective than Chordin in neural induction assays in Xenopus (Lamb et al., 1993), and targeted null mutations in both Noggin and Chordin in mouse are required to reveal anterior neural deficits in vivo (Bachiller et al., 2000). Thus, the effects of Noggin alone may underestimate the role for BMP-mediated inhibition of neural stem cell colony formation. To determine more directly the effect of blocking BMP signaling, we utilized an ES cell line with a targeted null mutation in the Smad4 gene (Sirard et al., 1998), a critical intracellular transducer of multiple TGFβ-related signaling pathways (Wranas, 2000). Smad4<sup>−/−</sup> ES cells cultured in the presence of LIF generated a 3- to 4-fold increase in the numbers of colonies, compared to the wild-type E14K cell line used to generate the targeted mutation (Figure 5C). The baseline numbers of colonies generated by wild-type R1 ES cells (26.3 ± 5.4) and wild-type E14K ES cells (25.7 ± 6.7) cultured at 20 cells/μl were not significantly different (t = 0.08, p > 0.05). Interestingly, the rate of proliferation between wild-type and Smad4<sup>−/−</sup> cells in high or low serum concentration is similar, indicating that the increase in the number of colonies from mutant ES cells is likely not a result of a general increase in proliferation. Taken together, these results indicate that BMP4 signaling has a specific effect in limiting the numbers of single ES cells that differentiate into colony-forming neural stem cells and that inhibition of this pathway is sufficient to enhance neural stem cell colony formation. Importantly, the Smad4<sup>−/−</sup> primary neural stem cell–derived colonies did not passate at a greater rate compared to control primary neural stem cell colonies (data not shown), suggesting that the effect of the mutation is on the transition from ES cell to neural stem cell and not on the later symmetrical division of the neural stem cells.

The secreted factor Cerberus is a potent anterior neural inducer in Xenopus (Boeuf et al., 1996) (as is the mouse homolog Cerberus-like [Belo et al., 1997]) and acts by antagonizing BMP signaling (Pearce et al., 1999; Piccolo et al., 1999). To determine whether Cerberus can interfere with neural stem cell commitment in mammalian cells, we cultured primary ES cells in the presence of LIF in media containing supernatant collected from transiently transfected Neuro2a cell lines producing mouse Cerberus-like (mCer-l) protein. The presence of 20% (v/v) of mCer-l supernatant in 0.5 ml serum-free media plus LIF resulted in close to a 50% increase in the numbers of primary neural stem cell colonies generated, compared to control ES cell cultures containing equivalent proportions of supernatant from cell lines similarly transfected with the backbone vector without the mCer-l gene (Figure 5D). A similar increase in sphere colony formation was also observed when using supernatant collected from a transiently transfected COS7 cell line (data not shown). Again consistent with the default model, mCer-l–mediated inhibition of BMP signaling can enhance the frequency with which single ES cells differentiate into colony-forming neural stem cells. Moreover, ES-derived neural colonies in the presence of mCer-l–enriched media, but not in the presence of the control media, express Otx-1 (data

Figure 5. TGFβ Signaling Can Modulate Neural Stem Cell Differentiation from ES Cells

(A) BMP4 inhibits neural colony formation compared to controls (t = 4.45, p < 0.05). ES cells were cultured at 20 cells/μl in the presence of LIF + FGF2 + heparin alone or in the presence of BMP4. Sphere colonies were quantified after 7 days in vitro. Data represent the average of 6 cultures per group from 2 separate experiments.

(B) Under similar conditions, Noggin (100 μg/ml) enhances neural colony formation compared to controls (t = 4.78, p < 0.05). Sphere colonies were quantified after 7 days in vitro. Data represent the average of 6 cultures per group from 2 separate experiments.

(C) A null mutation in the Smad4 gene enhances neural colony formation compared to wild-type controls (t = 2.67, p < 0.05). Smad4<sup>−/−</sup> and wild-type E14K ES cells were cultured at 20 cells/μl in the presence of LIF and sphere colonies were quantified after 7 days in vitro. Data represent 5–12 cultures per group from 3–5 separate experiments.

(D) mCer-l enhances neural colony formation compared to controls (t = 2.4, p < 0.05). ES cells were cultured at 20 cells/μl in the presence of LIF and B27 in the presence of 20% (v/v) in 0.5 ml culture wells of media supernatant from Neuro2a cell lines transiently expressing a mCer-1 transgene or V2 plasmid control. Sphere colonies were quantified after 7 days in vitro. Data represent an average of 6 cultures per group from 2 separate experiments.

from the neural default model. Given that BMP4 and BMP-receptor-1 are expressed by undifferentiated ES cells (Elefanty et al., 1997), we tested whether BMP could inhibit ES sphere colony formation by adding BMP4 (5 ng/ml) to ES cell cultures containing LIF and FGF2. We observed a >50% decrease in the number of colonies generated, and this effect appeared to be maximal since a 5-fold increase in BMP4 concentration did not further significantly attenuate the number of sphere colonies generated (Figure 5A). Addition of the BMP4 protein antagonist Noggin (100 μg/ml) to the primary ES cell cultures caused a 50% increase in the number of sphere colonies generated (Figure 5B). This increase appeared to be maximal since an increase in Noggin concentration from 10 μg/ml to 100 μg/ml resulted in no additional increase in the numbers of colonies generated.
not shown), indicating that mCer-l also may anteriorize the neural colony cells.

**Neural Cell Fate Is Rapidly Established from ES Cells in the Absence of Exogenous Factors**

When grown under their optimal feeder layer conditions, none of the ES cells express the neural marker Nestin (see last paragraph of Results). We predicted that if ES cells were acquiring a neural identity by default, they would express neural markers at very early stages during the culture period after being transferred to low-density, serum-free, and feeder-free conditions. To test this, ES cells (seeded at 10 cells/μl) were allowed to adhere to a polyornithine substrate, and the proportion of ES cells that differentiated into neural cells was determined. After 24 hr in culture, 69.9% ± 4.6% of ES cells were nonviable in the absence of growth factors (estimated using Trypan blue exclusion, n = 4 separate cultures). Thus, in addition to TGFβ-related inhibition, the low frequency of ES cells differentiating into neural cells may be a result of extensive cell death in long-term culture assays. However, of the remaining 30% of viable cells, 82% were immunoreactive for the neuroepithelial marker Nestin in the absence of growth factors (Figure 6A). Interestingly, although the percentage of viable cells after 4 hr was significantly greater (90%), the frequency of Nestin+ cells at this earlier time point was similar (70%–80%). The majority of the Nestin+ cells had a relatively large, flattened, and irregular morphology with prominent filamentous immunolabeling within the cytoplasm. A smaller subpopulation of the Nestin+ cells were also immunolabeled for the neuronal markers βIII-tubulin (51%) and NeuN (29%), many of which had a relatively small soma with very little perinuclear cytoplasm resembling an immature neuronal morphology (Figure 6A). The addition of LIF and FGF2 to these culture conditions did not significantly alter ES cell differentiation (data not shown). These data indicate that within 24 hr, ES cells may be competent to directly differentiate into neural cells at low cell densities and serum-free conditions in the absence of exogenous growth factors.

A second prediction that can be made from the default model is that an increase in cell density will facilitate inhibitory intercellular communication (cells in close proximity) and attenuate the numbers of ES cells differentiating into neural cells. To test this, we cultured ES cells in identical conditions for 24 hr but increased the cell density by 5-fold (to 50 cells/μl). At this relatively higher cell density in the absence of growth factors, the proportion of Nestin+ cells was reduced from 82% to 40% (t = 2.98, p < 0.05) and the proportion of βIII-tubulin+ cells was reduced from 51% to 13% (t = 4.07, p < 0.05) (Figure 6A). LIF + FGF2 did not affect the reduction in ES cells expressing these neural markers at higher cell densities (data not shown).

To exclude the possibility that a subpopulation of ES cells at the start of the 24 hr culture period were already committed to a neural fate, we tested whether ES cells just prior to culturing expressed the ICM/ES cell nuclear marker Oct4, a POU-type transcription factor (Nichols et al., 1998). ES cells were gently removed from their feeder cell substrate (with either trypsin/EDTA or EDTA alone), washed in serum-free media, and then fixed in 4% paraformaldehyde. Subsequently, cells were either plated on a polyornithine substrate or maintained in suspension prior to immunolabeling with an anti-Oct4 antibody. Using either of these methods, all of the ES cells were immunoreactive for nuclear Oct4, but none expressed Nestin. As a control, forebrain-derived sphere colony cells expressed Nestin under this immunolabeling protocol, but were negative for Oct4 expression.

Next, we tested whether the remaining non-Nestin immunoreactive population after 24 hr in our low-density cultures retained their ES cell identity. All of the non-Nestin immunoreactive cells (17%) expressed nuclear Oct4 at cell densities of 10 cells/μl (Figure 6A). The Oct4+ cells had a rounded morphology with a thin rim of perinuclear cytoplasm that was distinct from the morphology of Nestin+ cells. Furthermore, we observed a trend toward an increase in nuclear Oct4 immunoreactivity (up to 26%) when ES cells were cultured at a 5-fold higher cell density (Figure 6A), which was inversely proportional to the relative decrease in Nestin and βIII-tubulin expression at the same high cell densities. Cells expressing the epidermal marker Cytokeratin were not observed under these conditions. Thus, increased cell density inhibits neural cell differentiation and may facilitate the maintenance of ES cells in an undifferentiated state instead of promoting an alternative epidermal fate.

**Neuronal Differentiation Is Enhanced in Smad4-/- ES Cells**

To determine whether TGFβ signaling influences the extent to which ES cells adopt a neuronal phenotype in the short-term differentiation assay, we cultured Smad4-/- ES cells at relatively high cell densities (50 cells/μl) for 24 hr and double immunolabeled for Nestin and βIII-tubulin. Under these conditions, neuronal differentiation from wild-type ES cells is relatively low. The numbers of Nestin+ cells that differentiated from Smad4-/- ES cells after a 24 hr culture period were increased to 71% compared to 58% in the E14K wild-type control ES cells. Furthermore, a significant increase in βIII-tubulin+ neurons (26%) was observed from the Smad4-/- ES cells, compared to the E14K control ES cells (10%; t = 2.62, p < 0.05), and a greater number of the Smad4-/- ES cells demonstrated a more elaborate neuritic morphology (Figure 6B). These results support the notion that TGFβ signaling represses neural differentiation. Thus, at a relatively high cell density, inhibition of the BMP signaling pathway resembles increased cell dilution in its effectiveness in facilitating neural cell differentiation from ES cells.

**Undifferentiated ES Cells and Differentiated Neural Cells Are Distinct Populations, Even in High-Cell Density, Serum-Containing Cultures**

It is evident that even at high cell density some ES cells can start to express neural markers (e.g., Figure 2B), raising the possibility that some of the Nestin expression we are detecting at low densities after 24 hr is normally present in undifferentiated ES cells at high densities. As reported above, morphologically distinct Nestin+ cells were separate from Oct4+ undifferentiated ES cells in serum-free, low-density conditions (Figure 6). However, we further tested whether undifferentiated ES cells also
expressed Nestin when cultured at high cell densities (100 cells/μl) on a gelatin substrate in the presence of 15% FCS and LIF. After 24 hr in culture, many circumscribed undifferentiated ES cell colonies were observed, as well as separated cells distributed diffusely between the clusters. We found that the clusters of cells with the typical undifferentiated ES cell morphology do not express Nestin (Figures 7A and 7B). However, separate Nestin+ cells (16.1% ± 3.4% of the cells/well) were observed between clusters and their morphology resembled Nestin− cells from the low cell density cultures. In contrast, cells within the clusters expressed SSEA-1 (Solter and Knowles, 1978), an ES cell specific marker (65.3% ± 3.9% of the cells/well) (Figures 7C and 7D). Importantly, the population of cells expressing SSEA-1 did not overlap with the population of cells expressing Nestin, confirming the results we obtained in serum-free, low-density conditions. These findings may indicate that there is a direct phenotypic change from ES cells to neural cells. This direct phenotypic change is substantially inhibited at high cell densities, confirming our earlier experiments. There were significantly fewer (t = 9.3, p < 0.05) Nestin+ cells in these high-density cultures than the 82% Nestin+ cells seen in our low-density ES cultures after 24 hr (see above), again confirming our previous results of inhibition of neural differentiation with increasing cell density after 24 hr.

It is possible that, in the absence of a feeder cell substrate, some ES cells can escape neural inhibition especially in cell-sparse regions of the culture. To determine whether feeder cells can maximally inhibit the neural differentiation of ES cells, we cultured CFP ES cells on a feeder layer substrate at high cell densities in the presence of 15% FCS and LIF (our typical ES cell propagation and maintenance culture conditions). Greater than 98% viability of ES cells was observed when cultured under these conditions prior to immunostaining. Furthermore, the CFP ubiquitously expressed in the ES cells allowed us to unambiguously distinguish positive immunoreactivity between ES cells and feeder cells. Under these conditions, all of the CFP+ ES cells expressed nuclear Oct4, but were negative for Nestin expression. Similar results were observed using the SSEA-1 antibody. However, cells from embryonic forebrain-derived sphere colonies cultured for 2 days on feeder cells were positive for Nestin expression, but negative for Oct4 and SSEA-1 expression. Thus, under optimal culture conditions, ES cells maintain an undifferentiated ES cell phenotype and express ICM/ES specific markers, but do not express Nestin. We conclude that in the absence

Figure 6. Neural Cell Fate Inhibition Is Attenuated in Relatively Low-Cell Density Cultures
ES cells were cultured on a polyornithine substrate for 24 hr at 50 cells/μl or 10 cells/μl in the absence of exogenous growth factors or, where indicated, in the presence of LIF + FGF2 + heparin.

(A) Cells expressing Nestin, βIII-tubulin, NeuN, and Oct4. Cultures were counterlabeled with Hoechst nuclear dye to facilitate cell quantitation.

(B) In a similar manner, Nestin and βIII-tubulin expression were assessed in Smad4−/− ES cells, compared to the E14K wild-type controls, plated at 50 cells/μl. Data represent the average proportion of phenotype-specific cells (positively immunolabeled) per total numbers of cells (Hoechst labeled) obtained from 4–6 random standardized areas (using an ocular grid) at 20 × objective magnification from 3–6 separate cultures. Scale bar: 20 μm. ND, not determined.

<table>
<thead>
<tr>
<th>% Immunolabeled Cells</th>
<th>Cell Line</th>
<th>50 cells/μl</th>
<th>10 cells/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>40.0 +/- 13.6</td>
<td>82.0 +/- 4.0</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>13.3 +/- 2.3</td>
<td>51.0 +/- 8.9</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>n.d.</td>
<td>29.0 +/- 2.5</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>26.2 +/- 3.4</td>
<td>19.3 +/- 3.9</td>
<td></td>
</tr>
</tbody>
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of feeder-derived or serum-derived factors and at low cell densities, ES cells undergo a direct phenotypic change toward a neural fate, which is consistent with a default mechanism of neural fate specification.

Discussion

A Primitive Stage in the Neural Stem Cell Lineage

The ontogenesis of tissue-specific mammalian stem cells is not well understood. In the present study, we identified a novel cell type in the neural lineage based on the degree of neural commitment and growth factor responsiveness in vitro and the potential to give rise to neural and non-neural progeny in vivo. This cell type may be suitably described as a primitive neural stem cell. This term has been used previously to describe a stem cell that is primarily tissue-specific, but that retains a certain degree of pluripotency during a restricted early period of development (Morrison et al., 1997).

Neural colonies generated from ES cells share some similar features to forebrain stem cell colonies. At a very low frequency (0.2%), single ES cells proliferate in a LIF- and FGF-dependent manner to form neural colonies that express multiple neural precursor markers (e.g., Nestin, Emx2, Hoxb1), even though the vast majority of ES cells upregulate Nestin expression and downregulate nuclear Oct4 expression within 24 hr. We previously demonstrated that the proportion of FGF-dependent neural stem cells isolated from Nestin-expressing precursors of the E8.5 anterior neural plate was similar (0.3%) (Tropepe et al., 1999), and forebrain neural stem cell colonies express similar region-specific patterning genes (present study; unpublished data). Thus, the mechanism for segregating a subpopulation of colony-forming neural stem cells among a larger population of neural cells may be recapitulated during neural fate specification from ES cells. This raises the question of whether the first neural cell to arise in the nervous system is a neural stem cell or whether the first neural derivative is a general neural precursor cell that precedes (or is generated simultaneously with) the emergence of the neural stem cell lineage (van der Kooy and Weiss, 2000).

Figure 7. Undifferentiated ES Cells Do Not Express Nestin at High-Cell Densities

A small proportion of ES cells cultured at high cell densities (100 cells/μl) on a gelatin substrate in the presence of LIF and 15% FCS display a relatively large flattened morphology and express Nestin (arrowheads in [A]). In phase contrast images, these Nestin-positive cells are almost exclusively found between clusters of small rounded cells in areas of minimal cell to cell contact (arrowheads in [B]). The well-circumscribed clusters of cells, which do not express Nestin (arrow in [A] and [B]) resemble typical undifferentiated ES cell colonies. These aggregated cells express the undifferentiated ES cell–specific marker SSEA-1 (arrowheads in [C] and [D]). Moreover, the relatively large cells that resemble Nestin-positive cells do not express SSEA-1 (arrow in [C] and [D]). (E) A model depicting the establishment of the early neural lineage from ES cells. Totipotent ES cells derived from the E3.5 ICM directly differentiate (limited by the inhibitory control of TGFβ molecules) to give rise to LIF- and FGF-dependent pluripotent primitive neural stem cells that undergo relatively few symmetric (expansionary) divisions. These primitive neural stem cells can generate neurons and glia, but under appropriate environmental conditions (chimeric embryos) have the potential to generate cells with the capacity to differentiate into various cell types. As development proceeds, primitive neural stem cells give rise to FGF-dependent (and not LIF-dependent) neural stem cells that are present at the neural plate stage at E8.5 (Tropepe et al., 1999). The FGF-responsive neural stem cells initially undergo mostly asymmetric divisions, but at later stages divide symmetrically to expand their population. By E14.5, FGF-responsive neural stem cells also give rise to a relatively separate EGF-responsive neural stem cell population, both of which have the potential to generate neurons and glia (Tropepe et al., 1999; Martens et al., 2000). Scale bar: 40 μm.
Neural stem cells derived from ES cells also display other features than those derived from the embryonic forebrain, which may indicate an earlier primitive stage in the neural lineage. First, in contrast to neural stem cells isolated from embryonic or adult tissues, where either exogenous FGF or EGF is sufficient for colony formation and self-renewal, LIF and FGF are critical for ES-derived neural stem cell colony formation and subsequent stem cell self-renewal. The nature of the LIF effect on the ES to neural transition is not completely understood but may act as a survival factor (reviewed in Mehler and Kessler, 1997). The survival-promoting function of LIF is distinct at different stages of ontogenesis and, along with FGF and EGF, growth factor requirements may be sequentially modified from a primitive neural stem cell stage (Figure 7E). Second, expression of neural genes and at least one non-neural gene (GATA4) and the ability to extensively colonize various embryonic tissues in chimeric embryos in vivo indicates that the colony-forming neural stem cell retains a certain degree of pluripotency that is drastically reduced in forebrain stem cells.

In contrast to the ICM and subsequent epiblast cells in vivo, ES cells can express the neural precursor marker Nestin and the neuronal markers βIII-tubulin and NeuN within 24 hr when dispersed in culture in the absence of exogenous factors. The onset of Nestin expression in vivo occurs at approximately E7.5 within the neuroepithelium of the presumptive neural plate (Lendahl et al., 1990) and neuronal differentiation begins thereafter. One possibility that emerges from our findings is that the potential for cells within the ICM or epiblast to behave like primitive neural stem cells in vivo is actively suppressed. For example, epiblast cells in vivo may be competent to differentiate into neurons, but the absence of neurons prior to neurulation (even after a neural fate has been specified) suggests these cells may be inhibited from precocious neuronal differentiation.

Lineage Restriction in Developing Neural Stem Cells May Be Reversible
To what extent can the microenvironment dictate the identity of neural stem cells and their ability to produce different progeny? We demonstrate that ES-derived primitive neural stem cells can produce progeny that colonize neural and non-neural tissues in chimeric mice in vivo. In contrast, we were unable to generate chimeras using neural stem cell colonies derived from either the early embryonic or adult forebrain. This difference would suggest that primitive neural stem cells transiently retain their pluripotency, but through development neural stem cells become restricted in their ability to generate non-neural cell types. This restriction, however, may be reversible.

Clarke et al. (2000) recently demonstrated that a very low percentage (1%, n = 600 embryos) of adult neural stem cell colony cells could contribute to neural and non-neural tissues in a similar mouse chimera paradigm. An increase in the frequency of chimeras was observed when undissociated stem cell colonies were injected into the mouse blastocoel or chick amniotic cavity (Clarke et al., 2000). Consistent with our findings, the degree to which definitive forebrain neural stem cell-derived progeny can contribute to non-neural tissue in mouse is very restricted, compared to the proportion of ES cell-derived primitive neural stem cell progeny that contribute to neural and non-neural tissues in the present study (92%, n = 24 embryos). It is clear from their in vitro analysis of the inductive influence of EB on adult neural stem cell colonies that appropriate inductive signals can reveal the potential of neural stem cells to give rise to non-neural cells independent of the in vivo environment (Clarke et al., 2000). However, it is equally clear that this occurrence is rare, which may explain why we did not observe this phenomenon in our sample size. One intriguing possibility is that these inductive cues could enable some of the definitive neural stem cells to revert to a pluripotent primitive neural stem stage. Thus, the delineation of a pluripotent primitive neural stem cell stage during neural stem cell ontogenesis provides a basis for further investigations into the mechanisms governing this remarkable cellular plasticity.

Default Neural Cell Fate Specification during Mammalian Development
Once the primordium of the embryo proper is established (i.e., the segregation of ES cells in the ICM from extraembryonic tissues), the formation of the neural lineage is under inhibitory control. Our present findings suggest that in isolation at relatively low cell densities, ES cells have an autonomous tendency to differentiate into neural cells, but that this tendency is partially mitigated by intercellular signals (stronger at higher densities) that inhibit neural differentiation. In vivo, where cell density and neural inhibition are maximal, the differentiation of the neural stem cell lineage is highly dependent on the suppression of neural inhibition. We showed that blocking TGFβ-related signaling can augment the proportion of either primitive neural stem cell colony formation or neuronal differentiation, consistent with similar evidence for TGFβ-dependent neural inhibition obtained from experiments using several vertebrate species (Sasai et al., 1995; Fainsod et al., 1997; Hoodless and Hemmati-Brivanlou, 1997; Wilson et al., 1997; Grinblat et al., 1998). Thus, our observations reveal a striking similarity between mammals and other vertebrates in the propensity of uncommitted precursor cells to directly adopt a neural fate, which supports a default model of neural specification (Hemmati-Brivanlou and Melton, 1997).

The inhibition of the ES default to neural stem cells may occur even at lower doses of TGFβ signaling. At relatively low cell densities, ES cells may secrete TGFβ-related neural inhibitors (e.g., BMP4) in an autocrine fashion to limit the proportion of cells adopting a primitive neural stem cell phenotype. This may explain why a decrease in cell density did not increase the proportion of ES-derived primitive neural stem cell colonies after 7 days. However, a cell density-dependent change in neural differentiation of ES cells was observed after 24 hr. It is possible that although these low-density conditions alleviate some neural inhibition (mediated primarily by TGFβ signaling), colony-forming primitive neural stem cells may be more sensitive to very low concentrations of TGFβ. Thus, in the absence of all TGFβ signaling (e.g., Smad4−/− ES cells), enhanced primitive neural col-
ory is observed. Furthermore, if a default mechanism is solely responsible for neural stem cell fate specification, then other antagonists of neural inhibition in addition to TGFβ inhibitors may be required under our culture conditions in order to maximally promote an ES-to-neural default.

Default neural specification in mammalian ES cells is similar to the default neural specification that has been observed in amphibian-dissociated animal cap experiments. However, in amphibians, ectodermal cells differentiate into epidermis as their alternate fate when neural differentiation is inhibited. In contrast, we did not observe epidermal cell differentiation. Instead, an undifferentiated ES cell phenotype appeared to be maintained in the absence of neural differentiation. Thus, although the establishment of a neural phenotype may be under inhibitory control, additional signals may be required to influence ES cell commitment toward various non-neural lineages, including intermediate cell types such as ectoderm or mesoderm. Recent evidence suggests that under the influence of a stromal cell line, ES cells can respond to neural inhibitors (in this case BMP4) by differentiating into epidermal cells (Kawasaki et al., 2000). These data suggest that the influence of the stromal cell line may facilitate ectodermal commitment in ES cells, which will then allow these cells to respond to neural inhibitors in a manner that is identical to amphibian ectodermal cells. Therefore, the alternative to a default neural cell fate may be dependent on the degree of commitment toward a particular lineage.

Other studies investigating the role of BMP inhibition in neural fate specification using avian epiblast cells have come to different conclusions. BMP inhibition (by Noggin or Chordin) was not sufficient for ectopic neural cell differentiation in extraembryonic tissue (Streit et al., 1998), and dissociated epiblast cells preferentially adopted a muscle cell phenotype in culture (George-Weinstein et al., 1996). Combinations of multiple BMP and (Wnt) inhibitors may be required for avian neural differentiation to occur, which is evident in other vertebrate species (Glinka et al., 1997; Bachiller et al., 2000). Culture conditions employed in some chick studies (George-Weinstein et al., 1996) indicate that the results may in fact be consistent with the neural default model. Although the epiblast cells were cultured at relatively low cell densities (15 cells/µl), they were pretreated at high cell densities (400 cells/µl) for up to 5 hr in the presence of serum and chick embryo extract (George-Weinstein et al., 1996) a condition likely to suppress neural cell differentiation. Interestingly, these authors reported that neurofilament-expressing chick neurons were found in relatively cell-dispersed regions of the cultures, whereas muscle cells were typically aggregated. Thus, these data provide clear examples of how neural differentiation can be inhibited in epiblast cells upon aggregation.

An ES Cell Paradigm for Neural Stem Cell Fate Specification

Several studies have demonstrated neural differentiation from EB-derived cells, with the addition of specific growth factors (Doetschman et al., 1985; Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995; Okabe et al., 1996; Brustle et al., 1999). Also, BMP4 has been shown to suppress neuronal differentiation of EB-derived cells (Finley et al., 1999). Although these observations clearly demonstrate the potency of such factors to promote or attenuate neuronal differentiation, each experiment initially utilized EB cultures in the presence of serum. Here we present an alternative and specific paradigm for neural cell fate specification directly from ES cells in serum-free conditions in the absence of EB formation. This paradigm can facilitate, for instance, the discovery of systems that positively and negatively regulate the transition from an ES cell to a neural cell by utilizing an expression-based gene trap library of ES cell lines (Stanford et al., 1998; Seaberg et al., 1999). Thus, our present findings underscore the potential for using ES cell models of mammalian neural development.

Experimental Procedures

Propagation and Maintenance of ES Cells

The ES cell line R1 was grown on mitotically inactive fibroblast feeder layers or 1% gelatin and maintained in DMEM plus 15% FCS culture medium containing LIF (1000 U/ml) at low passage number (6–11) as previously described (Nagy and Rossant, 1993).

Culturing ES Cells

ES cells were washed, centrifuged, and resuspended in chemically defined serum-free media as previously described (Reynolds and Weiss, 1996). ES cells were plated at various cell densities in 24-well culture plates (Nunc) in the presence of LIF (1000 U/ml), FGF2 (10 ng/ml; Upstate Biotech or Sigma), and 2 µg/ml heparin (Sigma), EGF (20 ng/ml; Upstate Biotech or Sigma) individually, or in combination, or in the absence of any exogenous growth factors. For short-term (4–24 hr) neural differentiation, ES cells were plated in identical culture conditions in wells precoated with poly-L-ornithine (15 µg/ml, Gibco). Limiting dilution analysis was performed as previously described (Tropepe et al., 1999). The linear relationship observed between the cell density and the number of sphere colonies generated (regression coefficient R² = 0.99) can be accounted for by the clonal proliferation of a single rare population of cells. To assess colony formation at clonal densities, ES cells were plated in serum-free media containing LIF at 5 × 10³ cells per 94 mm Greiner hybridoma tissue culture dish (Greiner Labortechnik, Belco Glass, Vineland, NJ). Using this procedure, microwells contained ~15 viable cells per well (randomly assigned). Self-renewal of single primary colony-forming ES cells was assessed as previously described using growth factor concentrations as above (Tropepe et al., 1999). Cell viability after a 7-day culture period (sphere colony assay) or after 4–24 hr (short-term ES differentiation assay) was determined using Trypan blue exclusion (1:2 dilution of 0.4% trypan blue; Gibco). To determine the effect of a targeted null mutation in the Smad4 gene on neural colony formation, we used clones C8-13 and the wild-type E14K (+/+) ES cell lines (Sirard et al., 1998). There were no differences in colony formation between the various ES cell clones and thus the analysis included the pooled results from all of the clones. Human recombinant BMP4 protein (stock 0.812 mg/ml) was provided by Genetics Institute, and human recombinant Noggin protein (stock 1.05 mg/ml) was provided by Regeneron Pharmaceuticals.

Embryonic and Adult Dissections

Adult or pregnant CD1 mice (Charles River, Quebec) of gestational age 9.5 (E9.5) or E14.5 (see below) dissected as previously described (Chiaisson et al., 1999; Tropepe et al., 1999). In order to assess positive immunolabeling, E14.5 dissections of embryonic brain and skin were prepared as above and plated on a MATRIGEL substrate at high cell densities (100 cells/µl) in the same culture media containing 1% FBS. For RT–PCR analyses, tissues (e.g., brain, somite, liver, footpad epidermis) that served as positive controls were dissected and enzymatically treated in a similar fashion prior to RNA extraction.
Immunocytochemistry

Single sphere colonies were transferred to a well coated with MATRIGEL basement membrane matrix (15.1 mg/ml stock solution diluted 1:25 in serum-free media; Becton-Dickinson) in individual wells of a 24-well culture plate. Immunocytochemistry was performed as previously described (Tropepe et al., 1999). Primary antibodies were added as follows: anti-Nestin rabbit polyclonal (1:1000; a gift from Dr. R. McKay (Tohyama et al., 1982), anti-MAP-2 mouse monoclonal (IgG) (1:1000; Boehringer Mannheim), anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon), and anti-04 mouse monoclonal (IgM) (1:40; Boehringer Mannheim). FITC, TRITC, and DTAF (IgM) conjugated secondary antibodies were all diluted to 1:200 (Jackson ImmunoResearch). Cultures were incubated in Hoechst 33258 nuclear stain (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Boehringer Mannheim) to facilitate cell quantification. Control cultures were processed simultaneously using identical protocols except dilution solutions were devoid of primary antibodies. All controls were negative for immunolabeling. For short-term (24 hr) differentiation, ES cells were adhered to a poly-L-ornithine substrate (15 μg/ml; Sigma) and processed as above. Primary antibodies were as follows: mouse monoclonal anti-jii-tubulin antibody (1:1000; Sigma), anti-Nestin antibody (as above), rabbit anti-mouse Oct4 antibody (1:400; a gift from Dr. J. Cross), mouse monoclonal anti-NeuN (1:100; Chemicon), and mouse monoclonal anti-Cytokeratin (1:20; AE1/AE3, Boehringer Mannheim). For high cell density cultures of ES cells on gelatin, cells were fixed and washed as above and immunolabeled using Nestin (as above) and mouse monoclonal anti-SSEA-1 (1:500; MC-4B, Hybridoma Bank). FITC or TRITC secondary antibodies were diluted to 1:200; Jackson ImmunoResearch and cultures were counterlabelled with Hoechst (as above) or visualized with phase contrast and cells were quantified by counting 3–4 random standardized areas (using an ocular grid) at 20× objective magnification per culture. ES-derived or forebrain-derived sphere colonies were cryosectioned and mounted on gelatin coated slides as previously described (Tropepe et al., 1999) and processed for Nestin or Oct4 immunolabeling as above.

RT–PCR Analysis

Total RNA was isolated using the RNeasy extraction kit (Qiagen) and 1 μg was used to synthesize cDNA with oligo-d(T)16-25 primers and MuMLV reverse transcriptase (Superscript II; Boehringer-Mannheim) at 42°C for 1 h. For PCR, 1 μl cDNA was used to amplify specific sequences for the following genes: Emmx2, HoxB1, Otx1, Six3, Brachyury, GATA4, HNF4, Cytokeratin-17, and GAPDH. Primer sequences and PCR cycling conditions will be provided upon request. Amplified products were electrophoresed in 2% agarose gel containing ethidium bromide (25 μg/ml) and bands were visualized with UV light (DualLight Transilluminator, Fisher Biotech).

Expression of Mouse Cerberus-Like in Neuro2a Cells

Neuro2a cells were seeded at 1 × 106 cells per 100 mm petri dish and transiently transfected with 10 μg of plasmid DNA by means of LipofectAMINE ( Gibco) according to manufacturer’s instructions. After 6 h, the culture media was changed to 10 ml of DMEM plus 10% FBS ( Gibco). Twenty-four hours after transfection, culture media was changed to 10 ml of serum-free media. Seventy-two hours after transfection, cell supernatant was collected, aliquoted, and stored at −70°C. Addition of 4% (v/v) of supernatant (three separate experiments) resulted in a similar increase in colony formation compared to the addition of 20% (v/v) supernatant (two separate experiments), but this effect was considerably variable from one experiment to the next, compared to 20% (v/v). Thus data from the 20% (v/v) experiments were used for the analysis. The plasmids, pCS-V2 (gift from Dr. R. Moon) and pCS- cer-l (a gift from Dr. E. De Robertis) (Belo et al., 1997) were used.

Generation of Chimeras

ES sphere colonies were generated using ES cells harboring a YFP or CFP transgene (gifts from Drs K. Hadjantonakis and A. Nagy). Embryonic or adult forebrain-derived sphere colonies were generated from ES GFP transgenic mice (gift from Drs K. Hadjantonakis and A. Nagy) or ROSA mice (Jackson Laboratory) (Friedrich and Soriani, 1991). ES-derived or E9.5, E14.5, and adult forebrain-derived sphere colonies were aggregated with diploid CD1 morula stage embryos for 24 hr in vitro as previously described (Nagy and Rossant, 1993). Once integrated, the colony embryo aggregates were then transferred into pseudo-pregnant CD1 females, harvested at embryonic day E8.5–E9.5 and either stained for β-gal activity (for ROSA-CD1 chimeras) or visualized for fluorescence (GFP-CD1 chimeras). β-gal activity was detected using X-gal as previously described (Tropepe et al., 1999).

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References


