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Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences

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The mechanisms governing the emergence of the earliest mammalian neural cells during development remain incompletely characterized. A default mechanism has been suggested to underlie neural fate acquisition; however, an instructive process has also been proposed. We used mouse embryonic stem (ES) cells to explore the fundamental issue of how an uncommitted, pluripotent mammalian cell will self-organize in the absence of extrinsic signals and what cellular fate will result. To assess this default state, ES cells were placed in conditions that minimize external influences. Individual ES cells were found to rapidly transition directly into neural cells, a process shown to be independent of suggested instructive factors (e.g., fibroblast growth factors). Further, we provide evidence that the default neural identity is that of a primitive neural stem cell (NSC). The exiguous conditions used to reveal the default state were found to present primitive NSCs with a survival challenge (limiting their persistence and proliferation), which could be mitigated by survival factors or genetic interference with apoptosis.

Introduction

The emergence of the earliest neural cells during mammalian development and the mechanisms that govern this process remain incompletely characterized. Such cells are likely to be neural precursors or stem cells, though the ontogeny of the neural stem cell (NSC), which can be isolated from embryonic and adult forebrain (Weiss et al., 1996; Gage, 2000), has not been fully elucidated. During development, neural cells arise from the ectodermal germ layer, which also produces epidermis. According to the classical model of this process, conceptualized largely from amphibian embryology studies, nascent embryonic ectoderm receives a positive signal from a specialized group of dorsal mesodermal cells, termed the organizer, which instructs the adjacent ectodermal cells to adopt a neural fate (Harland and Gerhart, 1997; Weinstein and Hemmati-Brivanlou, 1999; Spemann and Mangold, 2001). The structural equivalent of the organizer in amniotes is the node. It was thought that organizer/node–derived signals were necessary for the process of neural induction and that in their absence the ectoderm would adopt an epidermal fate.

More recent data have challenged the validity of this classical model. Low-density cultures of dissociated ectodermal cells, in the absence of organizer tissue, were found to differentiate into neural cells (Grunz and Tacke, 1989; Sato and Sargent, 1989; Godsave and Slack, 1991). Furthermore, undissociated ectodermal explants expressing a dominant-negative receptor for activin (a member of the TGFβ family of growth factors), which effectively inhibited signaling of multiple TGFβ-related molecules (Schulte-Merker et al., 1994; Hemmati-Brivanlou and Thomsen, 1995), were shown to become neural when cultured in vitro (Hemmati-Brivanlou and Melton, 1994). Signaling molecules secreted from the organizer tissue, such as Noggin, Chordin, and Follistatin, were found to exert potent neuralizing effects (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995) and were thus initially thought to represent the instructive neuralizing signal. However, the mechanism by which they promoted neural differentiation of ectodermal cells was not entirely consistent with the existing positive induction model. The neuralizing effects of these factors were found to depend on inhibitory interactions with bone morphogenic proteins (BMPs), which are members of the TGFβ family of molecules that strongly inhibit neural differentiation (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). Thus, their mechanism of action appeared to be through prevention of BMP binding to their cognate receptors on ectodermal cells.

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Abbreviations used in this paper: *aif*, *apoptosis-inducing factor*; *apaf1*, *apoptotic protease activating factor 1*; BMP, bone morphogenic protein; *cas9*, *caspase 9*; cGMP, cyclic guanosine monophosphate; E, embryonic day; EB, embryoid body; ES, embryonic stem; ICM, inner cell mass; LIF, leukemia inhibitory factor; NAC, *N*-acetyl-L-cysteine; NFM, neurofilament-M; NSC, neural stem cell; NS, neurosphere; pCPT, 8-(4-chlorophenylthio).

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These findings led to the development of the currently more widely accepted model, the default model, which states that each individual ectodermal cell has an intrinsic default program to become a neural cell (Munoz-Sanjuan and Brivanlou, 2002). In the context of the intact embryo, this default program is being actively suppressed by ubiquitously expressed BMPs. Thus, the organizer tissue does not provide a positive inductive signal but rather secretes factors that antagonize BMP signaling, thereby disinhibiting the default neural program in proximal ectodermal cells.

Several subsequent studies have challenged the default model of neural fate acquisition. For example, experiments in chicks have suggested that BMP inhibition may not be sufficient to induce neuralization (Streit et al., 2000; Linker and Stern, 2004). However, it is uncertain how complete the BMP inhibition was in these studies, and it is possible that the activity of individual BMPs was insufficiently suppressed to allow neuralization to occur (and/or that some BMP subtypes or other neural inhibitors escaped blockade). It has also been suggested that other factors, such as FGF and Wnt signaling, are involved with neural specification in several vertebrates (Baker et al., 1999; Streit et al., 2000; Wilson et al., 2000, 2001), though whether they are required for the initial neural fate change or for the later expansion of this neural population is currently unresolved. Further, their mechanism of action may be through modulation of BMP gene transcription (Bainter et al., 2001), consistent with a model of BMP inhibition–mediated neuralization.

There are currently few published studies examining the neural default model in mammalian cells, and there is controversy over whether such a default neural mechanism exists in mammals. In an effort to determine whether a default mechanism underlies neural fate specification from uncommitted mammalian precursors, we undertook studies using mouse embryonic stem (ES) cells, which are derived from the inner cell mass (ICM) of the blastocyst-stage embryo and represent a model of the earliest pluripotent mammalian cell (Evans and Kaufman, 1981; Martin, 1981). ES cells are capable of generating entire viable mice in vivo (Nagy et al., 1993) and are able to produce most, if not all, cell types in vitro (Beddington and Robertson, 1989; Rathjen and Rathjen, 2001; Wobus, 2001). Use of ES cells to investigate neural determination can potentially provide many insights into the developmental process. Importantly, though, their use in assessing a default fate specification mechanism allows us to explore a more basic and fundamental issue, i.e., how an uncommitted, pluripotent mammalian cell will self-organize in the absence of extrinsic instructive or inhibitory signals and what cellular configuration/fate will result.

The standard methodology for the in vitro differentiation of ES cells typically involves the formation of embryoid bodies (EBs; Desbaillets et al., 2000), which are formed by aggregation of ES cells in the presence of serum and in the absence of leukemia inhibitory factor (LIF), a cytokine necessary for maintaining ES cells in an undifferentiated state. EBs contain many different cell types that are fated to produce cells of all three primary germ layers. Because there is complex intercellular signaling between the multiple cell types of an EB and they are generated in the presence of serum with its host of undefined factors, EB formation precludes a direct analysis of the mechanisms regulating the differentiation of a specific cell lineage. To assess a default state, we wanted to isolate single ES cells and minimize any exposure to extrinsic factors that might be either instructive or inhibitory to cell fate specification. Therefore, we used a system of chemically defined serum- and feeder layer–free culture conditions coupled with low cell densities (to abrogate intercellular signaling). In a previous study, we reported that these conditions appeared to favor neural determination of ES cells (Tropepe et al., 2001). Further, a novel colony-forming primitive NSC population arose under these conditions, one with characteristics intermediate to those of ES cells and forebrain-derived "definitive" NSCs. Here, we demonstrate default neural fate acquisition by ES cells, a process shown to be independent of potential instructive factors. FGFs were found to be important for the proliferation but not the generation of the default pathway–derived primitive NSCs. Further, we provide evidence that the default neural fate pathway specifically gives rise to primitive NSCs and that primitive NSC mortality resulting from a survival challenge, which could be mitigated by survival factors or genetic interference with apoptosis, was responsible for limiting the persistence and proliferation of these cells.

Results

ES cells rapidly acquire a default neural identity in minimal conditions

To assess the potential default fate of ES cells, we removed any factors that might be either instructive or inhibitory to cell fate specification. Therefore, single dissociated R1 ES cells were plated at low cell densities ($\leq$10 cells/μl; 2,600 cells/cm^2) in chemically defined serum- and growth factor–free media. As we reported previously (Tropepe et al., 2001), ES cells placed in these minimal conditions rapidly acquired a neural identity, with >90% of cells initiating expression of nestin, an intermediate filament protein associated with neural precursors (Lendahl et al., 1990), within 4 h (Fig. 1 A). The neural precursor identity of these cells was supported further by their expression of Sox1, one of the earliest transcription factors expressed in cells committed to the neural fate (Pevny et al., 1998; Fig. 1 C). Undifferentiated ES cell colonies did not exhibit expression of these markers (Fig. 1, B and D). At 4 h, no significant cell mortality was observed and <1% of the plated cells had proliferated, indicating that single ES cells began a direct transition to neural cells, without requirement for cell division. After an additional 20 h, most (77.5 $\pm$ 1.3%) of these ES-derived neural cells did not survive, as these minimal conditions were not very supportive. However, 99.3 $\pm$ 0.2% of the remaining viable cells expressed nestin and Sox1 and maintained some expression of Oct4, a transcription factor expressed in ES cells (Nichols et al., 1998; Fig. 1, E and F). To verify that the observed upregulation of nestin was not a nonspecific stress response, STO fibroblasts were placed in identical conditions; however,

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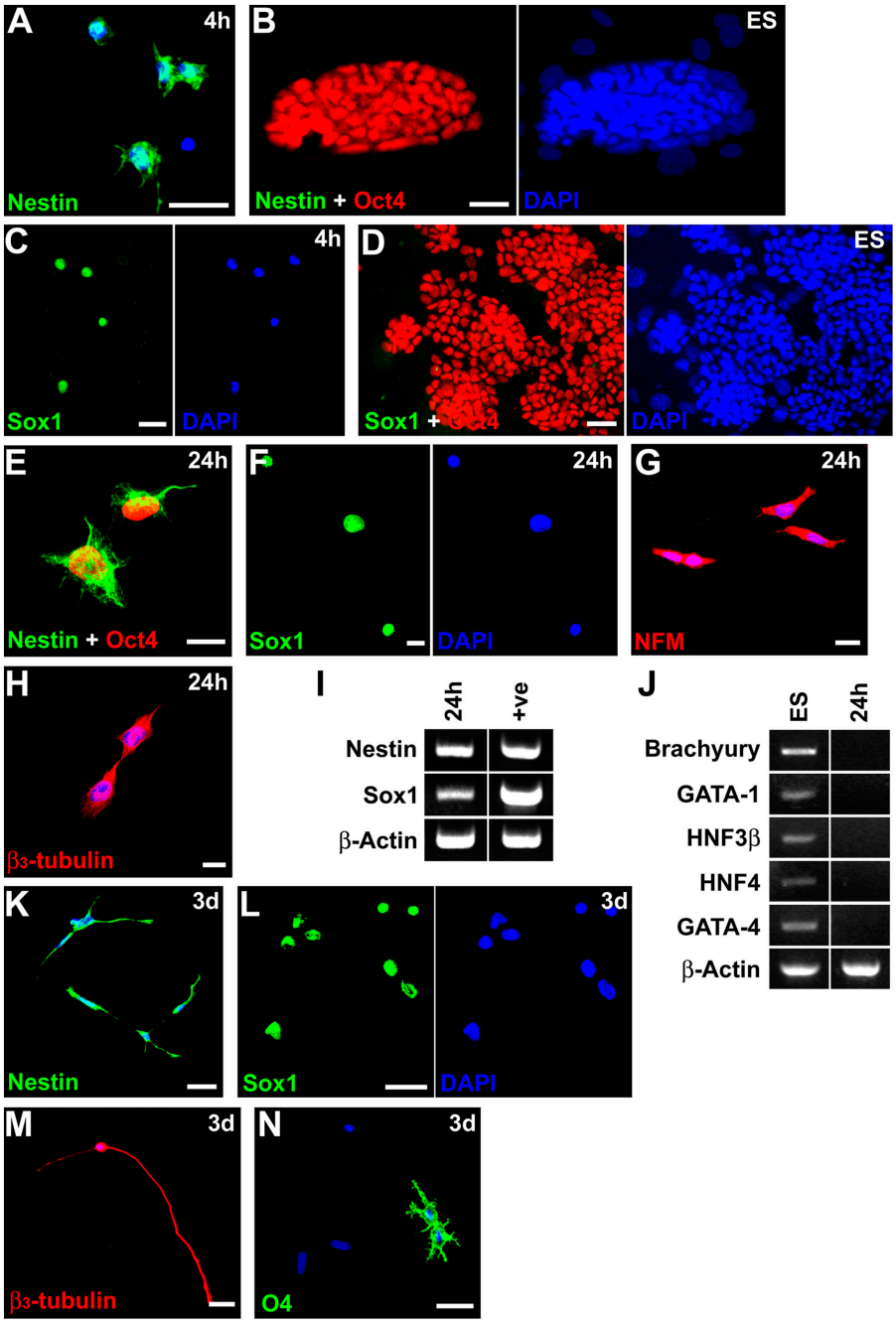

Figure 1. **ES cells rapidly transition into neural cells when placed in minimal conditions.** (A and C) Immunocytochemical labeling showed that ES cells placed in minimal media conditions (for 4 h) initiated pronounced expression of the neural precursor markers nestin (A) and Sox1 (C). The nuclear stain DAPI was used to identify all cell nuclei within the field. (B and D) Undifferentiated ES cells (growing on a fibroblast feeder layer) exhibited Oct4 (B) but not nestin or Sox1 (D). The large Oct4$^-$ nuclei are feeder cells. (E) By 24 h, almost all cells expressed nestin and maintained some nuclear Oct4 expression and Sox1 (F). (G and H) Most of the ES-derived neural cells express NFM (G), and many express the early neuronal marker β_3-tubulin (H; at 24 h). (I) Expression of nestin and Sox1 was confirmed by RT-PCR analysis of ES-derived neural cells (at 24 h). (J) Brachyury and GATA-1 (mesodermal markers), as well as HNF3β, HNF4, and GATA-4 (endodermal markers), were detected by RT-PCR in undifferentiated ES cells, though they were rapidly down-regulated within 24 h in minimal conditions. (K and L) After 3 d, most surviving ES-derived neural cells retain a neural precursor identity, maintaining expression of nestin (K) and Sox1 (L). (M and N) Differentiating cells with more elaborate morphologies were also apparent as β_3-tubulin$^+$ neurons (M) and O4$^+$ oligodendrocytes (N). Bars: (A–D and K–N) 25 μm; (E–H) 10 μm.

RT-PCR and immunostaining did not show any nestin expression either before or after culture in minimal media (unpublished data).

Further, 24 h after cell plating, 90.4 ± 1.7% of the surviving cells expressed neurofilament-M (NFM) and 47.5 ± 3.6% expressed the early neuronal marker β_3-tubulin (Fig. 1, G and H). RT-PCR analysis confirmed expression of nestin and Sox1 (Fig. 1 I). Additional confirmation of neural lineage commitment after 24 h was evidenced by the rapid down-regulation of brachyury and GATA-1 (mesodermal markers), as well as HNF3β, HNF4, and GATA-4 (endodermal markers), which were detectable in ES cells (Fig. 1 J). At 3 d, most surviving cells maintained a neural precursor identity, expressing nestin and Sox1 (Fig. 1, K and L). Cells with more elaborate neural morphologies were also evident, with the observation of neuronal and glial cells (Fig. 1, M and N). Cells with very advanced morphology, and positive for a neural cell subtype marker, typically displayed down-regulation of nestin expression (faint or absent immunostaining) and were Oct4$^-$ (unpublished data). By 7 d, viable cells were not observed, indicating that the exiguous nature of the media conditions was not supportive enough for the maintained survival of the neural cells. These data indicate that in the absence of extrinsic signals, ES cells rapidly begin to acquire a neural precursor identity, consistent with a default mechanism for an ES cell to transition directly into a neural cell.

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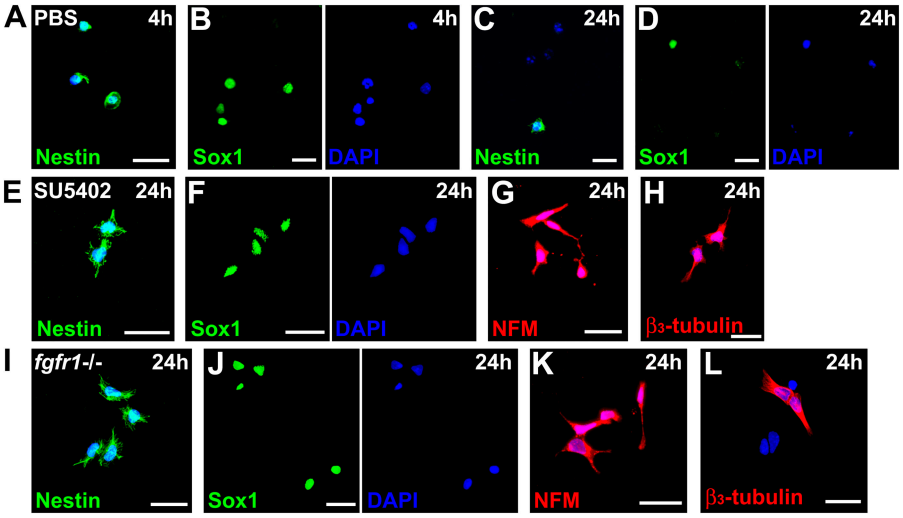

Figure 2. **The ES cell neural transition occurs by default without requirement for instructive factors.** (A and B) Initiation of nestin (A) and Sox1 (B) expression was observed by immunocytochemical labeling within 4 h when ES cells were placed in PBS alone. (C and D) After 24 h in PBS, the few remaining viable cells expressed nestin (C) and Sox1 (D). The fragmented nuclei (punctuate DAPI staining) represent dead cells. (E–H) Pharmacological inhibition of FGF signaling using SU5402 (5 μM) did not prevent the rapid acquisition of neural markers by ES cells placed in minimal conditions for 24 h, with expression of nestin (E), Sox1 (F), NFM (G), and β_3-tubulin (H) observed. (I–L) Similarly, ES cells harboring deletion of the *fgfr1* gene displayed the typically observed neural markers by 24 h, expressing nestin (I), Sox1 (J), NFM (K), and β_3-tubulin (L). Bars, 25 μm.

ES cell neuralization occurs by default without requirement for any exogenous instructive factors

Is this neural transition truly occurring by default? A protein component of the media formulation, transferrin, has been suggested to be required for ES cell neural fate initiation (Ying et al., 2003). We show that nestin and Sox1 expression was established after 4 h, even when ES cells were cultured in PBS alone, ruling out any requirement for media components in an instructive capacity (Fig. 2, A and B). After a 24-h culture period in PBS, very few cells remained viable, though they all expressed nestin and Sox1 (Fig. 2, C and D). The fragmented nuclei represent dead cells, which typically did not display immunoreactivity for the neural markers.

Though not exogenously required, autogenously produced FGFs have been proposed to be essential for ES cell neuralization, suggesting an instructive process (Ying et al., 2003). To the contrary, pharmacological inhibition of FGF signaling using the FGF receptor kinase antagonist SU5402 (5 and 10 μM) in our minimal conditions did not prevent the rapid acquisition of neural markers by ES cells (Fig. 2, E–H). Similarly, ES cells harboring deletion of the FGF receptor-1 gene (*fgfr1*; Ciruna et al., 1997) displayed the typically observed neural markers (Fig. 2, H–K). Further, SU5402 treatment or *fgfr1* deletion did not prevent the advanced neural morphologies and marker expression found at 3 d in culture (unpublished data). These data strongly suggest that neither media components (including transferrin) nor FGF signaling is required for ES cell neuralization, supporting the default nature of this transition.

Primitive NSCs emerge from the default neural pathway

What is the nature of the neural cells emerging from this default pathway? The early expression of nestin and Sox1 indicates that they are neural precursors. However, these markers are expressed both in true NSCs as well as in more restricted neural progenitor cells. Our previous work introduced a novel neural precursor arising under these conditions, the primitive NSC (Tropepe et al., 2001), identified with an assay analogous to the neurosphere (NS)-formation assay used to identify definitive forebrain-derived NSCs. When the cytokine LIF was included in our minimal condition assay, a very small percentage (0.18 ± 0.01%) of the initially plated ES cells became neural cells that proliferated over 7 d to form clonally derived floating sphere colonies, termed primitive NSs (Fig. 3 A). LIF was not required for early ES cell neuralization, as it could be added after 4 h without any decrement in primitive NS production (101 ± 4% of control). Rather, LIF appeared to prevent further differentiation down the neural lineage and thereby maintained these neural cells in an undifferentiated, proliferative state. This interpretation is supported by the findings that LIF addition after 2 d did not enable any primitive NS generation (unpublished data), indicating that there was an LIF-responsive temporal window, after which the neural cells had progressed to a non–LIF-responsive, unproliferative, and more differentiated state. These primitive NSs expressed both nestin and Sox1 (Fig. 3, B and C), similar to NSs generated by definitive NSCs, indicating that they are composed of neural precursors. The primitive NS cells also maintained some expression of Oct4 (by immunostaining and RT-PCR), as well as nanog (by RT-PCR), another transcription factor expressed by ES cells (Chambers et al., 2003; unpublished data). The neural precursor character of the primitive NSs was confirmed by expanded RT-PCR gene expression analysis. Various additional neural genes were expressed by primitive NSs, including Sox2, Sox3, Neurogenin1, NeuroD, Pax6, Nkx2.2, Mash1, Musashi-1, Otx2, and HoxB1, strongly supporting their neural nature (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200508085/DC1). Primitive NSs did not express CK-17 (an epidermal marker), nor did they express the mesodermal markers brachyury and GATA-1 or the endodermal markers HNF3β, HNF4, and Pax4. These multi–germ layer markers are typically found in EBs (Wassarman and Keller, 2003), and their absence further verifies that the primitive NS cells are specified to the neural lineage. Transcripts for most of these genes were found at detectable levels in undifferentiated ES cells, suggesting that there may be widespread, low-level, promiscuous gene expression in the uncommitted state and that

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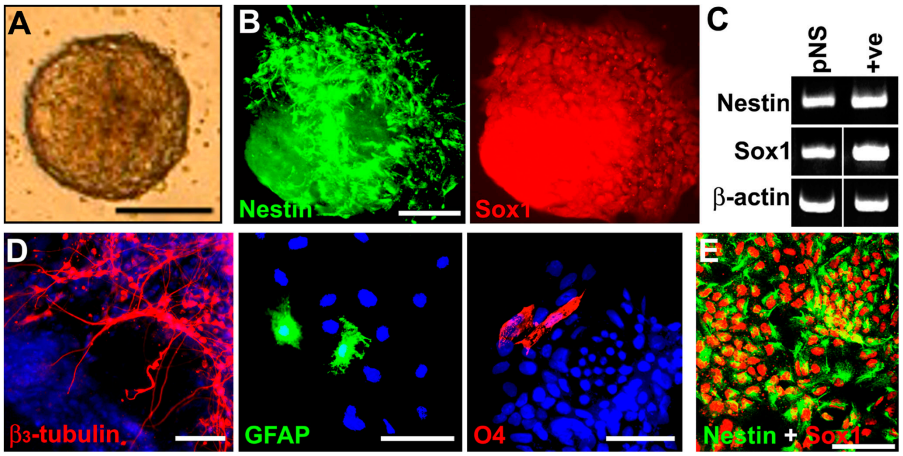

Figure 3. **Primitive NSCs emerge from the ES cell default neural pathway.** (A) When LIF was included in the minimal conditions, a small number of ES-derived neural precursor cells proliferated over 7 d to form clonally derived floating sphere colonies, termed primitive NSs. The primitive NS–initiating cell was termed a primitive NSC. (B) The primitive NS cells were found to coexpress nestin and Sox1 by immunocytochemical labeling, demonstrating their neural precursor identity. (C) RT-PCR analysis confirmed expression of nestin and Sox1 within primitive NSs (pNS). (D and E) When differentiated, primitive NSs yielded β_3-tubulin$^+$ neurons, GFAP$^+$ astrocytes, and O4$^+$ oligodendrocytes (D), whereas most cells retained a neural precursor phenotype, maintaining expression of nestin and Sox1 (E). Bars: (A) 100 μm; (B–D) 50 μm.

differentiation may involve the down-regulation of certain genetic programs with the maintenance and up-regulation of others. The primitive NS cells could be maintained in an undifferentiated state by subcloning/passaging in serum-free conditions to produce secondary, tertiary, and successive spheres. Interestingly, secondary and subsequent sphere formation was dependent on the addition of exogenous FGF2, similar to the FGF2-dependent proliferation of forebrain-derived NSCs. When transferred to conditions promoting differentiation, individual primitive NSs differentiated to produce neurons, astrocytes, and oligodendrocytes (Fig. 3 D), the three neural lineage cell types. Cells negative for differentiation markers maintained a neural precursor identity, expressing nestin and Sox1 (Fig. 3 E). Differentiation of the passaged spheres was similar to that of the primary primitive NSs (unpublished data). Though the cells present after in vitro differentiation of primitive NSs appeared limited to differentiated and immature/precursor neural cells, the maintained expression of some ES-like genes (Oct4 and nanog) in the undifferentiated primitive NS state suggested that cells constituting the primitive NS were not yet absolutely committed to the neural lineage. This is supported by our previous finding that primary primitive NSs could still manifest broader lineage potential when placed in the appropriate developmental environment, i.e., when used to generate morula-aggregation embryo chimeras (Tropepe et al., 2001). However, when the primitive NSs were passaged to produce secondary spheres (with FGF2 and without LIF), efficient chimera production was not observed (similar to our results obtained using NSs from forebrain-derived adult NSCs), suggesting that the secondary spheres were now fated exclusively to the neural lineage (Tropepe et al., 2001; unpublished data). Further, expression of Oct4 and nanog was not found by RT-PCR in the secondary spheres (unpublished data). Thus, a novel neural precursor, the primitive NSC, arose in these default conditions.

Primitive NSC proliferation is dependent on autogenously produced FGF signaling

Though not required for early default neural fate acquisition, autogenously produced FGFs were found to be necessary for primitive NS formation. Inclusion of 5 μM SU5402 in the primitive NS assay virtually eliminated primitive NS generation (reduced by 98 ± 1%; Fig. 4 A), and *fgfr1* −/− ES cells displayed dramatically diminished (by 91 ± 1%) primitive NS production (Fig. 4 B). This suggests that autogenous FGF signaling was important for the proliferation and/or survival of primitive NSCs. The proliferation interpretation is supported by results obtained from rescue experiments. When SU5402 was included in the primitive NS assay, minimal proliferation was observed by 3 d. If the drug was then removed and the assay was continued for 7 d, primitive NSC proliferation resumed and there was a large recovery of primitive NS formation (Fig. 4 C). Similarly, if the drug was maintained for a full 7 d and then removed, a substantial recovery of primitive NS formation was observed (Fig. 4 C). Further, if SU5402 was added on day 3 of the assay (at which point the cells have established their neural transition and primitive NSCs are proliferating), proliferation was impaired and there were consequently fewer primitive NSs observed after 7 d (Fig. 4 D). As FGF signaling was dispensable for the default neural fate switch in the short-term (4 and 24 h) experiments discussed previously, this suggests that primitive NSCs are still formed in the transition from ES cells, though their proliferation to form primitive NSs is dependent on signaling by autogenously produced FGFs. Sonic Hedgehog signaling, reported to be important in the regulation of some neural progenitors (Zhu et al., 1999), was found to be dispensable, as Sonic Hedgehog inhibition with cyclopamine had no inhibitory effect on primitive NS formation (unpublished data).

Cell mortality limits the number of default pathway-derived primitive NSCs that form primitive NSs

Given the low frequency of ES-derived neural precursors (~0.2%) that proliferate to form primitive NSs (which is our assay for the presence of a primitive NSC), the question remains as to whether the default fate pathway specifically gives rise to primitive NSCs or simply to a population of generalized and perhaps heterogeneous cells of the neural lineage. Though neural in nature, it is possible that this ES-derived population contains a very small subset of bona fide primitive NSCs, capable of long-term passaging and production of multilineage

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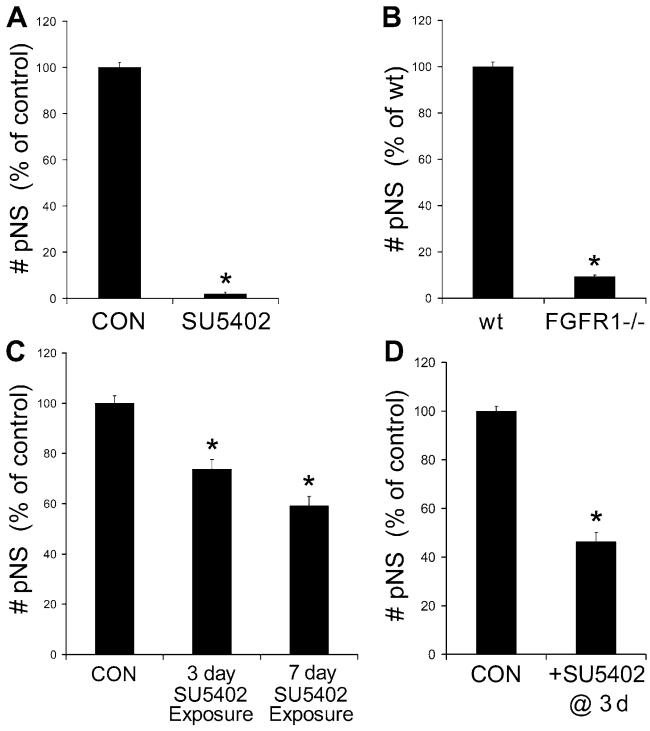

Figure 4. **Primitive NSC proliferation is dependent on autogenously produced FGF signaling.** (A) Pharmacological inhibition of FGF signaling throughout the primitive NS assay using SU5402 (5 μM) virtually abolished primitive NS formation (pNS). *, $P < 0.001$ versus control. (B) ES cells with a deletion of the *fgfr1* gene displayed drastically reduced primitive NS production. *, $P < 0.001$ versus wild type (wt). (C) When SU5402 was included for 3 or 7 d of the primitive NS assay and then removed, the primitive NSCs resumed proliferation, and a substantial recovery of primitive NS formation was observed over an additional 7 d. *, $P < 0.001$ versus condition with maintained SU5402 (not depicted). (D) When SU5402 was added on day 3 of the primitive NS assay, proliferation of already proliferating primitive NSCs was impaired and fewer primitive NSs ensued. *, $P < 0.001$ versus control.

progeny, whereas the remainder may represent more restricted or committed neural cells. If the default pathway does specifically give rise to primitive NSCs, there must be some inhibitory influences preventing the exhibition of the primitive NSC phenotype, i.e., primitive NS generation, in all of the individual neural derivatives of ES cells.

The early nestin$^+$ neural cells present after 4 h in minimal conditions subsequently underwent a survival challenge because of the exiguous nature of the media, resulting in extensive cell mortality (77.5 ± 1.3% by 24 h). In the absence of LIF, mortality progressively increased over 7 d, until no viable cells remained. In the presence of LIF, a few of the neural cells retained a proliferative character, surviving and propagating to form primitive NSs, though the vast majority of the early nestin$^+$ cells still died. Accordingly, primitive NSCs may have been produced but did not form primitive NSs because of the early survival challenge that they experienced. Thus, increasing cell viability should enhance primitive NS production. The survival factor *N*-acetyl-L-cysteine (NAC), which has multiple modes of action but is thought to act primarily through its antioxidant effects (Zafarullah et al., 2003), promotes survival of various neural cell types (Mayer and Noble, 1994; Ferrari et al., 1995; Yan et al., 1995). Inclusion of NAC dose-dependently increased primitive NS formation (up to ~35-fold), with toxic effects observed at very high concentrations (Fig. 5 A). We also explored the cAMP–protein kinase A pathway as a more physiological modulator of cell survival. cAMP signaling has been shown to enhance cell survival in several different neural cell systems (Rolletschek et al., 2001; Bok et al., 2003; Lara et al., 2003). Exogenous application of a membrane-permeable cAMP analogue, 8-(4-chlorophenylthio) (pCPT)-cAMP, was able to dose-dependently augment primitive NS formation (up to ~25-fold), with toxic effects observed at exceedingly supraphysiological concentrations (Fig. 5 A). Concentrations at which peak effects were observed were used in all subsequent experiments (1 mM NAC and 100 μM pCPT-cAMP). When added simultaneously, the effects of NAC and pCPT-cAMP were additive, if not synergistic, enhancing primitive NS formation (up to ~100-fold) such that ~20% of the initially plated ES cells became primitive NS–forming primitive NSCs (Fig. 5 B). This also suggests that the two compounds have distinct mechanisms of primitive NSC survival promotion (leading to subsequent primitive NS formation). RT-PCR analysis of primitive NSs derived in each or both factors demonstrated the expression of the neural precursor markers without detection of the multi–germ layer markers (discussed previously). Addition of NAC and/or pCPT-cAMP after 4 h of the primitive NS assay (at which point the cells have already undertaken their neural transition) revealed no decrement in their ability to augment primitive NS formation (NAC addition at 4 h was 98 ± 3% of NAC added from start, and pCPT-cAMP addition at 4 h was 101 ± 3% of pCPT-cAMP added from start), suggesting that they were not effecting the NS increase through an instructive role in early differentiation. Additionally, this demonstrated that the survival-promoting effects of NAC and cAMP were not directly on ES cells but rather on their nestin$^+$ clonal neural derivatives.

Viability assessment showed that NAC and pCPT-cAMP did indeed increase cell survival under minimal conditions. At 24 h, NAC increased survival of the initial 4-h nestin$^+$ cells from 22.5 ± 1.3 to 44.8 ± 2.3% ($P < 0.01$), whereas pCPT-cAMP increased survival to 43.6 ± 2.4% ($P < 0.01$). Though the survival factor–induced increases in viability relative to the control media were observed over the course of the complete 7 d, progressive mortality was still observed. Even with both factors present in the primitive NS assay, there was still 34.4 ± 2.6% mortality for the initial 4-h nestin$^+$ cells after 24 h and 54.3 ± 2.9% mortality by 3 d. There were very few cells that survived past the 7-d assay that were not in a primitive NS, suggesting that only cells that were in the context of a proliferating sphere were effective at long-term survival in these conditions. It is worth noting that although the survival factors greatly increased cell viability at 1 and 3 d in minimal media, the rapid acquisition of neural identity and the frequencies of marker-positive cells (discussed previously and in Fig. 1) were not altered (not depicted). Thus, these results indicate that the primitive NSC is the primary identity of cells derived from the default pathway but that cell mortality limits the number that

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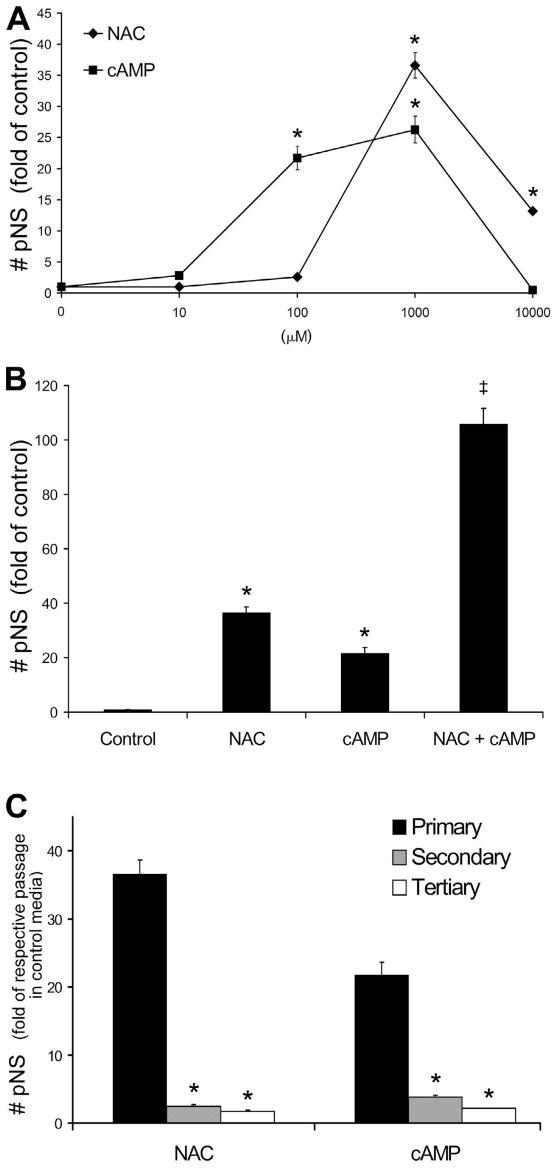

Figure 5. **A survival challenge attributable to the minimal culture conditions limits the number of ES-derived neural precursors that survive to form primitive NSs.** Extensive cell mortality was observed upon culture in the minimal conditions used for the default state assessment and primitive NS (pNS) assay. (A) Improving cell viability by inclusion of the survival factors NAC and the membrane-permeable cAMP analogue pCPT-cAMP (cAMP) dose-dependently enhanced primitive NS formation. *, $P < 0.01$ versus control. (B) Concurrent inclusion of 1 mM NAC and 100 μM pCPT-cAMP (a concentration at which peak effects were observed in A) in the primitive NS assay showed that they produced an additive, if not synergistic, effect. *, $P < 0.001$ versus control; ‡, $P < 0.001$ versus control and versus each factor alone. (C) The effectiveness of NAC (1 mM) and pCPT-cAMP (100 μM) in promoting NS formation decreased dramatically with passaging of NSs. *, $P < 0.001$ versus respective survival factor primary spheres.

can survive to form clonal primitive NSs and thereby be detected by our assay.

The ability of NAC and pCPT-cAMP to facilitate primitive NS formation decreased dramatically and progressively when the compounds were added during the derivation of secondary and tertiary spheres (Fig. 5 C). This suggests that the extensive survival challenge that limited primitive NS generation occurred only with the primary ES–derived primitive NSCs and not with the subsequently passaged NSCs, which are similar to forebrain-derived definitive NSCs (which also did not exhibit increases in NS formation with the survival factors; unpublished data). This effect highlights a fundamental difference between primitive NSCs and the passaged, more mature definitive NSCs. When individual primary spheres derived in the presence of pCPT-cAMP were passaged back into control media for secondary sphere formation, they generated 2.3 ± 0.2 times ($P < 0.01$) more secondary spheres compared with primary spheres that had been derived in control media. Spheres derived in NAC did not display any increased secondary sphere formation when passaged back into control media. This suggests that pCPT-cAMP may have had an additional effect, increasing the number of symmetric divisions that the primitive NSC underwent, thereby expanding the stem cell numbers within the primary sphere. Alternately, there may have simply been a prolonged survival enhancement in the pCPT-cAMP–derived primary sphere cells, even when they were placed back into media without pCPT-cAMP. Primitive NSs derived in NAC and/or pCPT-cAMP did not display any notable alterations in differentiation (unpublished data). Pharmacological modulation of the endogenous pathways showed that the cAMP pathway positively regulated and the cyclic guanosine monophosphate (cGMP) pathway negatively regulated primitive NSC survival and primitive NS formation (see online supplemental material).

Genetic deletion of apoptotic pathway components enhances primitive NS formation

To further substantiate that cell mortality limited the number of primitive NSCs that survived to proliferate and form clonal primitive NSs, we used ES cell lines with a survival advantage conferred by mutations in apoptotic signaling pathway components. *Apoptotic protease activating factor 1* (*apaf1*) and *caspase 9* (*cas9*) are components of the more common apoptotic pathway (Cai et al., 1998), whereas *apoptosis-inducing factor* (*aif*) is a mediator of an alternate, independent apoptotic pathway (Cande et al., 2002). The *apaf1*−/− (Yoshida et al., 1998), *cas9*−/− (Hakem et al., 1998), and *aif*−/Y (Joza et al., 2001) ES cells displayed substantially increased primitive NS formation relative to wild-type ES cells, with a more pronounced effect observed for the *apaf1* and *cas9* mutants (Fig. 6 A). Heterozygotes for *apaf1* and *cas9* displayed an intermediate effect, indicative of a gene dosage effect (Fig. 6 A). Differentiation of primitive NSs generated by these ES cell lines was similar to wild type (unpublished data). This demonstrates that, similar to the effect of survival factors, survival promotion through interference with apoptosis enhanced primitive NS formation. This also suggested that the primary apoptotic pathway responsible for mediating cell death in these cultures was the *apaf1–cas9* apoptotic pathway, although there was still some contribution from the *aif* pathway.

If the primitive NS–promoting effect of the survival factors NAC and pCPT-cAMP was achieved through a reduction in primitive NSC death mediated by these apoptotic pathways,

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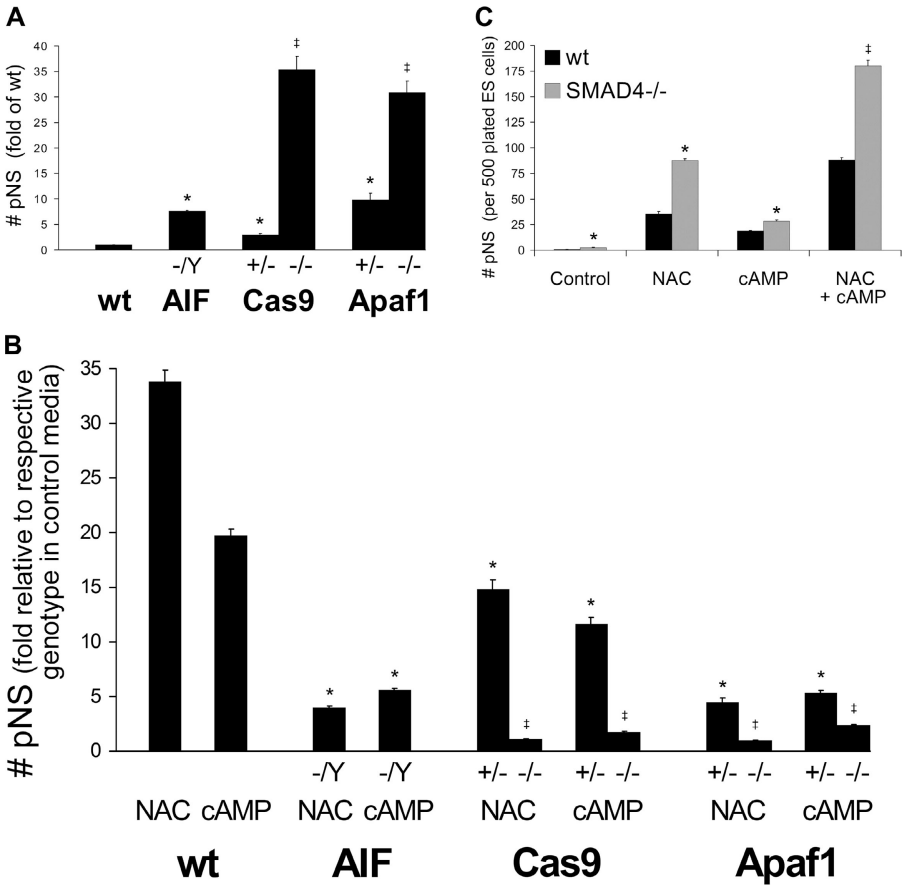

Figure 6. **Genetic interference with apoptotic pathways enhances primitive NS generation and TGFβ inhibition independently cooperates with survival factors to promote primitive NS production.** (A) ES cell lines with mutations in apoptotic pathway components *aif*, *cas9*, and *apaf1* displayed considerably enhanced primitive NS (pNS) production compared with wild-type (wt) ES cells. *, $P < 0.05$ versus wt; ‡, $P < 0.001$ versus wt and versus respective +/− genotype. (B) The primitive NS–promoting ability of NAC and pCPT-cAMP (cAMP) was assessed for each genotype of the apoptosis mutant ES cell lines. The effects of the survival factors were much reduced, though still considerable, in the *aif* mutant. Strikingly, the effects of NAC and pCPT-cAMP were drastically reduced in the *cas9*−/− and *apaf1*−/− ES cells. *, $P < 0.001$ versus wt in same survival factor, ‡, $P < 0.001$ versus wt in same survival factor and versus respective +/− genotype in same survival factor. (C) The *smad4*−/− ES cell line exhibited enhanced primitive NS production compared with wt ES cells. This independent enhancement was observed even in the presence of the survival factors NAC and pCPT-cAMP (cAMP), included individually or concurrently. *, $P < 0.05$ versus wt in same conditions; ‡, $P < 0.001$ versus wt in same conditions and versus *smad4*−/− in each factor alone.

then the survival factor effect should be reduced in these mutant ES cell lines. Typical robust stimulation was observed for the wild-type line, which was much reduced, though still considerable, in the *aif* mutant (Fig. 6 B). Strikingly, the effects of NAC and pCPT-cAMP were drastically reduced in the *apaf1*−/− and *cas9*−/− ES cells to the extent that there was no longer any significant effect of NAC and only an approximately twofold stimulation by pCPT-cAMP (Fig. 6 B). This attenuation or occlusion of the survival factor effects in these mutant ES cell lines verifies that NAC and pCPT-cAMP promote primitive NS formation through a reduction in apoptotic cell death, primarily mediated by the more dominant *apaf1–cas9* pathway.

TGFβ inhibition independently cooperates with the survival factors to promote primitive NS production

We have previously demonstrated that TGFβ-related signaling negatively regulates ES cell neuralization and the basal number of primitive NSs that form in our assay (Tropepe et al., 2001). To determine how survival promotion interacted with interference of the TGFβ pathway, the effects of NAC and pCPT-cAMP were assessed in an ES cell line that contains a mutation in *smad4*, a key downstream signaling molecule in multiple TGFβ-related pathways (Sirard et al., 1998). The *smad4*−/− ES cells displayed increased primitive NS generation relative to wild-type ES cells under both control media conditions as well as in the presence of the survival factors such that in the presence of both NAC and pCPT-cAMP ~35% of the initially plated ES cells became primitive NSCs that were able to survive and proliferate to form primitive NSs (Fig. 6 C). These results provide further support for the notion that the default pathway does indeed give rise specifically to primitive NSCs (though TGFβ signaling hinders this default to primitive NSCs), which then experience a separate survival challenge limiting the number of primitive NSCs that form primitive NSs.

Discussion

The present study demonstrates that in the absence of extrinsic influencing signals, ES cells will rapidly undergo a direct neural conversion, transitioning into neural precursor cells. This neuralization does not depend on any instructive factors but rather occurs by a default mechanism. The default neural pathway specifically gives rise to primitive NSCs, and because of the exiguous nature of the default conditions, primitive NSC mortality is primarily responsible for limiting the persistence, proliferation, and detection of these cells. Accordingly, increasing primitive NSC viability in these conditions with exogenous survival factors, activation of the endogenous cAMP pathway, or genetic interference with apoptosis enhanced primitive NS formation.

Studies using human ES cells have supported the concept of default neural differentiation (Schulz et al., 2004; Vallier et

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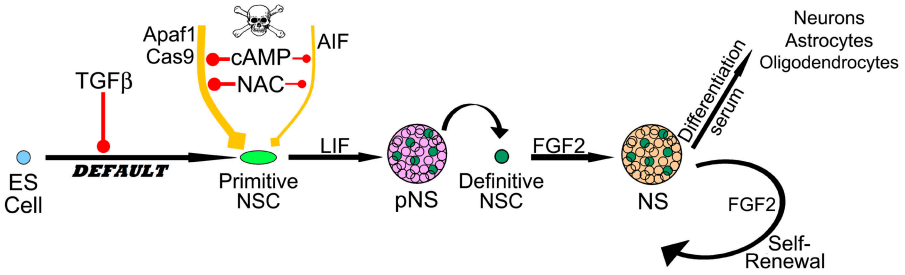

Figure 7. **ES cell–based model describing the ontogeny of NSCs.** When placed in minimal conditions, free of inhibitory influences, ES cells will acquire a primitive NSC identity through a default mechanism. This transition is negatively regulated by TGFβ signaling. The nascent primitive NSC then undergoes a separate survival challenge in these minimal conditions, with apoptosis mediated primarily by the Apaf1–Cas9 pathway as well as contribution by the AIF apoptosis pathway. Survival can be promoted by NAC and cAMP, which mediate their effects largely through abrogation of the Apaf1–Cas9 pathway. In the presence of LIF, the primitive NSC remains in an undifferentiated, proliferative state and generates a primitive NS containing a subpopulation of more mature (FGF2 dependent) definitive NSCs. Upon dissociation of the primitive NS, the definitive NSCs will proliferate in an FGF2-dependent manner to generate an NS. In the presence of FGF2, this self-renewal passaging can be performed repeatedly. With the addition of serum, the NS can be differentiated to produce neurons, astrocytes, and oligodendrocytes.

al., 2004), although clonal analyses were not performed. Other work examining the neural conversion of mouse ES cells without EB formation reported that most cells adopted a neural precursor phenotype in serum-free conditions; however, they found that widespread neuralization did not occur until at least 3 d (Ying et al., 2003). Further, Ying et al. (2003) suggest that the media component transferrin and autocrine FGFs were required positive instructive factors, in contrast to a pure default model. The present results demonstrate that neural specification commences over a matter of hours, even when ES cells were placed in PBS alone, ruling out any necessity for media components in an instructive capacity. Certainly, cells cannot be maintained long-term in PBS alone, as nutritive factors are required in a permissive role. Further, we provide evidence against any positive inductive role for autogenously produced FGFs by demonstrating that neither pharmacological FGF inhibition (using the same inhibitor as Ying et al., 2003) nor genetic deletion of the FGF receptor-1 effected any reduction in neuralization. Rather, such FGF signaling functions to allow the proliferation of the default pathway–derived neural precursors. Experiments of Ying et al. (2003) used cell densities up to ~20 times higher than those used in the present studies and in some cases allowed low-density plated ES cells to grow as colonies overnight in normal ES media (increasing the effective cell density) before removal of LIF and serum. These conditions are likely to facilitate the density-dependent accumulation and signaling of secreted neural inhibitors (e.g., BMPs), as we demonstrated previously (Tropepe et al., 2001), potentially explaining why their neuralization was delayed. They also reported the gradual increase in expression of BMP antagonists (e.g., noggin and follistatin) over 1–5 d in their conditions, which would then provide the BMP antagonism necessary to allow the default program to manifest. Furthermore, the extensive viability and exponential cell expansion in their conditions is in stark contrast to our findings, indicating that their high cell densities and/or richer media formulation produced an environment less minimal than that used in the current study.

The severely minimal nature of our culture conditions was necessary to effectively minimize potential extrinsic influences and thereby assess a default state. However, this was clearly responsible for the extensive cell mortality observed. In attempts to evaluate the default state, one encounters a catch-22. At some point, progressive removal of extrinsic factors will dispense with constituents essential for default fated cell survival, and there will be no experimental output to assess. Such factors are required for neuralization not in an instructive capacity but rather in a permissive, supportive one. In interpretation of our results, one might suggest that we were merely providing conditions that selected for the survival of neural cells while nonneural cells perished, and thus the default state was not assessed. Two observations argue against this interpretation. First, in the initial 4 h after ES cell plating, no significant mortality was observed, yet the vast majority of individual ES cells had already begun their neural transition. Second, with the use of both survival factors in the 24-h assay, viability was increased from 23 to 65%; however, a multiple marker–based analysis showed that there was no difference in the uniform cell neuralization and that there was no expression of other germ layer markers. If the conditions used were selecting against the survival of nonneural lineage cells, they should have been apparent when survival was so dramatically increased.

The primitive NSCs described here exhibited characteristics (i.e., proliferative sphere formation, gene expression, and differentiation potential) that are analogous to forebrain-derived NSCs. Although it is clear that the primitive NSCs commenced neural lineage specification, it was apparent that they retained vestiges of ES cell features, suggesting that the commitment was not yet absolute. This was evidenced by the broader differentiation potential observed in blastocyst chimera experiments (Tropepe et al., 2001). Passaging of the primitive NSs yielded more committed neural precursors, increasingly similar to NSs formed by forebrain-derived NSCs (e.g., they acquired dependence on exogenous FGF2, ceased expression of ES cell markers Oct4 and nanog, and lost blastocyst chimerism potential). Recent work from our lab has demonstrated that LIF-dependent sphere-forming cells can be isolated directly from the embryonic day (E) 5.5–7.5 mouse epiblast (Hitoshi et al., 2004). These embryo-derived spheres possessed similar characteristics to the ES-derived primitive NSs and were found to give rise to FGF2-dependent, NS-forming, definitive NSCs upon passaging in vitro (Hitoshi et al., 2004). Thus, in vivo there are two distinct sphere-forming NSC populations that are present at different stages of development from E5.5 to adult. In addition, corresponding sphere-forming NSC populations could be sequentially derived from ES cells. This suggests the in vivo relevance of an ES cell–based model describing the ontogeny

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of NSCs (Fig. 7). Pluripotent ES cells derived from the E3.5 ICM will commence a direct default transition in the absence of inhibitory influences (e.g., TGFβ-related signaling) to yield LIF-dependent primitive NSCs, similar to those that can be isolated from the epiblast (Hitoshi et al., 2004). The primitive NSs produced can be passaged to give rise to definitive, FGF2-dependent NSCs, analogous to the FGF2-dependent NSCs that can be isolated from the E8.5 neural plate (Tropepe et al., 1999). The in vitro–derived definitive NSCs can be extensively passaged to demonstrate long-term self-renewal, consistent with the effective isolation of definitive NSCs from the adult remnant of the embryonic brain germinal zone throughout the lifetime of the animal.

Is the propensity of ES cells to default to a neural identity developmentally relevant? ES cells have properties similar to the E3.5 ICM cells from which they are derived. Our data suggest that the ES cells can directly become neural without a discernible ectodermal intermediate phase. The developmental dogma states that neural tissue arises from the ectodermal germ layer, which is not defined until gastrulation (E6.5–8.5). ES cell differentiation has been suggested to recapitulate these stages (Pelton et al., 2002). It is possible that the neural tendency of the E3.5 cells never has the opportunity to manifest in vivo because of extensive antineural signaling via BMPs until after gastrulation, when node regions are formed to disinhibit the neural fate in what is now specified ectoderm. However, recent work in both *Xenopus laevis* and chicks has suggested that there may be some neural-fate acquisition even before gastrulation (Streit et al., 2000; Gamse and Sive, 2001). This may be attributable to preectodermal ICM-like cells that begin to express their default neural tendency after ineffective neural inhibition. In the context of the intact embryo, even with its milieu of secreted BMPs, it is conceivable that transient interruption or inefficiency in BMP delivery or activity would occur, allowing a cell or cells to escape neural inhibition and rapidly initiate the default neural program. In addition, the present data suggest that mouse ES cells can transition directly into neuronal cells; however, neurons are not produced in vivo until approximately E10. It is possible that neural precursors present in vivo before this stage are competent to form neurons but do not do so because of the presence of factors like LIF, which have been shown to prevent neural precursors from further differentiation down the neural lineage and maintain the undifferentiated primitive NSC state.

In addition to providing insights into the mechanisms of neural development, default fate studies using ES cells allow exploration of what is perhaps a more basic and fundamental issue, i.e., how an uncommitted, pluripotent mammalian cell will self-organize in the absence of extrinsic instructive or inhibitory signals and what cellular configuration/fate will result. Our data suggest that such uncommitted cells tend to self-organize in the configuration of a NSC, i.e., the default fate. What is meant by the term default? Clearly, such analyses begin with the definition of a system, and the default state represents the configuration that the system will autonomously acquire in the absence of extrinsic inputs to the system. However, this means that there can be different levels of analysis depending on the definition of the initial system. In the context of ES cell default neural fate, a fairly gross level of analysis would simply state that no other tissues or cells are required for an ES cell to become a neural cell, whereas all other factors (such as environmental/media components and endogenous/autocrine signaling) are included within the system. The present data would clearly satisfy this level of analysis. A finer level of analysis would state that a single ES cell would acquire a neural identity with only permissive molecules present (in the media) and without autocrine inductive signals. The present data seem to partially satisfy this level of analysis as well, as we have ruled out requirement for exogenously supplied instructive molecules and autogenous FGF signaling. However, we cannot yet definitively demonstrate that no other potential instructive endogenously supplied (and perhaps autocrinely acting) factors are present. An even finer level of analysis would be at the genomic level, with the system defined as the entire genome. Though comprehensive and definitive analysis of the genomic system in isolation is currently impossible (as the genome only exists in an active form in the context of living cells), a default neural state at this level would predict that the highest genes within the neural genetic program (i.e., a potential "master neural genes") would have only repressor elements within its regulatory regions. In this model, signals extrinsic to the genome would not be necessary to activate the neural genetic program; rather, they could serve only to inhibit it. Transcriptional repressors have been identified that are responsible for inhibition of the neural program, e.g., neuron-restrictive silencing factor/RE1-silencing transcription factor (NSRF/REST; Chen et al., 1998). In vivo inhibition of NSRF/REST leads to derepression of neural genes in both neural and nonneural tissues (Chen et al., 1998). Thus, it is possible that NSRF/REST, as well as other transcriptional repressors, is required to prevent the default neural genetic program from being activated in all cells.

Materials and methods

ES cell culture

ES cells were maintained on mitotically inactivated mouse embryonic fibroblast feeder layers in DME media containing 15% FCS and LIF (1,000 U/ml). The main ES cell line used was R1, as well as E14K, *aif−/Y* (Joza et al., 2001), *cas9+/−*, *cas9−/−* (Hakem et al., 1998), *apaf1+/−*, *apaf1−/−* (Yoshida et al., 1998), and *smad4−/−* (Sirard et al., 1998) ES cells (provided by J. Rossant, A. Nagy, T. Mak, and J. Penninger, University of Toronto, Toronto, Canada).

Animals and NSC culture

NSCs were isolated from the forebrain ventricular subependyma of adult CD1 mice (Charles River Laboratories) and cultured/maintained using the NS assay as previously described (Seaberg and van der Kooy, 2002).

ES cell minimal condition assays

ES cells were dissociated into a single-cell suspension and plated at ≤10 cells/μL on laminin/polyornithine–coated culture plates (Nunclon) in chemically defined, serum-free media (Tropepe et al., 1999, 2001), which consisted of DME/F-12 (1:1; Invitrogen) with 0.6% D-glucose, 5 mM Hepes, 3 mM $NaHCO_3$, 2 mM glutamine, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite. PBS used for cell incubation in the short-term neural conversion experiments included 20 mM glucose. Uncoated dishes were also tested, and though cells exhibited greatly decreased adhesion, no differences in the neural conversion assays were observed. Cells were fixed for immunocytochemical analysis or cells were collected for RT-PCR analysis

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at various time points over the course of a 7-d culture period. For assessment of the survival of the 4-h nestin$^+$ cells, ES cells were initially plated at 50 cells/well (48-well plate). At various time points thereafter, trypan blue exclusion was used to determine the number of viable cells or cell colonies (each representing the survival of a single 4-h nestin$^+$ cell). Survival factors (Sigma-Aldrich) were included as indicated in the text.

Primitive NS assays

A single-cell suspension of ES cells was plated at ≤10 cell/μL in uncoated 24-well culture plates (Nunclon) in serum-free media (as described in the previous section) containing LIF (1,000 U/ml). Over a 7-d culture period, clonal, floating sphere colonies (>75-μm diam) of neural precursors formed (primitive NSs; see online supplemental material for more details). For passaging, spheres were individually or bulk dissociated into single cells by trypsin/EDTA (Invitrogen) incubation followed by manual trituration. Cells were then replated in the same media with inclusion of 10 ng/ml FGF2 (Sigma-Aldrich) and 2 μg/ml heparin (Sigma-Aldrich) and allowed to form subsequent spheres for 7 d. Survival factors and other pharmacological agents were from Sigma-Aldrich, except SU5402 (Calbiochem), and were included as described in the text. For differentiation, individual spheres were removed from growth factor–containing media and transferred to Matrigel-coated plates in the same media formulation as noted previously (without growth factors) supplemented with 1% FCS and cultured for 7 d. Undifferentiated and differentiated NSs were fixed for immunocytochemical analysis or were collected for RT-PCR analysis.

Immunocytochemistry

Fixation and immunocytochemical analysis of cells was performed as described previously for NSs (Seaberg and van der Kooy, 2002). Primary antibodies used included anti-nestin rabbit polyclonal (1:2,000; a gift from R. McKay, National Institutes of Health Research, Bethesda, MD), anti-nestin rabbit polyclonal (1:50, a gift from R. Goldman, Northwestern University, Chicago, IL), anti-nestin mouse monoclonal (1:1,000; Chemicon), anti-Oct4 mouse monoclonal (1:500; BD Biosciences), anti-Sox1 rabbit polyclonal (1:150; Chemicon), anti-NFM rabbit polyclonal (1:200; Chemicon), anti-tubulin isotype III (β_3-tubulin) mouse monoclonal (IgG; 1:500; Sigma-Aldrich), anti-glial fibrillary acidic protein (GFAP) mouse monoclonal (IgG; 1:400; Sigma-Aldrich), and anti-O4 mouse monoclonal (IgM; 1:100; Chemicon). Nestin immunoreactivity in the short-term (1–3 d) assays and in primitive NSs was verified by immunostaining with at least two nestin antibodies. Secondary antibody–only wells were processed simultaneously using the identical protocol, except that solutions did not contain primary antibodies. All secondary-only controls were negative for staining. Cell nuclei were counterstained with the nuclear dye DAPI or Hoechst 33258. Fluorescent images were visualized using a motorized inverted research microscope (IX81; Olympus) with 20×/0.40 and 40×/0.60 objectives at room temperature and captured using Olympus MicroSuite Version 3.2 image analysis software (Soft Imaging System Corp.). Images were prepared using Photoshop 6.0 (Adobe) software.

RT-PCR

Total RNA was extracted from cells using an RNeasy extraction kit (QIAGEN) with inclusion of DNase to prevent genomic DNA contamination. RT-PCR was performed using a OneStep RT-PCR kit (QIAGEN) in a GeneAmp PCR System 9700 (Applied Biosystems) according to kit instructions. See Table S3 (available at http://www.jcb.org/cgi/content/full/jcb.200508085/DC1) for details of primer sequences and cycling conditions.

Statistics

Data are expressed as means ± SEM unless specified otherwise. Statistical comparisons between two groups were performed using a *t* test where appropriate or by one-way analysis of variance with Dunnett's posttest for comparing groups to control or the Bonferroni posttest for comparisons between groups where an acceptable level of significance was considered at $P < 0.05$.

Online supplemental material

Fig. S1 shows expanded RT-PCR analysis of primitive NSs, confirming their neural precursor nature. Fig. S2 shows that cAMP pathway activity promotes primitive neurosphere production, whereas cGMP pathway activity impairs it. Table S1 shows actual raw sphere counts obtained in one experiment, and Table S2 shows normalized sphere counts. Table S3 shows gene primer sequences and expected product sizes for RT-PCR. The Results section describes studies in which the cAMP and cGMP pathways were modulated. These studies showed that the cAMP pathway positively regulated and the cGMP pathway negatively regulated primitive NSC survival and primitive NS formation. The Materials and methods section contains additional details regarding the primitive NS assay and the counting and normalization of data. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508085/DC1.

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Supplemental Results

cAMP and cGMP pathways regulate primitive NS production

The endogenous cAMP pathway was examined for a potential role in primitive NSC survival leading to primitive NS formation. Stimulation of endogenous cAMP production via adenylate cyclase (AC) activation with forskolin had a similar, albeit lesser, effect than that of exogenous cAMP in enhancing primitive NS generation (Fig. S2 A). Interference with the cAMP–PKA pathway through inhibition of AC with SQ22536 or 2′,3′-dideoxyadenosine or inhibition of PKA with H89 resulted in a reduction in basal (Fig. S2 A), forskolin-stimulated, and pCPT-cAMP–stimulated primitive NS formation (not depicted). Use of the physiological ligands insulin or insulin-like growth factor I, which have been shown to elevate cAMP levels in mouse ES cells (Parkin et al., 1996), also augmented primitive NS production (by approximately threefold; not depicted). These results demonstrate that endogenously generated cAMP and downstream signaling via the canonical AC–cAMP–PKA signaling pathway modulate both basal and enhanced (by endogenously generated or exogenous cAMP) primitive NS generation.

The cGMP pathway was also examined for a potential role. A membrane-permeable cGMP analogue, pCPT-cGMP, and stimulation of endogenous cGMP production via guanylate cyclase activation with 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole, reduced basal primitive NS generation (Fig. S2 B). Inhibition of cGMP signaling with the antagonist Rp-pCPT-cGMP slightly enhanced primitive NS formation (Fig. S2 B). This suggests that the cGMP pathway has a negative regulatory effect on primitive NSC survival and primitive NS formation.

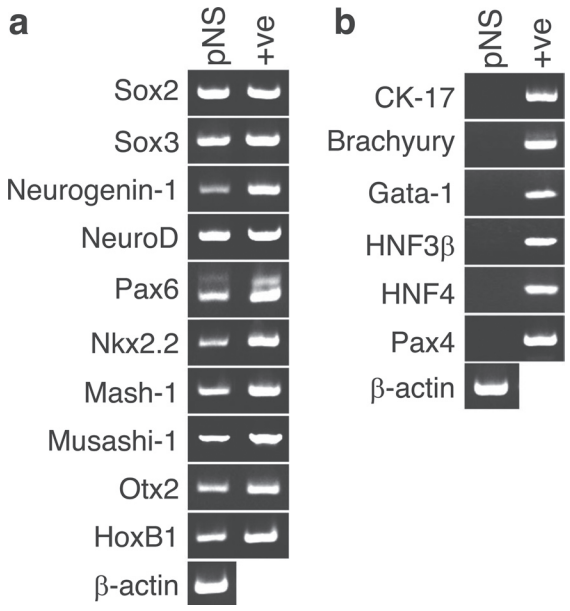
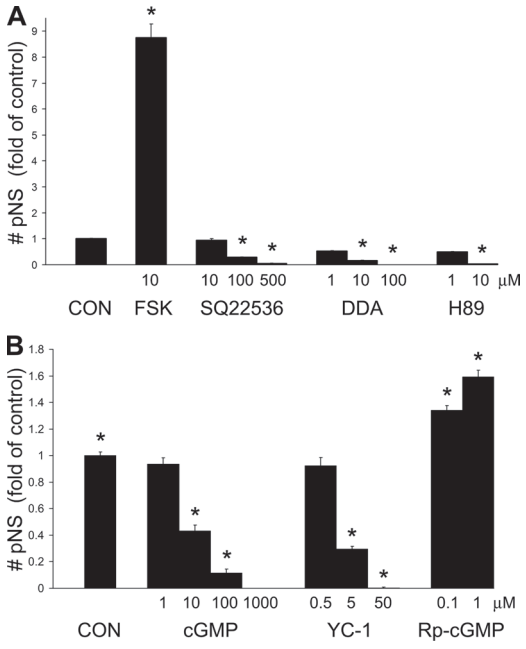


Figure S1. **Expanded RT-PCR analysis of primitive NSs confirms their neural precursor character.** RT-PCR was performed on primitive NSs, which demonstrated the expression of an array of neural and neural precursor genes (a), whereas epidermal, mesodermal, and endodermal marker expression was not observed (b). The β-actin band is not shown for the positive control lane, as various cell and tissue samples were used; however, all control sample β-actin bands were similar in intensity to the primitive (pNS) band.



Supplemental materials and methods

Primitive NS assay/counting

For quantification of primitive NS generation, single ES cells were typically plated at 10 cells/μl in 24-well plates, i.e., 5,000 cells/500 μl/well. Data were collected from four to seven independent experiments, each with five to six culture repeats (wells) per condition. To assess basal primitive NS production in the R1 ES cell line under control conditions, 269 spheres were counted from 30 culture repeats, which gives a mean of 8.97 ± 0.53

Figure S2. **cAMP pathway activity promotes primitive NS production, whereas cGMP pathway activity impairs it.** (a) Stimulation of endogenous cAMP production by activation of adenylate cyclase with forskolin (FSK) increased primitive NS (pNS) production. Inhibition of adenylate cyclase activity using SQ22536 or 2′,3′-dideoxyadenosine (DDA) or inhibition of PKA using H89 attenuated basal primitive NS production. *, $P < 0.01$ versus control. (b) Addition of the membrane-permeable cGMP analogue pCPT-cGMP (cGMP) or stimulation of endogenous cGMP production by activation of guanylate cyclase with YC-1 inhibited basal primitive NS generation. The cGMP signaling antagonist Rp-pCPT-cGMP (Rp-cGMP) slightly increased primitive NS formation. *, $P < 0.01$ versus control.

spheres per 5,000 initially plated cells. Thus, 0.18 ± 0.01% of the initially plated single cells gave rise to primitive NSs. When data is in the figures as "#pNS (% of control)" or "#pNS (fold of control)," for each individual experiment, the mean value of the control condition repeats was normalized to either 100 or 1, respectively, and the normalization factor was applied to all data points in all conditions within the experiment. For example, Table S1 shows actual raw sphere counts obtained in one experiment. The mean of the control repeats is 7.5, giving us the normalization factor of 7.5–1. In Table S2, we applied the normalization factor to each data point to produce fold changes. Such normalization was performed whenever data are expressed as folds or percentages.

RT-PCR

The OneStep RT-PCR kit (QIAGEN) was used with an annealing temperature of 58°C and 30 cycles. Primers sequences and expected product sizes are shown in Table S3.

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Table S1. **Raw sphere counts**

Control	NAC – 1 mM	CAMP – 100 µM	NAC + cAMP
6	253	184	709
8	278	190	856
5	217	156	882
9	293	212	834
9	267	207	927
8	259	176	821
MEAN: 7.5	261.1667	187.5	838.1667

Table S2. **Normalized sphere counts**

Control	NAC – 1 mM	CAMP – 100 µM	NAC + cAMP
0.800	33.733	24.533	94.533
1.067	37.067	25.333	114.133
0.667	28.933	20.800	117.600
1.200	39.067	28.267	111.200
1.200	35.600	27.600	123.600
1.067	34.533	23.467	109.467
MEAN: 1.000	34.822	25.000	111.756

Table S3. **Gene primer sequences and expected product sizes for RT-PCR**

Gene	Primer-1	Primer-2	Product Size
			bp
β-actin	ATCATGTTTGAGACCTTCAA	TCTGCGCAAGTTAGGTTTTGTC	825
Brachyury	TATGAACCTCGGATTCACATCG	AGCAGATGAATTGTCCGCATA	431
Gata-1	GGGAAGAGCAACAACACGTTC	GTTTGCTGACAATCATTCGCT	380
Gata-4	GGCCTCTATCACAAGATGAACG	GAGTGACAGGAGATGCATAGCC	522
HNF3β	CAGACCACGCGAGTCCTA	CATGATCCACTGATAGATCTCG	660
HNF4	CCATGGTGTTTAAGGACGTGC	TAGGATTCAGATCCCGAGCC	630
HoxB1	CTCCAACAAAACTCGGGGTA	GCACGGCTCAGGTATTTGTT	521
Mash-1	AAGAAGATGAGCAAGGTGGAGACG	CAGAACCAGTTGGTAAAGTCCAGC	257
Musashi-1	CTAAGATGGTCACTCGGACGA	GAGAGCCTGTCCCTCGAACTAC	560
Nestin	AGATGTGGGAGCTCAATCGAC	GCTGCACCTCTAAGCGACTCT	650
NeuroD	CTGATCTGGTCTCCTTCGTACAG	GATGCGAATGGCTATCGAAAG	540
Neurogenin-1	TGCATCTCTGATCTCGACTGC	AGATGTAGTTGTAGGCGAAGCG	406
Nkx2.2	CTCTTCTCCAAAGCGCAGAC	AACAACCGTGGTAAGGATCG	510
Otx2	ACAAGTGGCCAGTTCAGTCC	CTGGGTGGAAAGAGAAGCTG	347
Pax4	GTGAGCAAGATCCTAGGACGC	CGGGGAGAAGATAGTCCGATT	375
Pax6	TCACAGCGGAGTGAATCAGC	TATCGTTGGTACAGACCCCCTC	377
Sox-1	AATCCCCTCTCAGACGGTG	TTGATGCATTTTGGGGGTA	224
Sox-2	GGAGTGGAAACTTTTGTCCGA	TTCATGTAGGTCTGCGAGCTG	420
Sox-3	AGACTGAACTCAAGAACCCCG	GTCCTTCTTGAGCAGCGTCTT	435