Endothelial Cells Stimulate Self-Renewal and Expand Neurogenesis of Neural Stem Cells

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Neural stem cells are reported to lie in a vascular niche, but there is no direct evidence for a functional relationship between the stem cells and blood vessel component cells. We show that endothelial cells but not vascular smooth muscle cells release soluble factors that stimulate the self-renewal of neural stem cells, inhibit their differentiation, and enhance their neuron production. Both embryonic and adult neural stem cells respond, allowing extensive production of both projection neuron and interneuron types in vitro. Endothelial coculture stimulates neuroepithelial cell contact, activating Notch and Hes1 to promote self-renewal. These findings identify endothelial cells as a critical component of the neural stem cell niche.

Stem cell expansion and differentiation are regulated in vivo by environmental factors encountered in the stem cell niche (*1*). In the adult, neural stem cells lie close to blood vessels: in the hippocampus (*2*), the subventricular zone (SVZ) (*3*), and the songbird higher vocal center (*4*). In the developing central nervous system (CNS), ventricular zone cells produce vascular endothelial growth factor, which attracts vessel growth toward them (*5*). Thus, vascular cells are close to CNS germinal zones throughout life (fig. S1), and it has been suggested that they form a niche for neural stem cells (*2*).

To examine a possible functional interaction, we cocultured neural and vascular cells (Fig. 1A). Neural stem cells from mouse cerebral cortex from embryonic day 10 to 11 (E10-11) were plated at clonal density on the base of culture wells. The upper transwell compartment was seeded with purified vascular-associated or other feeder cells: primary bovine pulmonary artery endothelial (BPAE) cells, a mouse brain endothelial (MbEND) cell line, vascular smooth muscle (VSM) cells, NIH3T3 fibroblasts, or as a control, high-density age-matched cortical cells (CTX). $CD31^+$ (platelet endothelial cell adhesion molecule c-1, PECAM-1) endothelial cells were never found in the lower compartment when BPAE or MbEND cells were plated in the transwell upper compartment (Fig. 1B), confirming that the feeder cells could not migrate through the 0.4-μm-diameter membrane pores.

As expected (*6*), embryonic stem cell clones cocultured with CTX began producing neurons within a day. Most neuron production was over by 7 days, and growth after this time was largely in glial lineages. Clones cocultured with BPAE or MbEND cells behaved differently (Fig. 1D and fig. S2), growing into sheets of largely flat-

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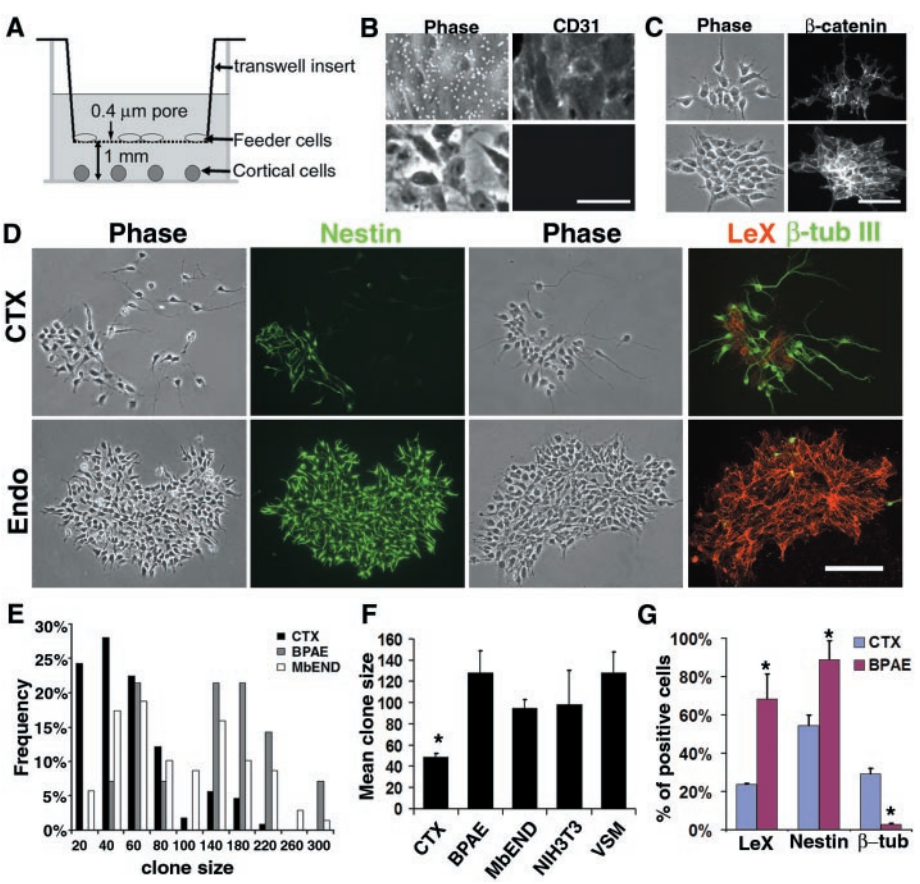

Fig. 1. Endothelial cell–derived soluble factors stimulate cortical stem cell expansion and delay differentiation. (**A**) The coculture system. (B to G) Results from E10-11 cortical stem cells. (**B**) CD31 stains endothelial cells in the transwells (top), but no $CD31^+$ cells are detected below in the cortical cell compartment (bottom). Scale bar, 25 μm. (**C**) Cortical stem cells generate larger, cohesive clones of flattened progeny with stronger junctional β-catenin staining in endothelial (bottom) compared to cortical (top) coculture. Scale bar, 50 μm. (**D**) Neural stem cell clones grown for 7 days with endothelial (Endo) cells were sheet-like and had more $Nestin^+$ and LeX^+ and fewer β-tubulin-III^+ progeny than control clones. Scale bar, 100 μm. (**E**) Histogram of clone size (defined by number of progeny) frequency at day 7 in culture. (**F**) Mean clone size with different feeder layers at 7 days in culture [analysis of variance (ANOVA), * indicates $P < 0.01$ by post-hoc tests]. (**G**) Percentages of cells with progenitor and neuronal markers per stem cell clone (*, $P < 0.05$, *t* test).

tened progeny that maintained tight cell-cell contact (illustrated in Fig. 1C by strong junctional β-catenin staining), with only a few immature, neuron-like cells appearing on top of the sheets. Neural stem cell clones grown with endothelial cells were larger, with more primitive progeny [expressing the progenitor markers Nestin and LeX (*3*)] and fewer neurons (expressing β-tubulin-III), than were clones grown with CTX (Fig. 1, D to G). Hence, endothelial factors facilitate expansion of cortical stem cell clones and inhibit their differentiation. VSM and NIH3T3 cells also promoted neural stem cell proliferation (Fig. 1F), but clones were less cohesive and included more glial-like progeny than those in endothelial coculture.

When the transwells were removed, endothelial-expanded stem cell clones continued to proliferate but also began to differentiate (Fig. 2A), and within 4 days they produced β-tubulin-III$^+$ neurons (Fig. 2B), which were almost all microtubule-associated protein 2 (MAP-2$^+$). About 30% of the neurons had acquired the later neuronal marker NeuN (*7*). The clones contained up to ~10,000 progeny, and on average 31% were neurons. In contrast, in control CTX cocultures 4 days after transwell removal, stem cell clones ranged up to 4350 cells, and on average only 9% were neurons. Similarly, E11 cortical cells cultured as neurospheres for 7 days then differentiated in adherent culture for 4 days produced only 7% neurons. Many more stem cell clones growing in BPAE cocultures contained a high percentage of neurons, up to 64%, compared to clones grown in CTX coculture (Fig. 2, D and E), and neuron production was prolonged (supporting online text and fig. S3). Increased neurogenesis from endothelial cocultured neural stem cells did not occur at the expense of gliogenesis: The percentage of glial fibrillary acidic protein (GFAP$^+$) astrocytes generated was similar, and although oligodendrocyte differentiation (indicated by staining with the early oligodendrocyte marker O4) was reduced in BPAE cocultures compared to CTX cocultures, the difference could not account for the enhancement of neuron generation (Fig. 2, C and E). NIH3T3 cells enhanced oligodendrocyte generation. Coculture with VSM or NIH3T3 cells reduced neurogenesis compared to CTX (Fig. 2E), showing that the endothelial effect is cell-type specific.

Endothelial cells stimulate proliferation and neurogenesis of neural stem cells from a variety of embryonic CNS regions (*7*) and from different stages. E15.5 cortical and adult SVZ stem cells grown in endothelial coculture generated sheets of LeX$^+$, Nestin$^+$ cells. After differentiation, E15.5 endothelial-expanded cortical cells and adult SVZ cells produced more neurons compared to control cells (Fig. 2, F and H).

Neurosphere-expanded stem cells responded to endothelial factors. E15.5 cortical cells grown as neurospheres in fibroblast growth factor 2 (FGF2) for 7 days were plated in adherent conditions and cocultured for 3 days with endothelial cells or with age-matched cortical cells, then differentiated by withdrawal of feeder cells for 4 days. Stem cells exposed to endothelial factors produced 22% neurons, compared to 2% neurons in control CTX cocultures (Fig. 2G).

In vivo, most projection neurons are born in the early embryonic period, whereas glia and interneurons arise later; adult stem cells are primed to generate interneurons (*8*, *9*). To examine the neuron subtypes generated from E10-11 cortical stem cells expanded in endothelial coculture, differentiated clones were stained for glutamic acid decarboxylase (GAD67), a GABAergic marker typically expressed in interneurons, or Tbr1, an early pyramidal neuron marker that preferentially labels projection neurons (*10*) (Fig. 3A). More stem cell clones growing in BPAE coculture made Tbr1$^+$ projection neurons, compared to CTX-cocultured clones (Fig. 3B). BPAE-cocultured stem cells generated more Tbr$^+$ neurons than neurosphere-expanded E10 cells that were subsequently differentiated in adherent culture (9.95% versus 2.41%). Thus, endothelial cell coculture supports development of both projection neurons and interneurons.

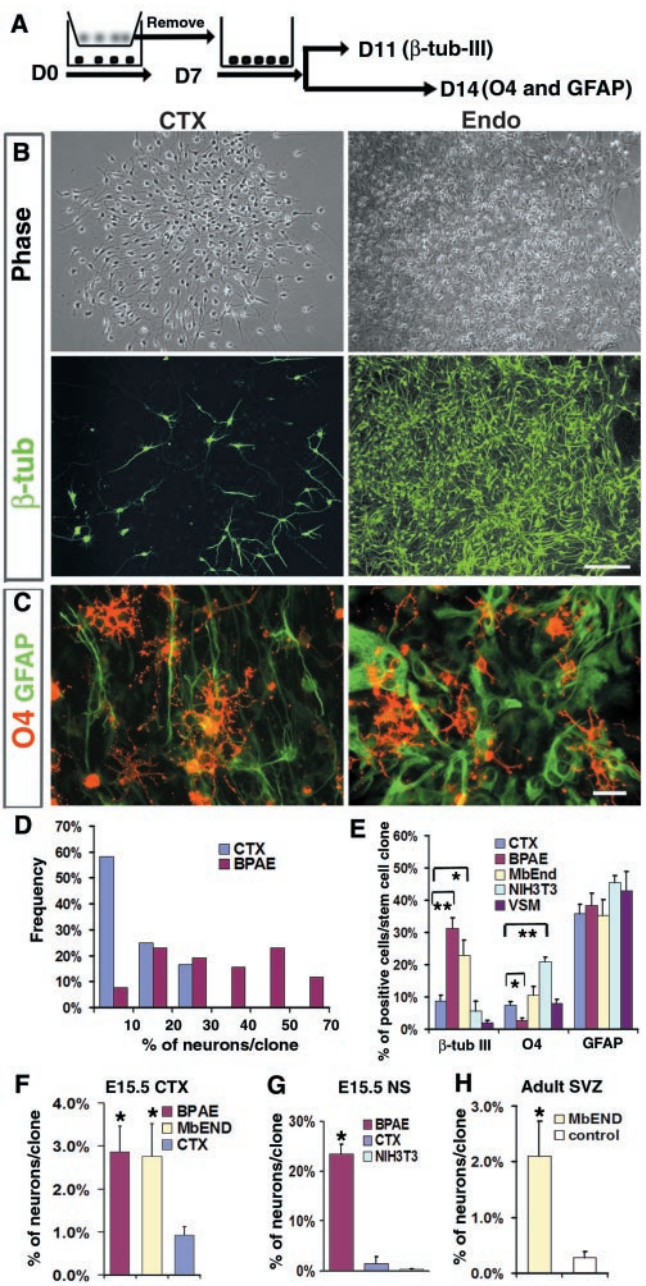

Fig. 2. Endothelial-expanded stem cells show enhanced neuron production. (**A**) Schematic of the experiment. (B to E) Results from E10-11 cortical stem cells. (**B**) Endothelial-cocultured stem cells (Endo) had massive neuron production compared to control-cocultured cells (CTX), shown by β-tubulin-III expression at day 11 (D11). Scale bar, 200 μm. (**C**) Oligodendrocytes and astrocytes, shown by O4 (red) and GFAP (green) labeling, respectively, are present in both endothelial and CTX cocultured clones at D14. Scale bar, 100 μm. (**D**) Histogram of neuron generation per stem cell clone. (**E**) Enhancement of neuron production from stem cells does not occur at the expense of glial cell production. Neuron production is specifically enhanced by endothelial cell coculture, compared to coculture with other cell types (*, $P < 0.05$; **, $P < 0.001$; ANOVA, Newman-Keuls test). (F to H) Forebrain stem cells from older embryonic and adult stages show enhanced neurogenesis with endothelial cell coculture. (**F**) Neurons per E15.5 cortical cell adherent clone (CTX) (*, $P < 0.05$, ANOVA, Newman-Keuls test). (**G**) Neurons per E15.5 cortical neurosphere (NS) (*, $P < 0.001$, ANOVA, Newman-Keuls test). (**H**) Neurons per adult SVZ adherent clone (*, $P < 0.01$, *t* test).

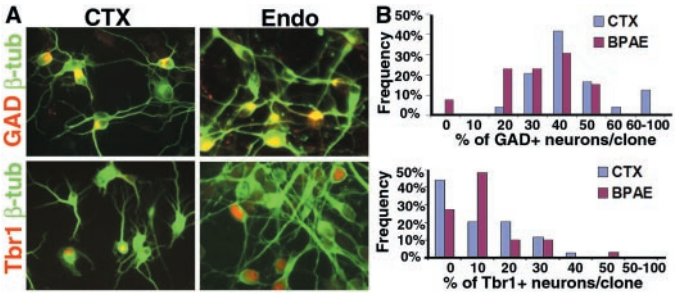

Fig. 3. Endothelial-expanded E10 stem cell clones retain the ability to generate Tbr1$^+$ projection neurons as well as GAD$^+$ interneurons. (**A**) GAD (cytoplasmic, red), Tbr1 (nuclear marker, red) and β-tubulin-III (green) staining. (**B**) Histograms showing the frequency of GAD$^+$ and Tbr1$^+$ neurons in stem cell clones.

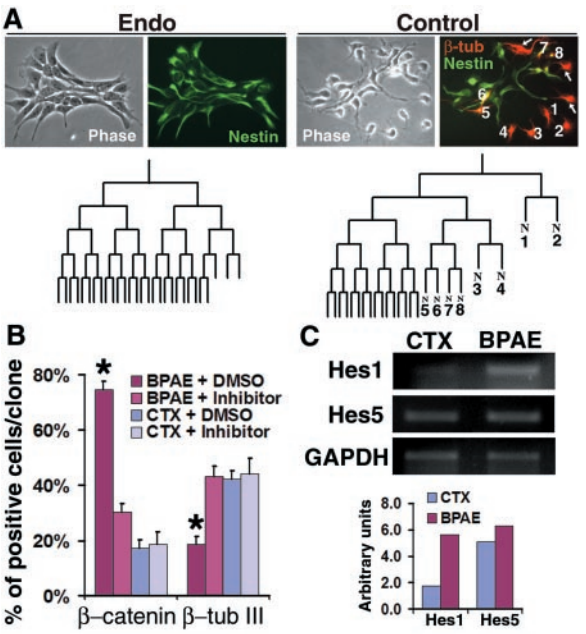

Fig. 4. Endothelial factors stimulate self-renewal of neural stem cells. (**A**) Comparison between typical lineage trees reconstructed from time-lapse video recordings of single E10 cortical stem cells grown with endothelial cells and those grown under control conditions. In endothelial coculture, the cortical stem cell divided symmetrically and did not make neurons during the recording period (all progeny were Nestin$^+$ as shown in the fluorescence and phase images of the final clone). In contrast, a cortical stem cell grown under control conditions generated an asymmetric lineage tree, generating neurons [β-tubulin-III$^+$ (red), designated as N in the lineage tree] as well as Nestin$^+$ progenitor cells (green). Neuronal progeny are numbered to show the match of cells in the final clone to the lineage tree. Arrows indicate neurons in the field that did not originate from this clone. (**B**) After treatment of 4-day-old cocultures with γ-secretase inhibitor II for 6 hours, β-catenin staining is significantly decreased and β-tubulin-III staining significantly increased in BPAE cocultured clones, whereas there was no effect on CTX cocultured clones (ANOVA; *, $P < 0.01$ by post-hoc tests). DMSO, dimethyl sulfoxide. (**C**) Hes1 is up-regulated after endothelial coculture, but Hes5 expression was similar to that in control coculture. Reverse transcription–polymerase chain reaction gel band densities were normalized to expression levels of glyceraldehyde phosphate dehydrogenase (GAPDH).

That projection neurons typical of the early embryo arise in E10-11 cocultures after many cell divisions suggests that endothelial factors promote stem cell self-renewal and inhibit the normal progression in which older stem cells preferentially produce glia or interneurons. We found few Tbr1$^+$ neurons produced from E15.5 stem cells and none from adult SVZ cells, indicating that endothelial factors are permissive, not instructive, for this fate: They cannot reverse the restriction.

Supporting the hypothesis that endothelial factors promote stem cell self-renewal, time-lapse video recording of dividing clones reveals that stem cells grown with endothelial cells underwent symmetric, proliferative divisions generating Nestin$^+$ progeny, in contrast to the asymmetric division patterns seen in control conditions (*6*, *11*) (Fig. 4A). Cortical stem cells cocultured with endothelial cells for 4 days generated more secondary stem cell clones, neurospheres, and neuron-generating progenitor cells than did those cocultured with CTX cells (fig. S4).

The most obvious effect of endothelial factors is that they promote neural stem cell growth as epithelial sheets with extensive junctional contacts (Fig. 1C), which could promote self-renewal by influencing β-catenin signaling pathways (*12*, *13*), mode of cell division (*14*), and Notch activation (*15*). Indeed, stem cells cocultured with endothelial cells and then exposed to γ-secretase inhibitor II, which inhibits Notch1 activation (*16*), showed a similar extent of cell-cell contact, division, and differentiation to those in CTX cocultures (Fig. 4B and fig. S5). In neural stem cells cultured with endothelial factors, the Notch effector Hes1 was up-regulated, but Hes5 was not (Fig. 4C), consistent with involvement of Hes1 in neural stem cell self-renewal (*17*, *18*).

Our results identify endothelial cells as critical components of the neural stem cell niche, as they secrete soluble factors that maintain CNS stem cell self-renewal and neurogenic potential. Thus, although FGF2 promotes neural stem cell proliferation, it cannot alone maintain their self-renewal; endothelial factors acting with FGF2 accomplish this.

In the presence of endothelial cells, a neural stem cell undergoes symmetric, proliferative divisions to produce undifferentiated stem cell sheets that maintain their multipotency and, upon endothelial cell removal, generate neurons as well as astrocytes and oligodendrocytes. No CD31$^+$ cells were detected in clones, showing that, at least under these circumstances, neural stem cells do not generate endothelial progeny.

Growth with endothelial cell–derived factors may be an important tool for promoting neural stem cell self-renewal and neurogenesis, allowing efficient production of neural stem cells and a variety of CNS neurons for use in replacement therapies.

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