Vascular Endothelial Growth Factor Directly Inhibits Primitive Neural Stem Cell Survival But Promotes Definitive Neural Stem Cell Survival

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There are two types of neural stem cells (NSCs). Primitive NSCs [leukemia inhibitory factor (LIF) dependent but exogenous fibroblast growth factor (FGF) 2 independent] can be derived from mouse embryonic stem (ES) cells in vitro and from embryonic day 5.5 (E5.5) to E7.5 epiblast and E7.5–E8.5 neuroectoderm in vivo. Definitive NSCs (LIF independent but FGF2 dependent) first appear in the E8.5 neural plate and persist throughout life. Primitive NSCs give rise to definitive NSCs. Loss and gain of functions were used to study the role of vascular endothelial growth factor (VEGF)-A and its receptor, Flk1, in NSCs. The numbers of Flk1 knock-out mice embryo-derived and ES cell-derived primitive NSCs were increased because of the enhanced survival of primitive NSCs. In contrast, neural precursor-specific, Flk1 conditional knock-out mice-derived, definitive NSCs numbers were decreased because of the enhanced cell death of definitive NSCs. These effects were not observed in cells lacking Fli1, another VEGF receptor. In addition, the cell death stimulated by VEGF-A of primitive NSC and the cell survival stimulated by VEGF-A of definitive NSC were blocked by Flk1/Fc-soluble receptors and VEGF-A function-blocking antibodies. These VEGF-A phenotypes also were blocked by inhibition of the downstream effector nuclear factor κB (NF-κB). Thus, both the cell death of primitive NSC and the cell survival of definitive NSC induced by VEGF-A stimulation are mediated by bifunctional NF-κB effects. In conclusion, VEGF-A function through Flk1 mediates survival (and not proliferative or fate change) effects on NSCs, specifically.

Key words: neural stem cell; embryonic stem cell; VEGF-A; cell survival/death; neurosphere; NF-κB

Introduction

Neural stem cells (NSCs), which exist in the embryonic brain germinal zone (and its postnatal remnant), can be expanded and their progeny induced to differentiate into neurons and glia in vitro and in vivo under the control of growth factors (Gage, 2000; van der Kooy and Weiss, 2000; Seaberg and van der Kooy, 2003). There are two major populations of NSCs: an early primitive NSC (p-NSC) population that gives rise to a later definitive NSC (d-NSC) population during embryonic development (Hitoshi et al., 2004). p-NSCs can be derived from undifferentiated embryonic stem (ES) cells and also can be isolated from the embryonic day 5.5 (E5.5) epiblast stage until the E8.5 neuroectoderm in mouse embryos (Hitoshi et al., 2004). d-NSCs can be derived from p-NSCs in vitro and are present in the E8.5 neural tube and persist into the senescent adult brain (Hitoshi et al., 2004). Both types of NSCs have similar potential to produce secondary and tertiary clonal colonies (neurospheres), and their clonal progeny can differentiate into neurons and glial cells. p-NSCs require exogenous leukemia inhibitory factor (LIF) for their maintenance and proliferation in vitro, but exogenous fibroblast growth factor (FGF) 2 is not required. In contrast, d-NSCs require only FGF2 or epidermal growth factor (EGF) (and not LIF) for their maintenance and proliferation (Hitoshi et al., 2004).

Vascular endothelial growth factor (VEGF) is known to be essential for both angiogenesis and hematopoiesis (Shalaby et al., 1995, 1997; Ema et al., 2003). Early hematopoietic cells and angioblasts express a number of genes in common, including type-1 VEGF receptor (commonly known as Flt1) and Kdr (commonly known as type-2 VEGF receptor or Flk1) (Dumont et al., 1995; Fong et al., 1996). Only mutation of Flk1 leads to the complete absence of hematopoietic and endothelial cell differentiation in vivo (Shalaby et al., 1995). A close anatomical relationship between proliferating hippocampal neural progenitor cells and pro-
ERG activity, 33258 (Roche, Welwyn Garden City, UK) for nuclear staining. To inhibit stained with a Nestin antibody (Chemicon, Temecula, CA) and Hoechst for 4 or 24 h. Cells were fixed with 4% paraformaldehyde (PFA) and for 4 or 24 h. Cells were fixed with 4% PFA and stained with either anti-mouse Ki-67 antibody (PharMingen, Franklin Lakes, NJ) or an in situ cell death detection kit (Roche).

Nuclear factor κB inhibitor treatment. SC-514 (100 μM; Calbiochem) or JSH-23 (100 μM; Calbiochem) were added to ES-derived p-NSC or mouse E14.5 d-NSC neurosphere cultures for 7 d in the presence or absence of 100 ng/ml human recombinant VEGF-A.

Reverse transcription-PCR from bulk spheres. Total RNA was isolated using the RNeasy extraction kit mini (Qiagen, Hilden, Germany) and 1 μg was used to synthesize cDNA with oligo(dT)(12–18) primers and MuMLV reverse transcriptase (SuperScript II; Roche) at 42°C for 1 h. For PCR, 1 μl of cDNA was used to amplify specific sequences for Nestin (forward, 5'-ATTTTGAAGAGCCGATGCACTGAG-3'; reverse, 5'-AGGCTCAG-CACTGTCTTACG-3'), Sox1 (forward, 5'-GCCGGATCTCTGTT-CAAGT-3'; reverse, 5'-TACAGAGCCGCGACTCAGTC-3'), Brachyury (forward, 5'-AGATGAAATTCCATGATCTC-3'; reverse, 5'-CCCCCTTTGTT-TACAAGTTCTC-3'), Flk1 (forward, 5'-TCAGTATTACGAGGAAACAGG-3'; reverse, 5'-AAACATTTTGTGGCACTCAGGG-3'), VEGF-A (forward, 5'-GACCTCGGATCTCTGGT-CAAGT-3'; reverse, 5'-GTTGAGGCTTACGACTCAGGG-3'), Flt1 (forward, 5'-GGACTAT-ACTGATTCTGTTG-3'; reverse, 5'-GAGAGTGACGCCACCCACA-3'), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-TCATTGACCTCACAAGTGGT-3'; reverse, 5'-AATGCAAGTTTGTGTGTGTG-3').

Single-sphere reverse transcription-PCR. Single-sphere reverse transcription-PCR (RT-PCR) followed the OneStep RT-PCR kit (Qiagen) protocol. Total RNA was extracted from single clonal sphere colonies and eluted in 15 μl of H2O. One microliter of the RNA preparation was used for quantification, and the remainder (containing ~0.1 μg of RNA) was used for RT-PCR.

Statistical analyses. Results are expressed as mean values ± SEM. ANOVA or t tests were used to analyze data as appropriate. Significant ANOVA factors were followed by post hoc comparisons of individual means using Tukey’s method where appropriate. Significance was defined as two-tailed p < 0.05.

Results

Lack of VEGF-A Signaling Enhances Primitive Neural Stem Cell Numbers

Initially, we examined the expression of Flk1 and VEGF-A in undifferentiated ES cells by RT-PCR. Wild-type Flk1+/− undifferentiated ES cells showed weak expression of Flk1 mRNA, and
as expected, undifferentiated Flk1−/− ES cells had no Flk1 expression (Fig. 1A). Undifferentiated Flk1+/+ and Flk1−/− ES cells each expressed VEGF-A mRNA and another VEGF receptor, Flt1 mRNA (Fig. 1A). We tested Flk1 function in the clonal formation of p-NSC-derived neurospheres by using Flk1-deficient ES cells in which the endogenous Flk1 gene is replaced by a β-galactosidase (lacZ) gene. Flk1−/− and Flk1−/− ES cells have one and two alleles replaced by the lacZ gene, respectively (Shalaby et al., 1995; Ema et al., 2003). Undifferentiated ES cells were suspended in LIF-containing, serum-free media conditions under which clonal p-NSC-derived neurospheres arose after 7 d (Tropepe et al., 2001). Interestingly, Flk1−/− ES cells produced a threefold greater number of sphere colonies than the control Flk1+/+ ES cells at the standard starting clonal cell density (10 cells/µl) and at a lower density (1 cell/µl) (Fig. 1B). Flk1+/+ p-NSC spheres formed in similar numbers to Flk1−/− p-NSC spheres (data not shown). In contrast, both the numbers (Fig. 1B) and diameters (Flk1+/+, 211 ± 36 µm; Flk1−/−, 226 ± 21 µm; t5 = 0.4; p > 0.5) of p-NSC clonal spheres derived from Flk1 knock-out ES (Flk1−/−) cells (Fong et al., 1999) were not different from the p-NSC clonal spheres derived from the control wild-type R1 ES cells. This suggests that VEGF-A signaling specifically through Flk1 receptors (not Flt1) regulates p-NSC formation. Moreover, when spheres were plated on Matrigel-coated dishes under differentiation conditions, the numbers of neurons and glial cells produced by both Flk1+/+ and Flk1−/− p-NSC spheres were similar (Table 1). These data suggest that loss of Flk1 expression produces only a p-NSC increase with no effect on p-NSC multipotency. Furthermore, Flk1+/+ and Flk1−/− p-NSC sphere diameters were similar (Flk1+/+, 205 ± 12 µm; Flk1−/−, 212 ± 17 µm; t5 = 0.4; p > 0.5), suggesting the Flk1 deficiency affects the p-NSC itself and not the proliferation of its neural progenitor progeny that comprise most of the cells in each sphere.

An initial gene expression characterization of the bulk sphere colonies was performed using RT-PCR. Flk1 mRNA expression was observed only in Flk1+/+ and not in Flk1−/− p-NSC spheres (Fig. 1C). Flk1−/− p-NSC spheres expressed Brachyury mRNA (a mesodermal marker), in addition to the neural markers Nestin and Sox1, whereas wild-type Flk1+/+ p-NSC spheres expressed only Nestin and Sox1 (Fig. 1C). This result suggests that Flk1−/− p-NSC colonies may have broader non-neural potential than Flk1+/+ p-NSCs. Nevertheless, even wild-type p-NSCs have the ability to form non-neural cell types when integrated into embryos derived from blastocyst chimeras (Tropepe et al., 2001). Indeed, p-NSC colonies that arise from wild-type Flk1+/+ ES cells cultured at high density (100 cells/µl) also show weak expression of Brachyury mRNA (Fig. 1D).

The Brachyury mRNA expression in wild-type p-NSC colonies derived from high-cell-density wild-type Flk1+/+ cultures may be explained in two ways. First, clonal p-NSCs (which have the potential to produce non-neural cells and express Brachyury when placed back into the high-cell-density blastocyst) (Tropepe et al., 2001) also may reveal this potential in high-density culture in vitro as well (present results). Second, high-density ES cultures (although done in neural-inducing, serum-free media conditions) may produce culture conditions similar to embryoid body cultures, in which separate ES cells aggregate to produce the different cell lineages of all three germ layers. To test these clonal versus nonclonal possibilities, we cultured Flk1+/+ and Flk1−/− ES cells at low densities at which only clonal p-NSC colonies formed (Tropepe et al., 2001) and then analyzed clonal p-NSC sphere colonies by RT-PCR. Although all the clonal Flk1+/+ p-NSC spheres expressed only Nestin (16 of 16) and never Brachyury (0 of 16), some clonal Flk1−/− p-NSC spheres expressed Brachyury (6 of 16) in addition to Nestin (16 of 16) (Fig. 1E). This suggests that the potential to produce non-neural cells is enhanced in clonal Flk1−/− p-NSCs compared with clonal Flk1+/+ p-NSC colonies.

Table 1. Loss of Flk1 does not affect the differentiation of neurons and glia from the progeny of either p-NSCs derived from Flk1−/− ES cells or d-NSCs derived from E14.5 Nes−/−Flk1−/− conditional knock-out mice

<table>
<thead>
<tr>
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<th>MAP2− neuron (n)</th>
<th>GFAP− astrocyte (n)</th>
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<tbody>
<tr>
<td>p-NSC Flk1+/+</td>
<td>2.5 ± 0.6(4)</td>
<td>17.4 ± 5.9(4)</td>
</tr>
<tr>
<td>p-NSC Flk1−/−</td>
<td>2.2 ± 0.8(4)</td>
<td>18.7 ± 6.0(4)</td>
</tr>
<tr>
<td>d-NSC Flk1+/+</td>
<td>3.8 ± 1.1(5)</td>
<td>43.6 ± 5.7(5)</td>
</tr>
<tr>
<td>d-NSC Flk1−/−</td>
<td>3.4 ± 0.9(4)</td>
<td>38.3 ± 7.6(4)</td>
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The proportions of microtubule-associated protein 2 (MAP2)− and GFAP− cells were calculated from total cells.

Flk1−/− primitive neural stem cells show less cell death than control Flk1+/+ primitive neural stem cells

To test whether the initial fate change from single ES cells to single p-NSCs was affected by the Flk1 mutation, we observed Nestin expression 4 h after removal of serum and all growth factors from the ES cells, a time at which wild-type ES cells already have begun to express Nestin and default to p-NSCs (Tropepe et al., 2001). No Nestin expression was observed in undifferentiated ES cells (Fig. 2C). After 4 h in the minimal, neural default media, equal numbers of Nestin-positive cells were found in the Flk1−/− ES or Flk1+/+ ES cells (Fig. 1F). Moreover, the increase of non-YFP fluorescent Flk1−/− p-NSC spheres remained threefold higher than the number of non-YFP fluorescent Flk1+/+ p-NSC spheres (Fig. 1F). These data suggest that the Flk1−/− mutation works cell autonomously in the p-NSC-forming cell.
VEGF-A directly decreases primitive neural stem cell numbers

To investigate gain of Flk1 function effects on p-NSCs, VEGF-A was added to our minimal neuronal differentiation cultures. Flk1 is stimulated by VEGF-A in several kinds of cells during development (Cross et al., 2003). When undifferentiated ES cells were cultured with VEGF-A, p-NSC sphere numbers decreased significantly (Fig. 3A). However, VEGF-A treatment of these wild-type p-NSC spheres did not induce Brachyury mRNA expression and did not change Nestin and Flk1 mRNA expression (Fig. 4B). To check the specificity of VEGF-A action, two different VEGF-A inhibitors were added separately to p-NSC sphere cultures. The ability of VEGF-A to decrease p-NSC numbers was blocked by both of the VEGF-A antibodies and the Flk1/Fc chimeric soluble receptors (Fig. 3A). These results indicate that VEGF-A acts specifically on the Flk1 receptor in suppressing p-NSC formation. Moreover, VEGF-A inhibitors also increased p-NSC sphere numbers even in the absence of VEGF-A (Fig. 3A). This suggests that endogenous VEGF-A is released by the cells in culture and that the inhibitors are overcoming this endogenous effect.

Lack of VEGF-A signaling enhances primitive neural stem cell numbers in vivo

Flk1 conventional knock-out mice (Flk1<sup>−/−</sup> mice) die at around E8.5 (Shalaby et al., 1995). Although that is the earliest stage at which d-NSC can be isolated during development (Tropepe et al., 2001), p-NSCs can be isolated from the epiblast at E7.5 (Hitoshi et al., 2004). p-NSCs were isolated from E7.5 Flk1<sup>−/−</sup> and control wild-type (Flk1<sup>+/+</sup>) mice and cultured in LIF-containing media for 7 d. p-NSC-derived neurons from Flk1<sup>−/−</sup> epiblasts showed a 60% increase compared with numbers of p-NSCs derived from control Flk1<sup>+/+</sup> epiblasts (Fig. 3B). Thus, more p-NSCs are derived from both Flk1<sup>−/−</sup> ES cells and Flk1<sup>−/−</sup> embryos compared with wild-type controls. Together, these results imply that Flk1 acts to suppress p-NSC numbers.

Addition of VEGF-A increases the numbers of definitive neural stem cells
d-NSCs have formed in the E8.5 neural tube over a 1 d period from the p-NSCs of the epiblast and neural plate (Hitoshi et al., 2004). These d-NSCs persist in the embryonic and adult brain germinal zones throughout life. The proliferation and self-
Figure 2. A. Dissociated undifferentiated ES cells were plated on gelatin-coated chamber slides at 10 cells/μl in ES culture media containing 10% FBS. There were no significant differences in viable cells between Flk1+/+ and Flk1−/− undifferentiated ES cells at 4 h after plating. Although cell numbers were increased at 24 h after plating compared with 4 h, Flk1+/+ and Flk1−/− undifferentiated ES cells showed similar numbers of viable cells (at 4 h, t₀ = 0.2; p > 0.5; at 24 h, t₀ = 0.3, p < 0.5). B. Short-term differentiation assay from undifferentiated ES cell to p-NSC. Equal numbers of cells were present in both Flk1+/+ and Flk1−/− cultures at the 4 h after plating. However, fewer cells were found in Flk1+/+ than Flk1−/− cultures at 24 h after plating. A few Nestin-negative cells were observed only in Flk1−/− cultures at both 4 and 24 h after plating. C. RT-PCR of short-term differentiated ES cells. Nestin mRNA expression was detected in both Flk1+/+ and Flk1−/− p-NSC cells after 24 h of differentiation. Data are mean ± SEM.

VEGF-A signaling in definitive neural stem cells acts to increase cell survival

Conditional inactivation of VEGF-A in the developing neuroectoderm [using the Nestin-Cre transgenic line (Tronche et al., 1999) and mice that carry a conditional null VEGF-A allele (Gerber et al., 1999)] resulted in dramatic phenotype involving loss of vascularity and subsequent hypoxia, resulting in the degeneration of the cerebral cortex and neonatal lethality (Haigh et al., 2003; Raab et al., 2004). To distinguish between the paracrine vascular role of VEGF-A and a potential direct neuronal autocrine role of VEGF-A contributing to this severe brain phenotype, Flk1 (through Nestin-Cre action) was conditionally inactivated in neural precursor cells (Haigh et al., 2003). Surprisingly, despite the deletion of Flk1 in the developing neural precursor cells, no major lethality was observed compared with the dramatic degenerative phenotypes associated with the conditional inactivation of VEGF-A in the nervous system. To more specifically address the role of Flk1 in NSC function, we investigated the effects of loss of VEGF-A signaling on d-NSCs by using the previously reported nervous system Flk1 conditional knock-out mice (NesCre;Flk1−/−) (Haigh et al., 2003). Forebrain germinal...
zone tissue from adult Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− mice was dissociated in FGF2- and EGF-containing, serum-free media. d-NSC-derived clonal neurosphere numbers in the adult Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− mice showed a dramatic 85% decrease compared with control mice (Fig. 5A). The sizes (diameters) of individual clonal primary neurospheres from adult Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− mice (275 ± 26 μm) were not significantly different (t<sub>15</sub> = 0.7; p > 0.5) from these of control Nes<sup>Cre</sup>;Flk<sup>1+</sup>+/− mice (263 ± 35 μm). These results suggest that d-NSCs are less likely to survive to adult stages in Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− mice compared with control Nes<sup>Cre</sup>;Flk<sup>1</sup>+/− mice, but the proliferation of the Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− d-NSCs that do survive was not compromised, suggesting that Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− neural progenitor cells that make up most of the cells in the d-NSC spheres were unaffected by the Flk1 deficiency.

Even at E14.5, there is already a significant difference in the numbers of clonal d-NSCs derived from E14.5 Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− conditional knock-out mice compared with control Nes<sup>Cre</sup>;Flk<sup>1</sup>+/− mice (a decrease that is maintained through passing from primary to tertiary spheres in vitro) (Fig. 5B). The similar passing of the Flk1 knock-out and control adult neurospheres and the similar differentiation multipotentiality (into neuron and glia) of the single clonal adult neurospheres (Table 1) suggest that the loss of Flk1 has not modified these stem cell qualities (self-renewal and differentiation potential) of the d-NSCs, but rather affects the survival of the d-NSCs.

To ask whether the decreased neurosphere numbers in the Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− mice were caused by deficits in cell survival or cell proliferation, we acutely dissociated tissue directly from the adult mouse forebrain subependymal germinal zone and plated the cells on Matrigel-coated chamber slides. After 8 h incubation in serum-free, chemically defined media, cells were fixed and assessed for apoptosis and proliferation. Counts of total cell numbers revealed control Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− cells survived the overnight incubation significantly better than conditional knock-out Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− cells (Fig. 5C). Moreover, there were more apoptotic terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-positive cells in the overnight Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− cultures than in the control Nes<sup>Cre</sup>;Flk<sup>1</sup>+/− cultures (Fig. 5C). However, ~70% of the cells were proliferating, Ki-67-positive cells in both the Nes<sup>Cre</sup>;Flk<sup>1</sup>−/− and control cultures (Fig. 5C). These observations suggest that Flk1 signaling in neural cells directly isolated from the adult mouse forebrain germinal zone increases cell survival (but does not affect neural precursor proliferation) and imply that Flk1 signaling may increase d-NSC clonal colony numbers by increasing the cell survival of d-NSCs.

Lack of VEGF-A signaling decreases the definitive neural stem cells derived from the enhanced primitive neural stem cell population

The results so far suggest two separate effects of VEGF-A signaling: VEGF-A signaling decreases p-NSC survival and increases d-NSC survival. Because of the fact that ES-derived LIF-dependent p-NSCs can mature directly into FGF-dependent d-NSCs after single neurosphere suspension culture passaging (Troupepe et al., 2001; Hitoshi et al., 2004), we asked whether the two separate and opposite effects of VEGF-A signaling could be demonstrated within the same lineage in vitro. First, Flk1−/− primary clonal p-NSC spheres (derived directly from single mutant ES cells) showed the same threefold increase (that was described above) compared with primary p-NSC spheres derived directly from single Flk1−/− ES cells. However, when these ES cell-derived p-NSC clonal neural sphere colonies were dissociated and passaged as d-NSCs, Flk1−/− d-NSC neurosphere formation was significantly greater than Flk1−/− secondary d-NSC neurosphere formation in FGF2- and B27-containing media (Fig. 5D). A decrease in the number of secondary (d-NSC) neurospheres from the dissociation of single Flk1−/− compared with Flk1+/+ ES cell-derived clonal p-NSC neurosphere numbers (Fig. 5A), confirmed the bulk dissociation results. In contrast, the numbers of passaged clonal d-NSC neurosphere colonies derived from Flt1−/− p-NSCs were not changed compared with d-NSC colonies derived from Flt1+/+ p-NSCs (Fig. 5D), suggesting a specific action of Flk1 and not Flt1 on d-NSCs. Although Flk1 mRNA was expressed only in Flk1+/+ and not Flk1−/− cells, the mRNAs for the VEGF-A ligand and Flt1 were expressed both in the Flk1+/+ and Flk1−/− ES cell-derived p-NSC primary neurosphere colonies and also in the Flk1+/+ and Flk1−/− secondary d-NSC neurosphere colonies derived from primary p-NSCs (Fig. 5E).

The opposite effects of the loss of VEGF-A signaling on ES cell-derived primary p-NSC versus secondary d-NSC neurosphere formation within the same in vitro neural lineage parallel the opposite effects of loss of VEGF-A signaling on p-NSCs derived from E7.5 embryos (increased) versus d-NSC-derived from later embryos or adult brains (decreased).

NF-κB inhibits p-NSC survival and d-NSC cell death

NF-κB is a downstream effector of VEGF-A signaling (Santos and Dias, 2004) and has two effects on cell death (Barkett and Gilmore, 1999). NF-κB increases the survival of immature
B-lymphocytes (Sonenshein, 1997) but also induces cell death by activating expression of Fas ligand (and the tumor necrosis factor death receptor family) after T-cell receptor engagement (Matsui et al., 1998).

The expression of NF-κB was observed in both p-NSCs and d-NSCs derived from both Flk1+/−/− and Flk1−/− cells (Fig. 5E). To investigate whether NF-κB regulates cell death/survival downstream of VEGF-A signaling, NF-κB inhibitors were added to both p-NSC and d-NSC cultures. SC-514 inhibits the kinase activity of IkB kinase, a part of the NF-κB complex (Kishore et al., 2003). JSH-23 suppresses the DNA binding of NF-κB (Shin et al., 2004). These two types of NF-κB inhibitors (SC-514 and JSH-23) were added to cultures derived from wild-type R1 ES cells. The numbers of p-NSCs were significantly increased by both SC-514 and JSH-23 (Fig. 3C). These NF-κB inhibitors also increased p-NSC numbers in the absence of VEGF-A (Fig. 3C).

In contrast, E14.5 d-NSC neurosphere numbers were decreased by the two NF-κB inhibitors (Fig. 4E). The NF-κB inhibitors also decreased p-NSC numbers in the absence of VEGF-A (Fig. 4E). These two opposite direction effects of NF-κB inhibitors on p-NSCs versus d-NSCs parallel those seen with our manipulations of VEGF-A signaling.

**Discussion**

Single ES cells in minimal, serum-free media will default to p-NSCs (Tropepe et al., 2001; Hitoshi et al., 2004; Smukler et al., 2006). However, under these minimal culture conditions, many of the p-NSC then die. The present results show that VEGF-A signaling actually promotes the death of these p-NSCs (summary figure in supplemental material, available at www.jneurosci.org). On the other hand, both in vitro and in vivo, the survival of the embryonic d-NSCs that arise from p-NSCs (and the survival of adult d-NSCs) is promoted by VEGF-A signaling (summary figure in supplemental material, available at www.jneurosci.org). These data suggest cell death/survival effects of VEGF-A signaling on p-NSCs and d-NSCs, rather than cell proliferation or fate change effects. There is no evidence suggesting that VEGF-A regulates the transition between p-NSCs and d-NSCs. Indeed, VEGF-A signaling decreases the numbers of p-NSCs, so these...
fewer p-NSCs cannot give rise to the increased survival of d-NSCs that is produced by VEGF-A signaling. Manipulations of NF-κB (a known downstream effector of VEGF-A signaling) (Santos and Dias, 2004) also produce opposite effects on p-NSCs versus d-NSCs. Moreover, the effects of NF-κB manipulations were seen in the presence or absence of VEGF-A, suggesting that NF-κB acts downstream of VEGF-A signaling in the control of neural stem cell survival as well (summary figure in supplemental material, available at www.jneurosci.org).

The opposite effects of VEGF-A signaling on p-NSCs and d-NSCs both operate through a cell survival mechanism rather than through another cell biological process. VEGF-A signaling is not involved in the default fate change from an ES cell to a p-NSC. Four hours after being placed in serum- and growth factor-free media, similar numbers of Nestin-negative Flk1−/− and Flk1+/+ undifferentiated ES cells had defaulted to a Nestin-positive p-NSC fate. However, by 24 h in the same conditions, three times more Flk1−/− p-NSCs were alive than Flk1+/+ p-NSCs. Similarly, but in the opposite direction, many more apoptotic, TUNEL-positive NesCre;Flk1−/− than NesCre;Flk1+/+ adult neural precursor cells were observed 24 h after dissociation of the forebrain germinal zone. These data suggest that VEGF-A signaling controls the numbers of NSC through the regulation of cell survival. VEGF-A signaling does not appear to affect the proliferation of d-NSC because (1) the numbers of Ki-67-positive, primary adult neural precursor cells are similar in the NesCre;Flk1−/− and NesCre;Flk1+/+ short-term cultures, and (2) the individual neurospheres that proliferate clonally from both p-NSCs and d-NSCs of both the Flk1−/− and Flk1+/− genotypes are of similar sizes, and thus consist of similar numbers of cells.

p-NSC spheres are derived clonally from single p-NSCs (Tropepe et al., 2001; Smukler et al., 2006). Thus, the numbers of p-NSC spheres are taken as a direct measure of the number of p-NSCs. The p-NSC spheres are made up of many neural progenitors and only a few p-NSCs that passage as d-NSCs. Given that the Flk1+/+ and Flk1−/− p-NSC spheres are similar in size (and that most of the cells in the spheres are progenitors), this suggests that VEGF-A signaling had little effect on neural progenitor cell survival, as well as no effect on cell lineage transitions (Fig. 4B). Moreover, because it is not possible to passage clonal p-NSCs as p-NSCs at this point, we cannot test a role for VEGF-A signaling in the maintenance of p-NSCs.

Brachyury mRNA expression in p-NSCs derived from Flk1−/− (but not Flk1+/+) ES cells reveals not only a mesodermal
character, but we suggest also a less committed p-NSC phenotype. Although our data suggest that the loss of Flk1 affects both p-NSC survival and pluripotency, it is difficult to exclude the possibility that one phenotype is a direct effect of the other. The Brachyury expression in only some clonal Flk1−/− p-NSC spheres (Fig. 1E) might suggest that the survival and pluripotency effects of loss of VEGF-A signaling can be dissociated, but this also may reflect simple variation of Brachyury expression in the mutant clones. It remains possible that the Flk1−/− mutant p-NSC population is heterogeneous, but there is no evidence other than the variable expression of Brachyury that speaks to this point.

Although Notch signaling is essential for the maintenance of d-NSCs (Hitoshi et al., 2002, 2004), it is doubtful that VEGF-A signaling works through a Notch-like self-renewal mechanism, because Flk1 mutant d-NSCs do not show the significant decrease over in vitro passaging that is seen with the in vitro passaging of Notch pathway loss of function NSC mutants (Hitoshi et al., 2002). Furthermore, the similar neuronal and glial differentiation from Flk1−/− and Flk1−/− p-NSCs also suggests a different mechanism of action from Notch signaling in which increased neuronal and glial differentiation is seen from clonal NSCs (Hitoshi et al., 2002). Therefore, we conclude that the VEGF-A signaling effects on NSC survival provide the most parsimonious single factor, cell biological mechanism to explain VEGF-A pathway gain and loss of function effects.

Both clonal p-NSC and d-NSC colony formation were similar when starting with wild-type and Flt1−/− ES cells. These data suggest that the VEGF-A effects on p-NSCs and d-NSCs are working through Flk1, and not Flt1, receptors and reveal that Flt1 is less important than Flk1 in neural stem cell regulation.

Wild-type ES cell-derived p-NSCs have the potential to differentiate into not only neural tissues but also other non-neural lineages when they are used to make mouse E3.5 blastocyst chimeras (Tropepe et al., 2001). Similarly, in high-density ES cell culture, non-neural markers can appear in the wild-type p-NSC-derived neural spheres that only express neural markers when they derive from the low-density cultures. This suggests that the potential of wild-type p-NSCs to give rise to non-neural markers when they arise from the low-density cultures. This suggests that the potential of wild-type p-NSCs to differentiate into non-neural lineages is a particularly obvious advantage, because VEGF-A signaling suppresses the potential of clonal p-NSCs to differentiate into non-neural cell types, we suggest that the re-activation of VEGF-A signaling may be one of those especially advantageous conditions. Adult mouse brain-derived d-NSCs have been reported to differentiate into hematopoietic and other non-neural cells at very low frequencies (Bjornson et al., 1999; Clarke et al., 2000), but our results at least with wild-type NSCs have not replicated these d-NSC non-neural potentials (Morshead et al., 2002; T. Wada, unpublished data).

VEGF-A can be secreted by primitive and visceral endoderm as early as E5.5–E8.0 in the mouse embryo, at the same stage when p-NSCs can be observed in the epiblast and forming neuroectoderm (Miquerol et al., 1999). This result raises the possibility that VEGF-A released by non-neural epithelial cells may act on p-NSCs in vivo. However, our observation of VEGF-A and Flk1 mRNA expression in undifferentiated ES cells suggests even earlier embryonic effects, in which VEGF-A signaling is part of the mechanism (in addition to TGFβ signaling) (Tropepe et al., 2001) that suppresses p-NSC formation and survival in vivo before neural plate formation of Nestin+ cells is first detected at E7.5. Although undifferentiated ES cells in hypoxic conditions respond to VEGF-A as a survival factor (Brusselmins et al., 2005), this finding is different from the present results. The previous effects were seen only under hypoxic conditions and only on undifferentiated ES cells, whereas the present results were specific to primitive neural stem cells under nonhypoxic conditions. There also are several possible VEGF-A sources that might permit the endogenous regulation of d-NSC in both embryonic and adult brain. First, VEGF-A is expressed by d-NSC-derived clonal neurospheres (Maurer et al., 2003) and also acts as a survival factor for neural precursor cells (not distinguishing between stem and progenitor cells) in vitro and in vivo (Schanzer et al., 2004). Second, the endothelial cells of blood capillaries in the neighborhood of neural precursor cells (Palmer et al., 2000; Shen et al., 2004) allow for a paracrine action on d-NSCs (Haigh et al., 2003). Reactive astrocytes also are a source of VEGF-A when injury occurs (Chow et al., 2001). Certainly, non-VEGF-A-mediated signals from endothelial cells regulate d-NSC behaviors as well (Shen et al., 2004). The dramatic decrease of in vitro clonal d-NSC-derived neurosphere number from the brains of NesCre;Flk1−/− mice seems at variance with the finding of only relatively minor phenotypes in the embryonic brains of NesCre;Flk1−/− mice (Haigh et al., 2003). Presumably, the minimal culture conditions used to isolate NSCs in vitro emphasize the cell death effects of manipulating VEGF-A signaling. In contrast, such cell death effects in the brain of the NesCre;Flk1−/− mice in vivo may be compensated by other growth and survival factors.

Although there are differences in the growth factor dependency [LIF, FGF2, TGFβ (Tropepe et al., 2001) and Notch signaling (Hitoshi et al., 2004)] of p-NSCs compared with d-NSCs, the present report reveals completely opposite survival effects of a single growth factor (VEGF-A) on these two NSC types. Indeed, the separate VEGF-A survival effects on the p-NSCs versus d-NSCs appear to be independent of any direct effects on fate changes, proliferation, or self-renewal and appear to be specific effects on NSCs as opposed to other cells in the neural lineage.

References


