

# VEGF is a chemoattractant for FGF-2–stimulated neural progenitors

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**M**igration of undifferentiated neural progenitors is critical for the development and repair of the nervous system. However, the mechanisms and factors that regulate migration are not well understood. Here, we show that vascular endothelial growth factor (VEGF)-A, a major angiogenic factor, guides the directed migration of neural progenitors that do not display antigenic markers for neuron- or glia-restricted precursor cells. We demonstrate that progenitor cells express both VEGF receptor (VEGFR) 1

and VEGFR2, but signaling through VEGFR2 specifically mediates the chemotactic effect of VEGF. The expression of VEGFRs and the chemotaxis of progenitors in response to VEGF require the presence of fibroblast growth factor 2. These results demonstrate that VEGF is an attractive guidance cue for the migration of undifferentiated neural progenitors and offer a mechanistic link between neurogenesis and angiogenesis in the nervous system.

## Introduction

Migration of immature neurons during development is essential for the proper formation of the nervous system. In the mammalian brain, most neurons are generated within proliferative zones around the ventricle from where immature precursors migrate to specific sites in the cerebral wall (Marin and Rubenstein, 2003). A variety of clinical syndromes, including various forms of Lissencephalies, are related to deficient migration of neural cells (Ross and Walsh, 2001). The consequences of these malformations include mental retardation, epilepsy, paralysis, and blindness. Genetic studies of some of these perturbations have provided some understanding of the regulation of neuronal migration. The genetic repertoire that is required for neuronal migration has rapidly expanded over the past 10 yr (Rubenstein and Rakic, 1999; Ross and Walsh, 2001). In addition to playing a key role in early development, neuronal migration is also important for the adult brain. For example, in the brain of songbirds, neurogenesis and neuronal migration are required for structural plasticity and learning throughout adulthood (Goldman and Nottebohm, 1983). Recent evidence suggests

that undifferentiated multipotential progenitors also exist in the adult mammalian brain and during adult neurogenesis, as well as during the continuous neuronal replacement that occurs at specific sites in the rostral subventricular zone (SVZ)–olfactory bulb system and the dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Marshall et al., 2003). Finally, cell migration plays a central role in wound repair. Although the intrinsic capacity of the adult mammalian brain to replace lost or damaged neurons is very limited, migration of neural progenitors and cell replacement has been reported after administration of growth factors such as FGF-2 as well as after transplantation of purified progenitors (Kuhn et al., 1997; Arvidsson et al., 2002; Ben-Hur et al., 2003).

Considerable effort has recently been focused on understanding the factors and mechanisms involved in the navigation of immature neurons to their final destination. Highly conserved families of attractive and repulsive molecules are coordinately regulated to guide neurons to their final destination. These families include netrins, semaphorins, ephrins, Slits, and various neurotrophic factors (Marin and Rubenstein, 2003; Marin et al., 2003). Compared with migration of postmitotic immature neurons, little is known about the factors and mechanisms that direct the migration of neural stem cells and undifferentiated progenitors. In one study,

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Key words: VEGF receptors; live cell microscopy; directional guidance; cell motility; FGF-2

Abbreviations used in this paper: FMI, forward migration index; SVZ, subventricular zone; VEGFR, VEGF receptor.

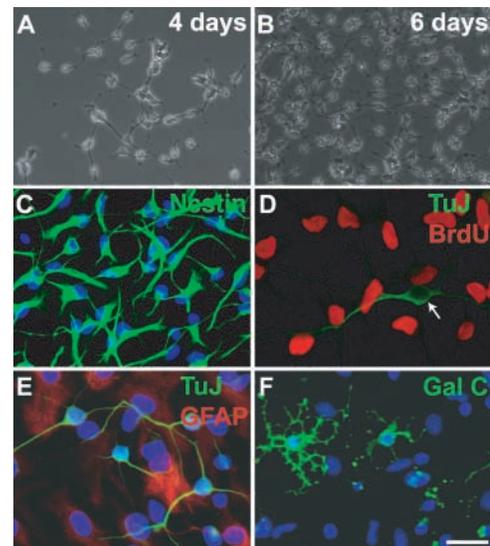
PDGF was shown to attract FGF-2-stimulated neural progenitors in a transfilter migration assay (Forsberg-Nilsson et al., 1998). Identifying candidate molecules that could play a role in this process is crucial not only for understanding proper tissue formation during development but also for developing methods for directing undifferentiated progenitors to achieve structural brain repair. Here, we report that VEGF-A is one such molecule.

VEGF belongs to a family of glycoproteins that plays an essential role in the development of blood vessels (vasculogenesis), the generation of new vascular networks from existing vessels (angiogenesis), and hematopoiesis (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001). The VEGF gene family has at least five members in mammals including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (Matsumoto and Claesson-Welsh, 2001). These secreted molecules bind with high affinity to tyrosine kinase receptors including VEGF receptor (VEGFR) 1 (Flt-1), VEGFR2 (KDR/Flk1), VEGFR3 (Flt-4), and neuropilin-1 and -2. As a result of the receptor activation and subsequent signal transduction, VEGF target cells may proliferate, migrate, or alter gene expression; e.g., of matrix metalloproteases or cytokines. Recent evidence suggests that VEGF is expressed in neural cells and plays a role in diverse aspects of brain development including axonal growth (Sondell et al., 1999, 2000), cell survival (Sondell et al., 1999; Jin et al., 2000; Ogunshola et al., 2002), and neuroprotection against glutamate toxicity (Matsuzaki et al., 2001; Svensson et al., 2002). Moreover, it has been suggested that VEGF can enhance neurogenesis in the SVZ *in vivo* as well as *in vitro* (Jin et al., 2002).

Angiogenesis and neurogenesis occur concurrently in the adult dentate gyrus (Palmer et al., 2000) and in the songbird brain (Louissaint et al., 2002). These observations raised the intriguing possibility that VEGF could function as a common factor to recruit both endothelial cells and neurogenic progenitors to specific sites. To test this hypothesis, we purified neural progenitors from the SVZ of newborn rats and expanded the cells with FGF-2. In a cell-based chemotaxis assay, we demonstrate that concentration gradients of VEGF induce directional sensing by neural progenitor cells. Chemoattraction by VEGF was mediated by signaling through VEGFR2. Moreover, we observed that FGF-2 increases expression of VEGFRs in neural progenitors, and that the chemotactic response of progenitor cells only occurs in the presence of FGF-2. Finally, cocultures of SVZ explants with cells secreting VEGF in a three-dimensional collagen gel matrix confirmed that VEGF acts as a chemoattractant for neural progenitors.

## Results

To investigate the potential role of VEGF in the regulation of FGF-2-stimulated neural progenitor migration, SVZ cells were isolated from newborn rats and cultured in the presence of FGF-2 in defined medium as described previously (Lim et al., 2000). 4 d after plating, the cells had an immature, round, or bipolar morphology (Fig. 1 A). Daily observations indicated that cells divided, formed loose colonies, and, by day 6, formed a monolayer (Fig. 1 B). At this stage,



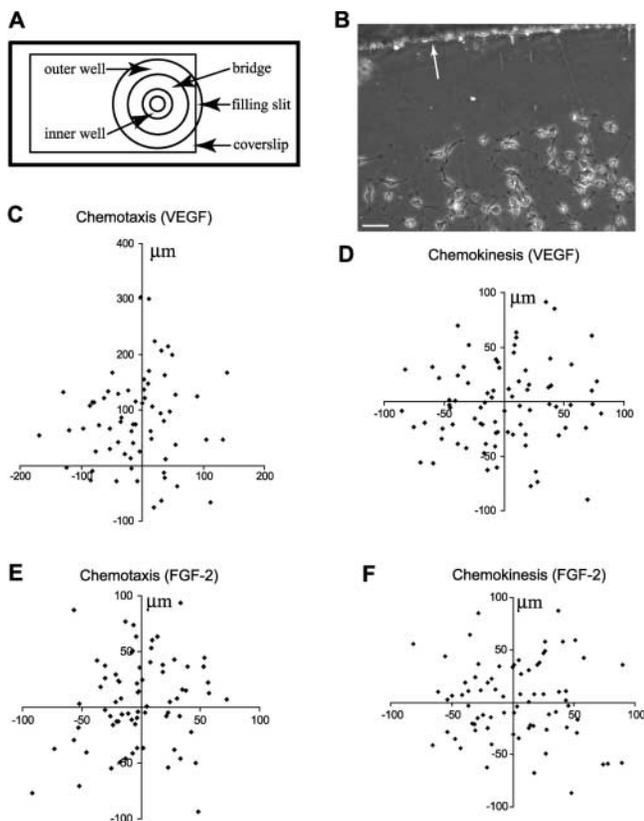
**Figure 1. Morphological and immunocytochemical characterization of neural progenitors in culture.** Neural progenitors were isolated and purified from the SVZ of newborn rat brains and cultured on matrigel-coated coverslips in the presence of 20 ng/ml FGF-2. (A and B) Phase-contrast images of neural progenitors at day 4 (A) and day 6 (B) in culture. (C) After 6 d in culture, the majority of cells are immunopositive for nestin, indicating that they are undifferentiated neural progenitors. (D) BrdU incorporation (red) showing that the majority of cells are proliferating. The rare cells that are positive for the neuronal marker (Tuj, green, arrow) are nonproliferative. (E and F) 5 d after the withdrawal of FGF-2, cells differentiate into GFAP-containing astrocytes (E, red), Tuj-positive neurons (E, green), and GalC-positive oligodendrocytes (F, green). Cell nuclei were counterstained with Hoechst 33342 in C, E, and F. Bar: (A and B) 80  $\mu$ m; (C) 30  $\mu$ m; (D) 19  $\mu$ m; (E and F) 30  $\mu$ m.

the vast majority (98%) of cells were stained with an Ab (Fig. 1 C), which is considered to be a marker of neural progenitors. Less than 3.2% of the cells expressed the neuronal marker Tuj; PSA-NCAM and BrdU incorporation showed that these cells did not divide (Fig. 1 D). Very few to no cells displayed immunoreactivity for GFAP or Gal C, markers for astrocytes and oligodendrocytes, respectively (unpublished data). With the exception of the few differentiated cells, progenitor cells maintained in the presence of FGF-2 did not display antigenic markers for neuron- or glia-restricted precursor cells including PSA-NCAM, doublecortin, NeuN, NG2, or A2B5 (unpublished data). In addition, nestin-positive cells were negative for endothelial markers such as von Willebrand factor and RECA-1 (unpublished data). These results indicate that the cultures consist of immature cells that do not yet possess cell lineage-specific markers for neurons or glial cells. When cultures were allowed to differentiate under conditions shown previously to stimulate both neuronal and glial differentiation (Palmer et al., 1997), >96% of the population displayed immunoreactivity for neuronal and astrocytic markers (Tuj+, 21%; GFAP+, 75%; Fig. 1 E). The remaining population was immunoreactive for oligodendrocyte markers A2B5 or Gal C (Fig. 1 F). These observations concur with earlier studies (Trapepe et al., 1999) showing that FGF-2-expanded cells are multipotential neural progenitors that can give rise to neurons, as-

trocytes, and oligodendrocytes, the three major cell types in the central nervous system.

### Chemoattraction of FGF-2-stimulated progenitors by VEGF

Next, we examined the response of neural progenitors to gradients of VEGF in the direct-viewing Dunn chemotaxis chamber (Zicha et al., 1991; Allen et al., 1998). This apparatus allows for direct monitoring of cell locomotion, including analysis of migration speed, turning behavior, and directionality (see Materials and methods). It has been established that chemoattractants added to the outer well of the Dunn chamber diffuse across the bridge to the inner well (Fig. 2, A and B) and form a linear steady gradient within  $\sim 30$  min of setting



**Figure 2. VEGF stimulates chemotaxis of neural progenitors.** (A) Schematic representation of Dunn chamber (top view) with the overlying coverslip, showing the position of the inner well, bridge, and outer well. (B) Cells over the annular bridge between the inner and outer wells of the chamber can be observed under phase-contrast optics. Arrow indicates the direction of the outer well of the Dunn chamber. Bar, 50  $\mu\text{m}$ . Cell migration was recorded continuously by time-lapse frame grabbing, and the migration tracks were plotted in scatter diagrams (C–F) as described in Materials and methods. The starting point for each cell is the intersection between the X and Y axes (0,0), and data points indicate the final positions of individual cells at the end of the 2-h recording period. Chemotaxis was tested by placing 200 ng/ml VEGF (C) or FGF-2 (E) in the outer well. The direction of the gradient is vertically upwards. Note that neural progenitors undergo chemotaxis and display a clear directionality of migration in the presence of a VEGF (C) but not an FGF-2 gradient (E). For chemokinesis (D and F), equal amounts of VEGF (20 ng/ml) or FGF-2 (20 ng/ml) were added in both the inner and outer wells of the chamber.

up the chamber (Zicha et al., 1991; Webb et al., 1996). The gradient remains stable for  $\sim 30$  h thereafter (Zicha et al., 1991; Webb et al., 1996). To study the chemotaxis of neural progenitors, the outer well of the Dunn chamber was filled with medium containing different concentrations of VEGF and the concentric inner well with medium only. Coverslips with progenitor cells were inverted onto the chamber, and cell locomotion (Fig. 2) was recorded over one part of the bridge region. We observed that progenitors at day 6 maintained in the presence of FGF-2 and exposed to concentration gradients established with 200 ng/ml VEGF displayed strong positive chemotaxis (Fig. 2 C). The scatter diagram of cell displacements in Fig. 2 C demonstrates a strong directional bias of migration toward the source of VEGF. In contrast, when VEGF was added to both inner and outer wells (chemokinesis conditions), cells remained motile but the population as a whole showed no clear preference for displacement (Fig. 2 D). In these experiments, 20 ng/ml FGF-2 was systematically included in the medium during the recording of neural progenitor chemotaxis or chemokinesis. However, FGF-2 had no chemotactic effect on these cells, irrespective of whether or not VEGF was present (Fig. 2, E and F). No difference was detected in the migratory behavior between cells exposed to an FGF-2 gradient (Fig. 2 E) and cells exposed to a uniform concentration of FGF-2 (Fig. 2 F).

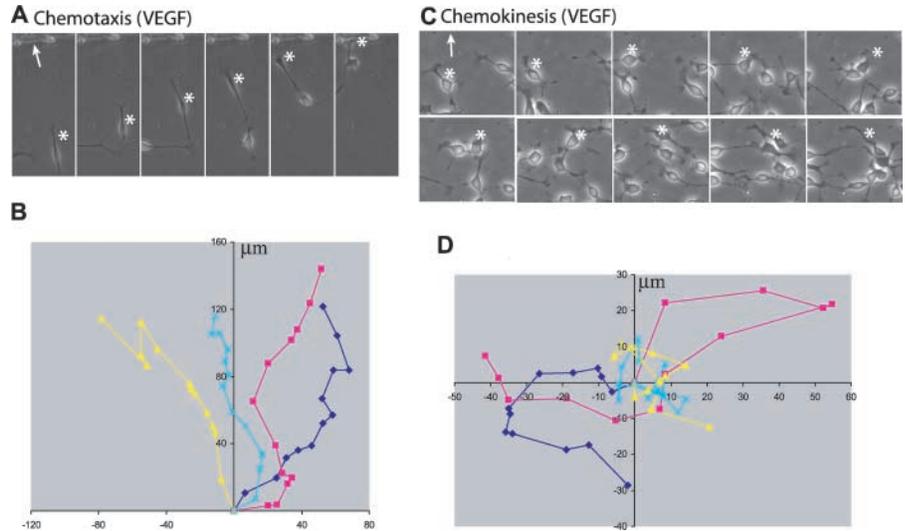
These observations were confirmed by the examination of individual cell tracks. As shown in Fig. 3, progenitors exposed to a VEGF gradient migrated efficiently toward the source of VEGF (Fig. 3, A and B), whereas those under conditions of chemokinesis (Fig. 3, C and D) or exposed to an FGF-2 gradient (not depicted) made random turns during migration.

To measure the efficiency of directed cell migration, we calculated each cell's forward migration index (FMI), i.e., the ratio of the most direct distance the cell progressed toward the gradient source (the outer well of the Dunn chamber) over its total path length. These quantitative analyses revealed that both the migration speed (Fig. 4 A) and the FMI (Fig. 4 B) of cells exposed to VEGF in the presence of FGF-2 were significantly greater than those of cells exposed to an FGF-2 gradient or to a uniform concentration of VEGF or FGF-2 (chemokinesis). The attractive effect of VEGF was similar on laminin-, poly-L-lysine-, or matrigel-coated coverslips. These data indicate that VEGF is attractant for FGF-2-stimulated neural progenitors, and that this effect is matrix independent.

### VEGFR expression in progenitors

Signaling receptors for VEGF include VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), both of which belong to the receptor tyrosine kinase superfamily (Matsumoto and Claesson-Welsh, 2001). To determine whether these receptors are expressed by FGF-2-stimulated progenitors, total cellular RNA was analyzed by RNase protection assay. This revealed that the cells express VEGFR1 and VEGFR2 when cultured in the presence of FGF-2. mRNA for VEGFR3 was not detected in these cultures (Fig. 5 A). Previous studies have indicated that in endothelial cells, FGF-2 regulates expression of VEGFRs (Pepper and Mandriota, 1998). To explore whether FGF-2 affects the expression of these receptors in FGF-2-stimulated progenitors, mRNAs for VEGFR1 and VEGFR2 were analyzed after 12-h starvation of FGF-2. We found that with-

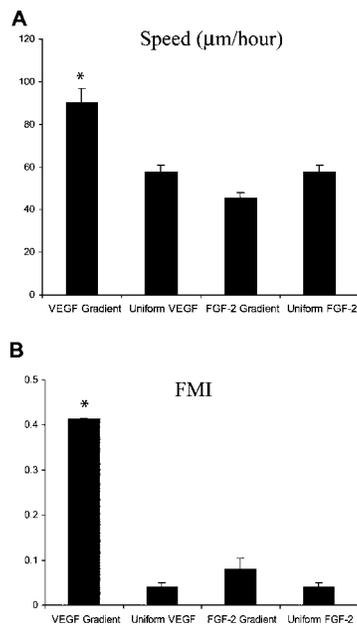
**Figure 3. Migration tracks of neural progenitors.** (A) Phase-contrast photos showing a representative cell (\*) migrating up a VEGF gradient. Arrow indicates the source of VEGF. (B) Migration tracks of four representative cells in the presence of a VEGF concentration gradient. The starting point for each cell is the intersection between the X and Y axes (0,0), and the source of VEGF is at the top. (C) Phase-contrast photos showing a neural progenitor that randomly migrates in a uniform concentration of VEGF. Arrow indicates the outer well of the Dunn chamber. (D) Migration tracks of four representative cells that migrate randomly under conditions of uniform VEGF distribution. The starting point for each cell is the intersection between the X and Y axes (0,0).



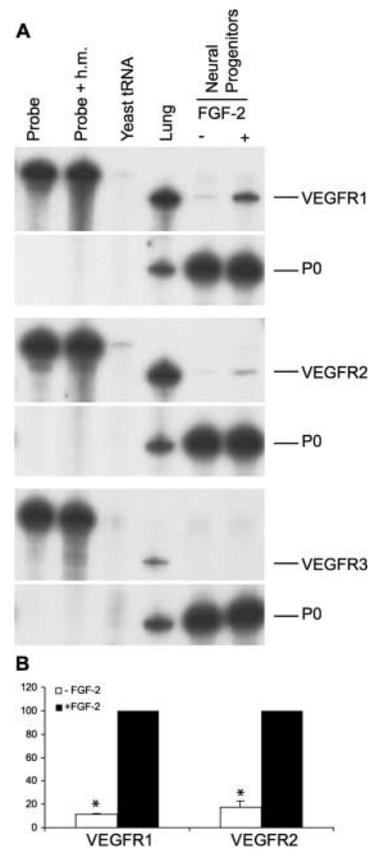
drawal of FGF-2 led to a marked, fivefold decrease in the level of VEGFR1 and VEGFR2 transcripts (Fig. 5, A and B). These results demonstrate that FGF-2–stimulated progenitors express mRNA for both VEGFR1 and VEGFR2 and that FGF-2 is required for this expression.

**VEGF-induced chemotaxis is mediated through VEGFR2**

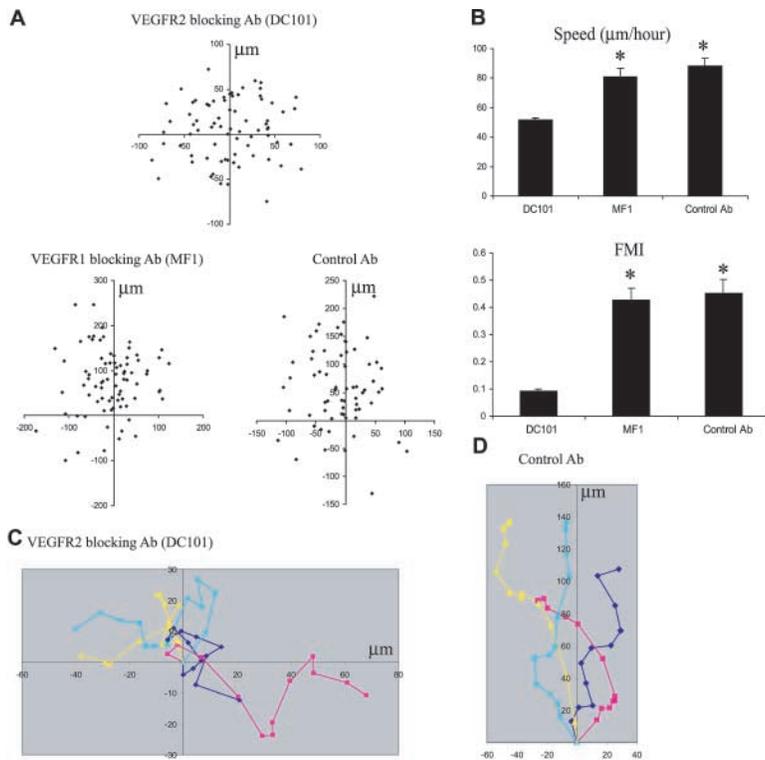
To identify the receptors involved in VEGF-induced chemotaxis, we analyzed the migratory behavior of cells toward



**Figure 4. The migration speed (µm/hr) and FMI values under different conditions.** (A) Cell migration speed was calculated for each time-lapse interval and the mean speed was derived for a period of 2 h. Data are shown as mean ± SEM from at least three independent experiments. FMI values (B), as described in Materials and methods, can be either positive or negative, depending on the direction in which the cells migrate. \*, P < 0.01 by two-tailed unpaired t test, significantly different from chemokinesis or an FGF-2 gradient.



**Figure 5. VEGFR expression in neural progenitors.** (A) Total cellular RNA was isolated and VEGFR mRNA expression was assessed by RNase protection analysis. Purified <sup>32</sup>P-labeled rat cRNA probes (Probe) were hybridized to hybridization mix (Probe + h.m.), yeast tRNA, or total RNA from cells grown in FGF-2 or starved of FGF-2 for 12 h. Rat acidic ribosomal phosphoprotein (P0) was used as an internal control. Rat lung was used as a positive control. (B) Quantitative analysis of VEGFR1 and VEGFR2 expression in cells cultured in the presence of FGF-2 or starved of FGF-2 for 12 h. Data are shown as the mean ± SEM from three independent experiments. \*, P < 0.01 by two-tailed unpaired t test, significantly different from cells in FGF-2 (n = 3).



**Figure 6. VEGF stimulates chemotaxis of neural progenitors through VEGFR2.** (A) Scatter plots showing the migration patterns of neural progenitors under control conditions or in the presence of VEGFR blockers. Cells treated with the VEGFR2-blocking Ab (DC101) lost the chemotactic response to VEGF. In contrast, the VEGFR1-blocking Ab (MF1) did not affect progenitor migration. (B) Speed and FMI under different migration conditions. Data are shown as the mean  $\pm$  SEM from three independent experiments. \*,  $P < 0.01$  by two-tailed unpaired  $t$  test, significantly different from DC101-treated cells. (C and D) Migration tracks of representative cells (four for each condition) exposed to a VEGF concentration gradient, in the presence of either VEGFR2-blocking Ab (C) or control (polysialic acid blocking) Ab (D). The starting point for each cell is the intersection between the X and Y axes (0,0), and the source of VEGF is at the top in the gradient condition.

VEGF in the presence of functional blockers (neutralizing Abs) of VEGFR1 or VEGFR2. As shown in Fig. 6, the chemotactic response of cells to VEGF was completely abrogated by the VEGFR2-blocking Ab DC101 (Fig. 6, A and C). In contrast, the VEGFR1-blocking Ab MF1 did not affect chemotaxis (Fig. 6 A). These observations were confirmed by measurements of speed and FMI (Fig. 6 B). In the absence of a VEGF gradient, addition of anti-VEGFR2 had no significant effect on progenitor migration. These experiments demonstrate that VEGF stimulates chemotaxis of progenitor cells through VEGFR2. This conclusion received further support from experiments in which concentration gradients of VEGF- $C_{\Delta\text{N}\Delta\text{C}}$  were used to induce chemotaxis. We observed that VEGF- $C_{\Delta\text{N}\Delta\text{C}}$  could efficiently induce chemotaxis of progenitor cells and that this effect was prevented by the VEGFR2-blocking Ab (unpublished data). Furthermore, because VEGF- $C_{\Delta\text{N}\Delta\text{C}}$  exerts its function through VEGFR2 and VEGFR3, and because VEGFR3 is not expressed by FGF-2-stimulated neural progenitors, these results strengthen the conclusion that signaling through VEGFR2 mediates chemoattraction of progenitor cells by VEGF.

### FGF-2 is required for VEGF to stimulate chemotaxis of progenitor cells

Inasmuch as FGF-2 is required for the expression of VEGFR2, the chemotactic response of progenitor cells to VEGF should also be dependent on the presence of FGF-2. To test this hypothesis, we examined the migratory response of progenitors to VEGF in the absence of FGF-2. Cells at 5 d of culture were starved of FGF-2 for 12 h, and then exposed to a VEGF gradient (Fig. 7 A). As shown in Fig. 7 B, starved cells failed to undergo chemotaxis in response to

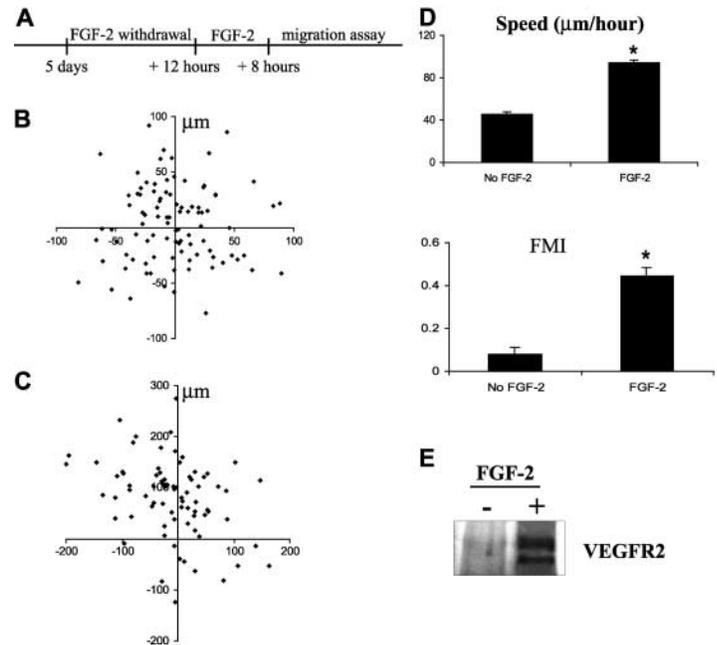
VEGF. Cells migrated randomly in a manner similar to when they were exposed to a uniform concentration of VEGF. In agreement with these results and confirming the data of the RNase protection assay (Fig. 5), Western blot analysis revealed little to no expression of VEGFR2 protein in the absence of FGF-2, whereas substantial expression was detected in the presence of FGF-2 (Fig. 7 E).

Next, we assessed whether the effect of FGF-2 withdrawal is reversible and whether cells could chemotactically respond to VEGF upon readdition of FGF-2 to the cultures. To this end, FGF-2 was included in the medium after a 12-h starvation period, and the cells were further cultured for 8 h. Diagrams of displacements of motile cells (Fig. 7 C) and a quantitative analysis of FMI and speed (Fig. 7 D) demonstrated that the loss of chemotaxis was rescued after an 8-h reincubation with FGF-2. Together, these data demonstrate that FGF-2 is necessary for the expression of VEGFR2 and for an adequate migratory response of progenitors to concentration gradients of VEGF.

### VEGF affects the migration of progenitor cells from the SVZ

To determine whether the effect of VEGF on FGF-2-stimulated progenitor migration is functionally relevant, we investigated the effect of VEGF on cell migration from SVZ explants. SVZ explants were cocultured in a three-dimensional collagen matrix with aggregates of control or VEGF-secreting murine  $C_2C_{12}$  myoblasts in the presence or absence of FGF-2. When explants were cocultured with aggregates of mock-transfected cells in the presence of 20 ng/ml FGF-2 (Fig. 8, B and D), migrating cells were symmetrically distributed around the explants (10/10 explants). When SVZ explants were cocultured in the presence of FGF-2, with

**Figure 7. FGF-2 is required for neural progenitors to chemotactically respond to a VEGF gradient.** (A) Experimental protocol: in the first group of cultures, FGF-2 was withdrawn at day 5 for 12 h, and then cells were exposed to a VEGF gradient (B). The second group was further cultured in the presence of FGF-2 after the 12-h starvation period for 8 h, and then tested in a VEGF gradient (C). The final positions of the cells after 2 h of migration were indicated, with the starting point for each cell at (0,0), and the source of 200 ng/ml VEGF at the top. (D) Speed and FMI were calculated as described in Materials and methods. Data are shown as mean  $\pm$  SEM from four independent experiments. After 12 h of FGF-2 starvation, cells lose their chemotactic response to the VEGF gradient. The starved neural progenitors resume their chemotactic response to VEGF upon readdition of FGF-2 to the cultures for 8 h (C). (E) VEGFR2 expression in neural progenitors cultured in FGF-2 or starved of FGF-2 for 12 h. Western blot analysis was performed on immunoprecipitates with an anti-VEGFR2 Ab. \*,  $P < 0.01$  by two-tailed unpaired *t* test.



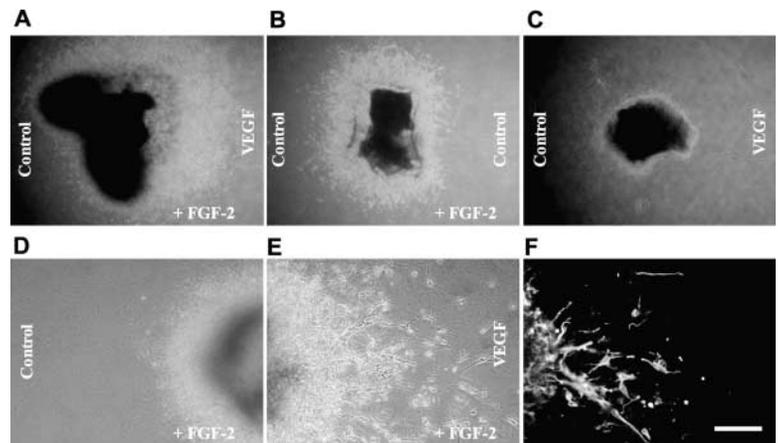
VEGF-expressing cells placed on one side and with mock-transfected cells on the other, cell migration was highly asymmetric (Fig. 8, A and E; 10/20 explants with cells migrating predominantly toward VEGF-secreting  $C_2C_{12}$  cells, and 10/20 explants with a symmetric migratory pattern). In contrast, when explants were cocultured with control or VEGF-expressing cells in the absence of FGF-2, no significant cell migration from SVZ explants was observed (Fig. 8 C; 10/10 explants). Similar results were obtained after application of VEGF in the absence of FGF-2 (4/4 explants). The application of VEGF and FGF-2 together or FGF-2 alone resulted in symmetric migration (12/12). To determine whether cells migrating in response to VEGF are immature progenitors, we performed immunocytochemical staining with an antinestin Ab. Migrating cells stained positively for nestin (Fig. 8 F) and were negative for PSA-NCAM (a marker for immature neurons; not depicted), confirming that they were indeed immature progenitor cells. Together,

these results indicate that immature progenitor cells migrate in response to VEGF gradients and that FGF-2 is required for this effect.

## Discussion

Previous studies have suggested that neurogenesis and angiogenesis are mechanistically linked in the nervous system (Palmer et al., 2000; Louissaint et al., 2002). Here, we report that VEGF, a major angiogenic factor that has been identified as a guidance factor for endothelial progenitors and hematopoietic cells (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001) is a chemoattractant for immature neural progenitors. We show that although FGF-2-stimulated progenitor cells express both VEGFR1 and VEGFR2, signaling through VEGFR2 appears to specifically mediate the effect of VEGF. Moreover, VEGFR2 expression, and consequently the capacity of cells to respond

**Figure 8. Effect of VEGF on neural progenitors migrating from SVZ explants.** SVZ explants were cocultured with VEGF-secreting  $C_2C_{12}$  cells and/or mock-transfected  $C_2C_{12}$  cells in collagen gel matrices in the presence (A, B, D, E, and F) or absence (C) of FGF-2. (A) In the presence of FGF-2, neural progenitors migrate out of the SVZ explant in an asymmetric manner, with many more cells on the side of the VEGF-secreting  $C_2C_{12}$  cells than on the side of control  $C_2C_{12}$  cells. (B) Neural progenitors migrate out of the SVZ explant symmetrically when cultured with control  $C_2C_{12}$  cells on both sides. (C) In the absence of FGF-2, few to no cells migrate out of the SVZ explant. (D) High-power photograph showing the SVZ explant on the side of control  $C_2C_{12}$  cells. (E) High-power photograph showing many neural progenitors migrating out of the SVZ explant toward VEGF-secreting  $C_2C_{12}$  cells. (F) Cells migrating out of the SVZ explant are positive for nestin, a marker for undifferentiated neural progenitors. Bars: (A–C) 700  $\mu$ m; (D and E) 100  $\mu$ m; (F) 50  $\mu$ m.



to gradients of VEGF, critically depends on the presence of FGF-2. Finally, we demonstrate that migration of progenitors from SVZ explants in a three-dimensional collagen matrix is directed by VEGF. This effect also requires the presence of FGF-2. These results reveal an intriguing signaling mechanism that guides migrating neural progenitors in the central nervous system. They also support the hypothesis that a common guiding mechanism exists for neurogenic progenitors and endothelial cells.

Our immunocytochemical characterization revealed that progenitor cells maintained in the presence of FGF-2 do not display antigenic markers for neuron- or glia-restricted precursor cells, including PSA-NCAM, doublecortin, NeuN, NG2, or A2B5. Most remarkable is the absence of NCAM immunoreactivity in these cells. We found very few cells displaying differentiation markers such as tubulin, GFAP, O4, or Galc. Their presence is probably due to contamination of the initial cell population after isolation and purification of progenitors. In contrast, the vast majority (98%) of cells were stained with an antinestin Ab. Importantly, nestin-positive cells were negative for endothelial markers such as von Willebrand factor and RECA-1. Together, these results indicate that our culture model consists of progenitor or stem cells that do not yet possess cell lineage-specific markers for neurons or glial cells. It should be emphasized here that progenitor cells in the presence of FGF-2 do not generate neurospheres, but rather spread out evenly and form a monolayer. This may expose cells to FGF-2 more evenly and favor the formation of a homogenous population of undifferentiated progenitors as has previously been suggested (Wu et al., 2002). Because >96% of these progenitor cells displayed immunoreactivity for neuronal and glial markers after differentiation, we conclude that our cultures are composed essentially of multipotential neural progenitors or stem cells.

The Dunn chemotaxis chamber allowed us to show directly that concentration gradients of VEGF induce directional sensing by neural progenitor cells. Under basal conditions in the presence of uniform concentrations of FGF-2, progenitor cells were polarized and motile, and moved randomly across a two-dimensional substrate with a mean speed of  $\sim 58 \mu\text{m/hr}$  (Fig. 4 A). When cells were exposed to linear gradients of VEGF, their leading processes became stable over a substantial portion of the time course, and the cells continued to migrate up the VEGF gradient. In contrast, when cells were exposed to uniform concentrations of VEGF, no biased displacement was observed, and the mean displacement speed was not significantly different from that measured in the presence of FGF-2 alone. Thus, although VEGF specifically affects the direction of progenitor migration, it does not appear to stimulate migration rate per se. Similar results were obtained with VEGF- $C_{\Delta\text{NAC}}$ . VEGF- $C_{\Delta\text{NAC}}$  is the proteolytically processed form of VEGF-C (21 kD; which binds to and activates VEGFR2 and 3 (Joukov et al., 1997). We also demonstrated that the effect of VEGF and VEGF- $C_{\Delta\text{NAC}}$  was specific because concentration gradients of FGF-2 did not induce directional migration of neural progenitor cells. In line with these observations, our experiments with SVZ explants show that FGF-2-stimulated progenitors are invasive in a three-dimensional collagen matrix in which they migrate toward the source of VEGF.

We observed that FGF-2-stimulated progenitors express the two tyrosine kinase receptors for VEGF; namely, VEGFR1 and VEGFR2, but not VEGFR3. These findings confirm previous observations demonstrating that members of the VEGFR family are expressed not only by blood and endothelial cells but also by immature neurons from different regions of the nervous system (Carmeliet and Storkebaum, 2002; Jin et al., 2002; Louissaint et al., 2002; Ogunshola et al., 2002; Svensson et al., 2002). Our results extend these observations by showing that, similar to endothelial cells (Pepper and Mandriota, 1998), FGF-2 is a critical regulatory factor for VEGFR expression in progenitor cells. We demonstrate that the receptor is expressed in the presence of FGF-2, and that withdrawal of the growth factor leads to a significant down-regulation as measured by RNase protection assay and Western blotting. Consistent with these findings, we demonstrate that migratory responses to VEGF also require the presence of FGF-2 in the two-dimensional as well as three-dimensional migration models. It is unlikely that down-regulation of VEGFR expression and the lack of chemotactic responses are due to death or suffering of cells in the absence of FGF-2. The arguments for this are as follows: (a) after removal of FGF-2 for 12 h, cells maintained in neurobasal medium supplemented with B27 displayed no difference in morphology compared with control cultures; (b) Hoechst 33258 staining of cell nuclei did not reveal any difference between cultures kept in the presence or absence of FGF-2; (c) our video analysis revealed that cells in the absence of FGF-2 exhibited random migration with the same migration speed as control cells in the presence of FGF-2; and (d) FGF-2 starvation did not change the expression of acidic ribosomal phosphoprotein. In vitro, FGF-2 is known to stimulate mitotic activity in progenitor cells and to maintain these cells in an undifferentiated state (Palmer et al., 1997; Tropepe et al., 1999). Because withdrawal of FGF-2 from cultures is a standard procedure used to induce the differentiation of FGF-2-stimulated progenitors (Palmer et al., 1997; Tropepe et al., 1999), these results raise the intriguing possibility that the more differentiated progenitors lose VEGFR expression as well as the capacity to respond to VEGF. However, the effect of FGF-2 withdrawal was reversible upon the reapplication of FGF-2 to the medium after 8 h. It will be of particular interest to determine whether VEGFR expression can be induced by FGF-2 in differentiated neurons.

Although FGF-2-stimulated progenitors express both VEGFR1 and VEGFR2, only the latter appears to be required for directional migration in response to VEGF. We observed that by blocking receptor function with a specific mAb, we could completely block the migratory response to VEGF. In contrast, function-blocking Abs against VEGFR1 had no effect. These results demonstrate that VEGFR2 signaling, previously shown to regulate survival and proliferation of neuronal progenitors (Carmeliet and Storkebaum, 2002; Jin et al., 2002; Ogunshola et al., 2002; Svensson et al., 2002), is also involved in the migration of these cells. The respective roles of VEGFR1 and VEGFR2 in regulating cell migration are not well understood. Although similar to FGF-2-stimulated neural progenitors, the VEGFR2 pathway appears to be involved in the migration of endothelial

cells (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001); in other cells such as a neuroectodermal cell line from a human cerebellar tumor, and monocytes, VEGFR1 signaling dominates (Bagnard et al., 2001; Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Forstreuter et al., 2002).

The *in vivo* relevance of our findings remains to be determined. The observations presented here raise the intriguing possibility that VEGF signaling could be involved specifically in directing migration of multipotent neural progenitors rather than in guiding neuron- or glia-restricted precursors. One possibility is that VEGF signaling plays a role in the initial phase of neurogenesis, in particular in the formation of neurogenic matrix or neurogenic niches in association with angiogenic sites. Indeed, high levels of VEGF transcripts were detected in the neurogenic matrix of the ventricular zone and SVZ in the embryonic, as well as in the postnatal, rat brain (Breier et al., 1992). Moreover, receptors for VEGF are expressed in neuroproliferative zones *in vivo* (Breier et al., 1995; Jin et al., 2002). Recently, Palmer et al. (2000) showed that clusters of proliferating cells in the dentate gyrus of the adult mammalian brain contain dividing endothelial as well as neural progenitors. They also demonstrated that proliferative clusters are associated with growing capillaries. Some of the cells in the clusters were immunoreactive for VEGFR2, whereas VEGF immunoreactivity was seen in tissue surrounding the clusters. These observations were corroborated by experiments showing that neurogenesis and angiogenesis occur concurrently in the adult avian brain (Louissaint et al., 2002). In this system, immature neurons generated in the subventricular germinal zone migrate to reach active angiogenic spots in the adjacent tissue. These results are also consistent with studies showing the association of neural progenitors with blood vessels (Capela and Temple, 2002). Based on these results, Palmer et al. (2000) proposed a model in which neural and angiogenic progenitors are recruited to form proliferative niches either by common signaling mechanisms or by cues acting simultaneously. Our results lend support to this hypothesis by demonstrating that VEGF, previously described as a chemoattractant for endothelial progenitors and blood cells (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001), is able to direct neural progenitor migration. Thus, VEGF could be a common guidance cue for recruiting neural progenitors and endothelial cells to correct sites for differentiation. Such a role is consistent with the observation that VEGF promotes the survival of both immature neurons and endothelial cells (Sondell et al., 1999; Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001), stimulates the proliferation of both cell types (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Ogunshola et al., 2002), and protects them from injury *in vitro* (Jin et al., 2000; Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Matsuzaki et al., 2001) as well as *in vivo* (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Ogunshola et al., 2002). The coordinated regulation of neurogenesis and angiogenesis by VEGF highlights the importance of this signaling pathway in the morphogenesis of nervous tissue.

Our results could also be important in the context of repair after brain injury. Increasing evidence indicates that VEGF and its receptors are activated after various lesions in the brain, including stroke (Carmeliet and Storkebaum, 2002). For example, focal or global brain ischemia induces an acute up-regulation of VEGF expression that is consistent with the notion that the promoter region of VEGF contains hypoxia-responsive elements (Carmeliet and Storkebaum, 2002; Zhang and Chopp, 2002; Sun et al., 2003). In addition, several cytokines and growth factors known to be associated with ischemia up-regulate VEGF expression in many cell types. VEGF expression under these conditions appears to be important for initiating neovascularization and regenerating capillaries in the damaged zone. Injuries to the brain, including ischemia and seizure, also stimulate the mitotic activity of neural progenitors in neurogenic centers and enhance neurogenesis (Parent and Lowenstein, 2002; Parent et al., 2002; Kokaia and Lindvall, 2003; Iwai et al., 2003). Under these circumstances, VEGF could be an attractant guidance factor for neural progenitors, just as it is for endothelial progenitors and blood cells. Thus, VEGF and FGF-2 appear to be potential therapeutic molecular tools whose efficacy in directing the migration of endogenous or transplanted progenitors to injured regions of the brain must be assessed.

## Materials and methods

### Isolation and cultures of neural progenitors

All animal experiments were conducted in accordance with Swiss laws, previously approved by the Geneva Cantonal Veterinary Authority. The SVZ was dissected from coronal slices of newborn rat brains, dissociated mechanically, and trypsinized as described previously (Lim et al., 2000). SVZ progenitors were purified using Percoll gradient centrifugation as described previously (Lim et al., 2000) and seeded onto 0.24 mg/cm<sup>2</sup> matrigel- or laminin-coated coverslips. Isolated cells were allowed to grow in neurobasal medium supplemented with 20 ng/ml FGF-2 (human recombinant; provided by P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy), 1 × B27, 2 mM glutamate, 1 mM sodium pyruvate, 2 mM *N*-acetyl-cysteine, and 1% penicillin–streptomycin. Cultures were fed every 3 d with fresh medium containing 20 ng/ml FGF-2.

### Immunocytochemistry

Immunostaining of cultures was performed as described previously (Wang et al., 1996; Vutskits et al., 2001). The following primary Abs and dilutions were used: mouse mAb against nestin (Biogenesis; 1:300 dilution); mouse mAb against A2B5 (Eisenbarth et al., 1979; hybridoma supernatant; ATCC; 1:5 dilution); Men B (Meningococcus group B) mouse IgM mAb (1:500 dilution) that specifically recognizes  $\alpha$  2–8-linked PSA with chain length superior to 12 residues (Rougon et al., 1986); anti-GalC (Ranscht et al., 1982) mouse IgM mAb (culture supernatant; 1:5 dilution); Tuj mouse mAb directed against  $\beta$ -tubulin isotype III (Sigma-Aldrich; 1:400 dilution); a rabbit polyclonal Ab to GFAP (Dakopatts; 1:200 dilution); a rabbit polyclonal Ab against NG2 (Chemicon International; 1:400 dilution); a goat polyclonal Ab against Doublecortin (Santa Cruz Biotechnology, Inc.; 1:300 dilution); a mouse mAb against Neu N (Chemicon International; 1:100 dilution). The rabbit antiserum directed against the NCAM protein core was a site-directed Ab recognizing the seven NH<sub>2</sub>-terminal residues of NCAM (1:1,000 dilution; Rougon and Marshak, 1986). O4 mAb (hybridoma supernatant, 1:5 dilution; Eisenbarth et al., 1979) was used to identify undifferentiated oligodendrocytes. Hoechst 33258 was used to counterstain cell nuclei in some cases. Fluorescence was examined with a fluorescence microscope (model Axiophot; Carl Zeiss Microimaging, Inc.). Controls treated with nonspecific mouse IgM, IgG preimmune sera, or secondary Abs alone showed no staining. In double immunolabeling experiments, the use of only one primary Ab followed by the addition of both anti-mouse FITC and anti-rabbit TRITC-conjugated secondary Abs resulted only in single labeling. Proliferating cells were identified with an mAb against BrdU (Boehringer; 1:50 dilution) after 20-h incorporation.

### Migration assays using Dunn chamber

Chemotaxis of neural progenitors was directly viewed and recorded in stable concentration gradients of VEGF (human recombinant, 165-amino acid homodimeric form; PeproTech) using the Dunn chemotaxis chamber (Weber Scientific international Ltd.; Zicha et al., 1991; Allen et al., 1998). (Recombinant human VEGF-C<sub>ΔNAC</sub> [provided by M. Skobe, Cancer Center, Mount Sinai Medical Center, New York, New York] was used in some experiments.) This device is made from a Helber bacteria counting chamber by grinding a circular well in the central platform to leave a 1-mm-wide annular bridge between the inner and the outer wells. Chemoattractants added to the outer well of the device will diffuse across the bridge to the inner blind well of the chamber and form a gradient. This apparatus allows one to determine the direction of migration in relation to the direction of the gradient. Coverslips with cells were inverted onto the chamber and cell migration was recorded through the annular bridge between the concentric inner and outer wells, and a period of 2 h was chosen to assess cell migration. In this work, we applied a systematic sampling, and all cells within the migration region of the chamber were recorded and analyzed. Data were recorded every 10 min using a 10× objective (Carl Zeiss Micro-Imaging, Inc.) via a CCD video camera (Hamamatsu) using Openlab software. In these chemotaxis experiments, the outer well of the Dunn chamber was filled with medium containing 200 ng/ml VEGF and 20 ng/ml FGF-2 and the concentric inner well with only medium and FGF-2. For chemokinesis experiments, 20 ng/ml VEGF or 20 ng/ml FGF-2 was added to both the outer and inner wells of the Dunn chamber. MF1, a VEGFR1 blocking Ab, DC101, and a VEGFR2 blocking Ab (both added at 20 μg/ml; provided by D. Hicklin, ImClone Systems Inc., New York, NY) were used to block the function of the corresponding VEGFR. A polysialic acid blocking Ab was used as a control.

Directionality of cell movement was analyzed using scatter diagrams of cell displacement. The diagrams were oriented so that the position of the outer well of the chamber was vertical (y direction). Each point represents the final positions of the cells at the end of the recording period where the starting point of migration is fixed at the intersection of the two axes.

To determine the efficiency of forward migration during the 2-h recording period, the FMI was calculated as the ratio of forward progress (net distance the cell progressed in the direction of VEGF source) to the total path length (total distance the cell traveled through the field; Foxman et al., 1999). FMI values were negative when cells moved away from the source of VEGF. The cell speed was calculated for each lapse interval recorded during the 2-h period.

### RNA purification and RNase protection assay

Neural progenitors at 6 d of culture in FGF-2 or after 12-h starvation of FGF-2 were used for RNA preparation. Total cellular RNA was purified using TRIzol reagent (Invitrogen). RNase protection assays were performed using cRNA probes for rat VEGFR1 and VEGFR2 as described by Pepper et al. (2000).

### Immunoprecipitation and Western blotting

Neural progenitors from the normal cultures in FGF-2 or from cultures starved of FGF-2 for 12 h were lysed, and VEGFR2 protein was immunoprecipitated from cell lysates with a polyclonal Ab (sc-504; Santa Cruz Biotechnology, Inc.) recognizing amino acids 1158–1345 in the mouse VEGFR2 COOH terminus. Western blot was performed with a polyclonal anti-VEGFR2 Ab (sc-315; Santa Cruz Biotechnology, Inc.) recognizing the mouse COOH-terminal amino acids 1348–1367.

### SVZ explants cultures

The frontal lobes of the brains of 1-d-old Sprague-Dawley rat pups (Sizv) were isolated and cut into 300-μm-thick coronal sections with a McIlwain tissue chopper. From these slices, the anterior part of the SVZ was microdissected. SVZ explants were embedded in a collagen matrix and cultured for 7 d in chemically defined serum-free medium (50% DME [GIBCO BRL], 50% F12, Hepes, Tris-HCl, and complemented with 20 μg/ml transferrin human, 100 μM putrescine, 30 nM sodium selenite, 1 nM triiodothyronine, 0.5 μg/ml docosahexaenoic acid, 1 μg/ml arachidonic acid, and insulin 60 U/liter) under 5% CO<sub>2</sub>. The medium was changed every third day. For coculture experiments, SVZ explants were cultured in the presence of murine C<sub>2</sub>C<sub>12</sub> myoblasts that had been engineered to secrete VEGF (Rinsch et al., 2001). C<sub>2</sub>C<sub>12</sub> cells were suspended in a drop of collagen matrix that was placed at a distance of ~1,000 μm from the SVZ explant. As a control, mock-transfected cells of the same origin were placed into the collagen matrix in a similar manner and at the same distance, but on the opposite side of the explant.

Cell migration was assessed at the end of 7 d in culture. We established three categories: (1) no migration, occurring when no or only a few cells emigrated from the explants; (2) symmetrical migration, occurring when numerous cells had left the explants, the distance of the migrating front of the cells exceeded 50 μm in random directions; and (3) asymmetrical or directional migration, occurring when the distance of the migrating front was at least twice of that on the other side and exceeded 50 μm.

We wish to thank S. Chliate, C. Di Sanza, and M. Quayzin for technical assistance.

This work was supported by Swiss National Science Foundation grant 31-64030.00 to J.Z. Kiss and grant 31-64037.00 to M.S. Pepper.

Submitted: 7 August 2003

Accepted: 28 October 2003

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