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HEPATOCYTE GROWTH FACTOR ACTS AS A MITOGEN AND CHEMOATTRACTANT FOR POSTNATAL SUBVENTRICULAR ZONE-OLFACTORY BULB NEUROGENESIS

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Abstract

Neural progenitor cells persist throughout life in the forebrain subventricular zone (SVZ). They generate neuroblasts that migrate to the olfactory bulb and differentiate into interneurons, but mechanisms underlying these processes are poorly understood. Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor that influences cell motility, proliferation and morphogenesis in neural and non-neural tissues. HGF and its receptor, c-Met, are present in the rodent SVZ-olfactory bulb pathway. Using *in vitro* neurogenesis assays and *in vivo* studies of partially HGF-deficient mice, we find that HGF promotes SVZ cell proliferation and progenitor cell maintenance, while slowing differentiation and possibly altering cell fate choices. HGF also acts as a chemoattractant for SVZ neuroblasts in co-culture assays. Decreased HGF signaling induces ectopic SVZ neuroblast migration and alters the timing of migration to the olfactory bulb. These results suggest that HGF influences multiple steps in postnatal forebrain neurogenesis. HGF is a mitogen for SVZ neural progenitors, and regulates their differentiation and olfactory bulb migration.

INTRODUCTION

Most neurons that inhabit the adult mammalian brain are generated during embryonic stages. In the hippocampal dentate gyrus and forebrain subventricular zone (SVZ), however, neurogenesis persists into adulthood (Altman and Das, 1965; Altman, 1969; Lois and Alvarez-Buylla, 1994; Kuhn et al., 1996). The subgranular layer of the dentate gyrus, even in adult humans (Eriksson et al., 1998), continually generates new neurons that migrate a short distance into the granule cell layer and differentiate into dentate granule cells (Kuhn et al., 1996). The striatal SVZ of the lateral ventricles generates neuroblasts throughout life that migrate in the rostral migratory stream (RMS) to the olfactory bulb and differentiate into granular and periglomerular neurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994).

The RMS is a highly defined pathway in which neuroblasts migrate tangentially in chains surrounded by astrocytic “tubes” (Peretto et al., 1997). RMS neuroblasts express immature neuronal markers such as doublecortin (Dcx), polysialylated neural cell adhesion molecule (PSA-NCAM), calretinin and β -III-tubulin (Peretto et al., 1997, 1999; Gleeson et al., 1999; Kato et al., 1999; Wang et al., 2005), some of which influence their long-distance migration (Chazal et al., 2000; Koizumi et al., 2006). Various growth factors, neurotransmitters,

transcription factors, environmental manipulations and injuries also influence postnatal SVZ-olfactory bulb neurogenesis by modulating cell proliferation, migration, differentiation or survival (see Cayre et al., 2009 and Whitman and Greer, 2009 for reviews).

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor that induces cell motility, proliferation and morphogenesis in neural and other tissues through its receptor tyrosine kinase, c-Met (Maina and Klein, 1999). c-Met activation depends on the enzymatic cleavage of pro-HGF to functional HGF by tissue-type plasminogen activator (tPA) or urokinase plasminogen activator (uPA), the latter of which requires binding to the uPA receptor (uPAR) for functional activity. HGF promotes neural induction during early development (Stern et al., 1990). In later embryonic stages, it serves as a mitogen for cortical interneuron precursors that migrate from ventral to dorsal telencephalon (Powell et al., 2001). HGF also is a mitogen for Schwann cells (Krasnoselsky et al., 1994), stimulates the proliferation and migration of oligodendrocyte progenitors (Yan and Rivkees, 2002), and enhances survival and differentiation of sympathetic, cortical, hippocampal and midbrain dopaminergic neurons (Hamanoue et al., 1996; Maina et al., 1998; Korhonen et al., 2000). In addition, HGF is an axonal chemoattractant and survival factor for spinal motor neurons (Ebens et al., 1996). Interestingly, HGF promotes cell proliferation and neuronal differentiation of embryonic neural stem cells (Kokuzawa et al., 2003), but its effect on postnatal neural progenitors is poorly understood.

In the neonatal and adult rodent brain, olfactory interneuron precursors proliferate in the forebrain SVZ and migrate tangentially to the olfactory bulb. HGF, c-Met, tPA and uPA are present in the postnatal mouse SVZ, RMS or olfactory bulb (Dent et al., 1993; Jung et al., 1994; Thewke and Seeds, 1996; Garzotto et al., 2008; Nicoleau et al., 2009), and a recent study using *in vitro* experiments suggests that HGF acts as a chemoattractant for RMS neuroblasts (Garzotto et al., 2008). This work and a subsequent report, however, show conflicting results regarding potential mitogenic effects of HGF-c-Met signaling on SVZ neural progenitors (Garzotto et al., 2008; Nicoleau et al., 2009). Moreover, potential effects of HGF on *in vivo* neurogenesis in the SVZ-olfactory bulb pathway are largely unexplored. Given the spatial expression pattern of HGF, c-Met, uPA and tPA, and known function of HGF in tangential migration of embryonic interneuron progenitors (Powell et al., 2001), we hypothesized that HGF regulates the persistent development of neuroblasts in the SVZ-olfactory bulb pathway. Because HGF or c-Met knockout mice are embryonic lethal but uPAR and tPA null mice survive to adulthood (Bugge et al., 1995), uPAR and tPA null mice serve as models of partial HGF deficiency (Powell et al., 2001; Zhang et al., 2003; Wang et al., 2007). Here, we investigate the role of HGF in postnatal forebrain neurogenesis using neurosphere (NS) assays, explant cultures, and *in vivo* studies of uPAR and tPA knockout mice. We find that HGF influences multiple steps of the neurogenic cascade, including cell proliferation, migration and differentiation, in the postnatal murine SVZ-olfactory bulb pathway.

RESULTS

HGF and c-Met expression in SVZ-derived NS cultures

Neural stem cells from the postnatal rodent striatal SVZ expand *in vitro* when cultured with mitogens to form NS (Reynolds and Weiss, 1992; Gritti et al., 1996). Upon mitogen removal, the cells differentiate into neurons, astrocytes and oligodendrocytes. To investigate a potential role for HGF in postnatal neurogenesis, we used reverse transcriptase polymerase chain reaction (RT-PCR) to examine the expression of HGF and its receptor, c-Met, in primary and secondary NS derived from postnatal day (P) 15 mouse striatal SVZ. Both HGF and c-Met mRNAs were detected in primary and secondary NS, although expression was weaker in secondary NS (Figure 1A). To further determine whether postnatal neural stem

cells are capable of responding to HGF, the protein expression of c-Met in NS was examined by immunocytochemistry. After 3 hour differentiation, c-Met protein was detected in most NS cells and many of them expressed the neural progenitor marker nestin (Figure 1B). c-Met expression decreased over days 1-4 of differentiation, and by day 5 expression remained in some NS with many c-Met-immunoreactive cells expressing the stem cell/astrocyte marker, GFAP (>95% of cells; Figure 1C). NS differentiated for 7 days continued to show scattered c-Met expression; none of the c-Met+ cells expressed the neuronal marker β -III-tubulin (0%; Figure 1D). In Figure 1D, the c-Met positive cells are likely astrocytes as they express GFAP at 5d and do not express a neuronal marker at 7 d. These data suggest that neural progenitors produce and may respond to HGF, and that the expression of HGF and c-Met is developmentally regulated.

HGF stimulates SVZ progenitor cell proliferation

HGF promotes the proliferation of a variety of neural cells during development (Krasnoselsky et al., 1994; Yan and Rivkees, 2002; Kokuzawa et al., 2003). To examine whether HGF influences cell proliferation in postnatal neurogenic regions, P15 SVZ explants were dissociated and cultured to form primary NS for 7 days. NS were passaged and cultured with mitogens $\pm$ HGF (10 ng/ml) to form secondary NS for 5 days. In high-density culture, HGF increased secondary NS size (Figure 1E-G; sphere size > 0.2 mm: $77.34 \pm 1.31\%$ HGF vs. $68.37 \pm 2.43\%$ Vehicle, $p=0.04$) but not neurosphere number. At clonal density, HGF increased NS numbers by nearly 50% (Figure 1H). HGF alone was insufficient to replace the NS-forming effect of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF; data not shown). Because NS produce HGF, the differences between results from high-density and clonal cultures may relate to greater effects of endogenous HGF in high-density, but not clonal, cultures. At clonal density, endogenous levels should be low, and exogenous HGF may then promote the formation of NS. In high-density cultures, with NS exposed to higher “endogenous” levels, potential NS-forming effects may not be further increased by adding HGF, although it is still capable of facilitating NS expansion.

To further study proliferative effects of HGF, primary NS were dissociated, electroporated with enhanced green fluorescent protein (EGFP) control plasmid alone or with a dominant-negative construct for c-Met (dnMet) (Furge et al., 2001), and cultured for 5 days to form secondary NS. To more accurately quantify NS size, we first screened for GFP-positive NS under epifluorescence to include only those that were successfully electroporated, which was approximately 50% of NS (Figure 2 and data not shown), and then measured NS size under bright field. In control cultures, HGF (10 ng/ml) did not increase the size of GFP+ NS (Figure 2A, A'; Table). In dnMet-transfected cultures, GFP-positive NS appeared much smaller in size (Figure 2B, B', Table) and HGF treatment did not rescue this effect (Figure 2C, C'; Table). These findings suggest that dnMet blocks endogenous HGF signaling in the cultures. Furthermore, fewer secondary NS formed after dnMet electroporation (Control-Vehicle: 1241.75 ± 67.58 NS, Control-HGF: 1221 ± 40.01 NS, dnMet-Vehicle: 933.25 ± 44.82 NS, dnMet-HGF: 865.75 ± 26.72 NS; dnMet vs. Control, $p<0.001$). It should be noted that these NS were smaller than in the initial experiment (Fig. 1E-H vs. Fig. 2/Table), likely due to adverse effects of the electroporation procedure. Also, HGF did not increase NS size in this experiment, probably because the higher culture density needed to enhance cell survival after electroporation led to increased exposure of the progenitor cells to endogenous HGF synthesized by other NS cells. Together, these results suggest that HGF signaling is necessary for the normal expansion and self-renewal of NS derived from the postnatal SVZ.

We next examined HGF effects during differentiation of primary NS. NS were dissociated and induced to differentiate by growth factor removal and addition of 1% FBS in the presence of vehicle, HGF (10 ng/ml) or function-blocking anti-HGF antibody (1 μ g/ml)

(Powell et al., 2001) for 2 days. Fixed cultures were immunostained with an antibody against the cell proliferation marker Ki67. Compared to control, HGF treatment significantly increased Ki67-immunoreactive cell numbers (Figure 3A; $17.74 \pm 0.51\%$ HGF vs. $11.8 \pm 1.03\%$ Vehicle, $p=0.01$). Consistent with this result, function-blocking anti-HGF antibody increased the number of cells that exited cell cycle as identified by immunoreactivity for the cyclin-dependent kinase inhibitor p27 (Figure 3A; $76 \pm 0.87\%$ anti-HGF vs. $67.16 \pm 2.06\%$ Vehicle, $p=0.05$). HGF treatment also trended toward a decrease in p27-immunolabeled cell number (Figure 3A; $59.93 \pm 2.57\%$, $p=0.08$ vs. vehicle). HGF therefore may act to expand SVZ progenitors by maintaining them in a proliferative state.

To further test for proliferative effects of HGF on postnatal SVZ cells in culture conditions that more closely model the *in vivo* environment (Wang et al., 2005), we prepared parasagittal brain slices through the SVZ-olfactory bulb pathway from P2 mice and treated them with vehicle, HGF (10 nM) or anti-HGF function-blocking antibody (1 $\mu\text{g/ml}$) for 2 days. BrdU was added to the culture media for 2 hours prior to fixation. Explants were resectioned and immunostained for BrdU. HGF treatment increased BrdU-labeled cell numbers in the SVZ by 40%; function-blocking antibody treatment, in contrast, decreased BrdU labeling (Figure 3E-H; BrdU+ cells as a % of control: $140 \pm 9.57\%$ for HGF, $88.51 \pm 1.6\%$ for anti-HGF; HGF vs. Vehicle, $p=0.01$; anti-HGF vs. Vehicle, $p=0.007$). These findings further support a mitogenic influence of HGF on postnatal SVZ cells.

HGF modulates neural differentiation of SVZ-derived neurospheres

We next used the NS assay to examine whether HGF signaling influences the differentiation of postnatal SVZ-derived NS. Secondary NS were exposed to HGF or vehicle for 5 days during expansion and then differentiated without HGF exposure. Compared to control cultures, HGF pre-treatment delayed or decreased neuronal differentiation by 60% (Figure 3B, right side; $6.64 \pm 1.39\%$ for HGF vs. $11.37 \pm 0.46\%$ for Vehicle, $p=0.02$) without changing glial differentiation (Figure 3B; $63.4 \pm 10.26\%$ for HGF vs. $60 \pm 7.87\%$ for Vehicle, $p=0.64$). The effects of adding HGF during neural differentiation were assayed next. Primary NS were dissociated and cultured in differentiation media plus HGF or vehicle for 5 days. HGF treatment led to fewer β -III-tubulin-positive neurons (Figure 3B-D; $25.1 \pm 3.4\%$ for HGF vs. $41.29 \pm 1.5\%$ for Vehicle, $p=0.03$) and more GFAP-positive astrocytes ($51.88 \pm 3.1\%$ for HGF vs. $37.37 \pm 1.45\%$ for Vehicle, $p=0.03$). No significant effects on cell death or total cell number were found with HGF treatment (data not shown).

To explore whether the HGF mediated decrease in neuronal differentiation was due to delayed differentiation, primary NS were dissociated and cultured with function-blocking anti-HGF antibody in differentiation conditions for 2 days. Nestin immunocytochemistry was used to identify neural progenitors as its expression in NS is rapidly downregulated with differentiation. Anti-HGF decreased Nestin+ cell numbers (Figure 3A; $63.17 \pm 2.02\%$ with anti-HGF vs. $71.34 \pm 0.99\%$ with Vehicle, $p=0.04$), suggesting premature differentiation. Although increased cell death due to blockade of HGF signaling is also possible, immunostaining for activated caspase-3 was not increased with anti-HGF treatment (data not shown). Since most c-Met expressing cells in NS are Nestin+ (Figure 1B), these results suggest that HGF signaling maintains the neural progenitor state and slows differentiation.

HGF influences the olfactory bulb migration of SVZ neuroblasts

HGF is a motogen for interneuron precursors migrating from the ventral to dorsal telencephalon during development (Powell et al., 2001). We therefore suspected that HGF influences cell migration in the postnatal SVZ-olfactory bulb pathway. To assay for migration, P2 mouse brains were electroporated with a CMV-EGFP construct and sagittal brain slices were cultured for 4 days with vehicle or function-blocking HGF or c-Met (anti-

c-MET) antibodies (Powell et al., 2001). Electroporation across the ventricle selectively labels SVZ cells, which then migrate through the RMS toward the olfactory bulb in this assay (see Wang et al., 2005). Slices with obvious off-target injections were excluded. In vehicle-treated slices, most GFP+ cells remained in the proximal part of the SVZ-olfactory bulb pathway (Figure 4A), having migrated a short distance from the injection site in the anterior SVZ (upper left corner in Figure 4A). Surprisingly, anti-HGF or anti-c-Met antibody treatment increased the migration of GFP+ cells to the olfactory bulb (Figure 4B, C). Quantification of maximum migration distance of the five furthest migrating cells of each group showed significant increases in cultures treated with function-blocking antibody (Figure 4F, left side; 1.742 ± 0.09 mm for anti-HGF vs. 1.011 ± 0.05 mm for Vehicle, $p < 0.01$).

Because of the unlikely possibility that both anti-HGF and anti-c-Met antibodies activated HGF signaling in the cultures, we performed additional experiments using alternative means to inhibit HGF signaling. First, we co-electroporated P2 SVZ cells with dominant-negative c-Met (dnMet; Furge et al., 2001) and EGFP constructs. RMS neuroblasts in the slices were identified by calretinin immunoreactivity (Kato et al., 1999; Wang et al., 2005). Consistent with the function-blocking antibody experiments, we found that dnMet promoted SVZ cell migration to the olfactory bulb (Figure 4D-F; 1.662 ± 0.11 mm for dnMet vs. 0.899 ± 0.05 mm for Control, $p < 0.01$).

We next examined migration in slices derived from $tPA^{-/-}$ mice, which have a partial defect in HGF signaling. GFP electroporation into SVZ cells in brain slices from P2 $tPA^{-/-}$ mice showed enhanced migration of GFP-labeled cells to the olfactory bulb compared to slices from wild-type mice (Figure 4F-H; 1.688 ± 0.04 mm for $tPA^{-/-}$ vs. 1.357 ± 0.06 mm for wild-type, $p < 0.01$). Addition of HGF to the $tPA^{-/-}$ slices rescued this effect, with maximum migration distance decreasing to levels similar to those in wild-type explants (Figure 4F, H, I; 1.688 ± 0.04 mm for Vehicle-treated $tPA^{-/-}$ slices vs. 1.089 ± 0.08 mm for HGF-treated $tPA^{-/-}$ slices, $p < 0.01$). Interestingly, GFP+ cells often were more dispersed and also found in ectopic locations outside of the SVZ-olfactory bulb pathway, such as the septum and striatum, when HGF signaling was blocked either by antibody, dnMet or in $tPA^{-/-}$ slices (Figure 4C, H and data not shown). The extent of ectopic migration likely varied due to variable effectiveness of the treatments used to block HGF signaling. These results suggest that HGF signaling constrains SVZ cells to their proper locations in the migratory pathway.

A potential difficulty in interpreting the slice culture experiments is that treating the entire slice with loss or gain of function reagents may lead to effects of altered HGF signaling on other parts of the SVZ-OB pathway that secondarily influence neuroblasts migration. To more directly examine migratory effects of HGF signaling, including migration during exposure to an HGF gradient, we next used a 3-dimensional culture assay. SVZ neuroblasts display their typical chain migration pattern in Matrigel cultures (Wichterle et al., 1997). P7 mouse SVZ explants cultured in Matrigel for 2 days showed β -III-tubulin-immunoreactive neurons migrating out of the explants, while cells expressing the glial marker GFAP remained within the explants (Figure 5E and data not shown). SVZ explants cultured in Matrigel in the presence of either HGF or function-blocking antibody added to the culture media did not display altered migration patterns (data not shown). However, when SVZ explants were co-cultured with HGF-transfected Cos7 cells, emerging cells preferentially migrated towards the HGF-expressing cell aggregate compared to Cos7 cells transfected with GFP alone (Figure 5A, C, E). The ratio of cells migrating away from the explant side nearest the Cos7 cell aggregate (proximal) versus those migrating out of the side opposite (distal) was significantly increased in co-cultures with HGF-secreting cell aggregates compared to control aggregates (Figure 5F). Addition of anti-HGF antibody, but not control

IgG, abolished the chemoattractant effect of the HGF-transfected Cos7 cells, while anti-HGF had no effect on co-cultures with control Cos7 cells (Figure 5). Occasionally, migrating neuroblasts even invaded the HGF-expressing cell aggregate (Figure 5E). Taken together, our findings from the co-culture and slice culture experiments indicate that HGF acts as a chemoattractant for SVZ neuroblasts to maintain them in the SVZ-olfactory bulb pathway. When HGF signaling is blocked, neuroblasts appear to migrate out of the normal pathway and end up in ectopic locations.

Partial HGF deficiency alters *in vivo* SVZ-olfactory bulb neurogenesis

To determine whether HGF signaling through c-Met might influence postnatal SVZ-olfactory bulb neurogenesis *in vivo*, we first examined the expression of HGF and c-Met by immunostaining for these proteins as well as cell-type specific markers in neonatal mice. c-Met was expressed throughout the SVZ-olfactory bulb pathway (Figure 6A-H and data not shown) as described previously (Garzotto et al., 2008; Nicoleau et al., 2009). We found weak c-Met immunoreactivity in the SVZ in GFAP+ stem-like cells, and stronger expression in a subset of SVZ and RMS neuroblasts that co-expressed doublecortin (Figure 6A-H). HGF was also expressed in the SVZ, RMS and olfactory bulb (Figure 6I and data not shown), consistent with findings by others (Thweke and Seeds, 1996; Garzotto et al., 2008; Nicoleau et al., 2009). Like many secreted growth factors, the antibodies do not work very well for immunostaining, and we were unable to double immunolabel to determine which cells were expressing HGF. However, some of the cells appear to have a glial morphology and are likely RMS astrocytes (Figure 6I, inset).

We next examined cell proliferation and neurogenesis in the SVZ-olfactory bulb pathway of postnatal tPA and uPAR null mice as *in vivo* models of partial HGF deficiency (Powell et al., 2001; Wang et al., 2007). Consistent with this idea, P5 uPAR null mice showed decreased HGF expression compared to wild type in the SVZ, RMS and OB by immunohistochemistry (Figure 6I, J, and data not shown). At P14, the overall brain morphology generally was normal in uPAR and tPA null mice, although enlarged ventricles were seen occasionally. In addition, both uPAR and tPA null mice had smaller olfactory subependyma and SVZ by Nissl staining compared to wild-type (data not shown). We used Ki67 and BrdU immunolabeling to examine SVZ cell proliferation. At P8 (Figure 7A-C) and P14 (data not shown), both tPA and uPAR null mice had fewer Ki67+ cells in the dorsolateral SVZ (P8: 3640 ± 251 cells/mm² in tPA^{-/-} vs. 5579 ± 492 cells/mm² in wild-type, $p=0.01$). In P8 wild type and tPA^{-/-} mice injected with BrdU 1 day earlier, more BrdU+ cells were present in the SVZ of wild type mice than in mutants (data not shown). Combined with our *in vitro* findings, these data indicate that HGF stimulates SVZ cell proliferation. Delayed migration of proliferating cells out of the SVZ also likely contributes to this effect (see below).

HGF modulation of SVZ-olfactory bulb migration *in vivo* was studied next. P7 wild-type, uPAR null and tPA null mice were injected with BrdU (100 mg/kg) and sacrificed one day (P8) or one week (P14) later to examine changes in BrdU labeling in the SVZ-olfactory bulb pathway over time. Newly generated olfactory periglomerular interneurons originate mainly from cells recently dividing in the RMS, and BrdU+ cells typically reach the glomerular layer presumably within one day after BrdU administration (Hack et al., 2005). Consistent with our *in vitro* findings, tPA null mice showed a 1.7 fold increase of BrdU+ cells in the olfactory glomerular layer compared to wild-type mice at P8 (Figure 8A, B, E; 2063 ± 175 /mm² for tPA^{-/-} vs. 3474 ± 200 /mm² for wild-type, $p=0.003$). Most BrdU-labeled cells migrated away from the SVZ toward the olfactory bulb by P14 in wild-type, tPA null and uPAR null mice (data not shown).

By P14, uPAR and tPA null mice had more BrdU⁺ cells throughout the bulb compared to wild-type (Figure 8C, D, and data not shown). Quantification of olfactory subependymal BrdU-labeled cells revealed increases in uPAR^{-/-} mice versus controls (Figure 8F; 244.67 ± 17.16 cells for uPAR^{-/-} vs. 142.83 ± 12.52 cells for wild-type, $p=0.002$). Together with our *in vitro* findings, these data suggest that SVZ cells exit the cell cycle and begin to migrate earlier to the olfactory bulb when HGF signaling is diminished. The former conclusion, early cell cycle exit, was also supported by observations that uPAR or tPA null mice tended to have more intensely BrdU-immunoreactive cells than wild type (data not shown). In control mice, SVZ progenitor cells are actively proliferating. BrdU content may be diluted below detectable levels by multiple cell divisions after only a single BrdU injection seven days earlier, making detection less sensitive. In mutant mice, progenitor cells begin migrating and leave the cell cycle earlier, and thus do not dilute the BrdU content leading to increased immunoreactivity and the appearance of more BrdU-positive cells in the OB due to greater BrdU content.

Increased BrdU-immunoreactive cell numbers also appeared in regions surrounding the SVZ, such as the striatum, in uPAR null mice at P14 compared to wild type (data not shown). To directly examine SVZ neurogenesis, sections from wild type, uPAR^{-/-} and tPA^{-/-} mice were immunolabeled for doublecortin (Dcx) at P14 to identify neuroblasts. Consistent with decreased SVZ cell proliferation, uPAR and tPA null mice had less Dcx immunoreactivity in the dorsolateral and ventral SVZ compared to controls (Figure 7D, E and data not shown). Interestingly, we also found more Dcx-immunolabeled cells in the striatum of tPA^{-/-} mice than in controls (Figure 7D-F; 218 ± 33 cells/mm² in tPA^{-/-} vs. 104 ± 9 cells/mm² in wild-type, $p=0.008$). Although the source of the striatal Dcx-positive cells is uncertain, their close proximity to the SVZ and our *in vitro* data showing ectopic migration of SVZ cells in slice cultures after inhibition of HGF signaling suggest that they originated in the SVZ. Our findings thus support a model in which HGF acts as a chemoattractant to maintain SVZ neuroblasts in the SVZ-olfactory bulb pathway. With depletion of HGF signaling, cells either migrate farther into the olfactory bulb or ectopically into surrounding structures such as the striatum.

We next investigated the effect of HGF on neuronal differentiation *in vivo*. We reasoned that if HGF maintains the neural progenitor state, loss of HGF signaling would accelerate neuronal differentiation and lead to more newly generated neurons in the postnatal olfactory bulb. We administered BrdU to P7 wild type, uPAR^{-/-} and tPA^{-/-} mice, sacrificed animals a week later, and double labeled olfactory bulb sections for BrdU and the mature neuronal marker NeuN. We found that the uPAR^{-/-} mice had over 7-fold more NeuN/BrdU double-labeled cells in the olfactory periglomerular layer (Figure 8G, H; $53 \pm 8.55\%$ for uPAR^{-/-} vs. $7.14 \pm 7.15\%$ for wild-type, $p=0.05$) and a non-significant 2.4-fold increase in the granular layer ($50.74 \pm 8.64\%$ for uPAR^{-/-} vs. $21.25 \pm 8.75\%$ for wild-type, $p=0.14$) compared to wild type littermates. Taken together, these results suggest that HGF signaling maintains neural progenitors in an undifferentiated state or slows neuronal differentiation.

DISCUSSION

We used *in vitro* neurogenesis assays and *in vivo* studies of partially HGF-deficient mice to show that HGF regulates neurogenesis in the postnatal SVZ-olfactory bulb pathway. The effects of HGF are pleiotropic and appear to involve promoting cell proliferation, maintaining SVZ neural progenitors in an undifferentiated state, and serving as a chemoattractant to restrict migrating neuroblasts to the SVZ-olfactory bulb migratory stream.

In the mammalian brain, HGF is found in neurons, astrocytes and microglia, while c-Met is expressed in neurons (Honda et al., 1995; Yamagata et al., 1995; Hamanoue et al., 1996; Yamada et al., 1997). Transcripts for both are present in the postnatal SVZ-olfactory bulb pathway (Jung et al., 1994; Thewke and Seeds, 1996; Garzotto et al., 2008; Nicoleau et al., 2009), and we find both HGF and c-Met expression in the postnatal SVZ-OB pathway and by NS cells derived from P15 mouse SVZ. c-Met is expressed mainly by nestin⁺ and GFAP⁺ neural progenitors in NS cultures. *In vivo*, we find c-Met weakly expressed by GFAP-immunoreactive SVZ progenitors and more strongly expressed by doublecortin-positive cells in the SVZ and RMS. These findings may in part explain the discrepant results in the literature, as one group found c-Met expression in cells co-expressing PSA-NCAM but not GFAP (Garzotto et al., 2008), while another described cMet expression by Nestin- and GFAP-positive but Dcx-negative cells (Nicoleau et al., 2009). Although the precise source of HGF in our study is undefined, we detected it in SVZ-derived NS by RT-PCR assay and others have found HGF mRNA in microdissected SVZ (Garzotto et al., 2008) and HGF immunoreactivity in the SVZ after blocking its release *in vivo* (Nicoleau et al., 2009). The latter report described HGF in nestin-positive, GFAP-negative cells suggesting that it is expressed by transit amplifying or neuroblast populations in the SVZ. We found HGF expression in a subset of RMS cells that we speculate are astrocytes based on morphology, but further study is required to better delineate the specific cell types expressing HGF. Nonetheless, based upon these findings and published *in situ* hybridization data (Thewke and Seeds, 1996), HGF expressed in the SVZ-olfactory bulb pathway likely regulates neurogenesis by acting on c-Met-expressing neural progenitors. We also find that HGF delays the neuronal differentiation of SVZ progenitors. We speculate that the GFAP⁺ stem-like cells in the SVZ may downregulate c-Met during early differentiation into transit-amplifying cells, and then re-express higher levels of c-Met as they become migratory neuroblasts.

We showed that HGF signaling *in vitro* expands NS cultures and increases SVZ cell proliferation and neurogenesis *in vivo*, most likely by keeping SVZ cells in active cell cycle. Correspondingly, HGF appears to slow neuronal differentiation *in vitro* and *in vivo*, as evidenced by fewer cells exiting cell cycle and more cells expressing neural progenitor markers *in vitro*. These findings are consistent with previous findings in neonatal rat SVZ-derived neurosphere cultures (Nicoleau et al., 2009). Garzotto and colleagues (2008) failed to detect an effect of HGF on SVZ proliferation in neonatal mice, perhaps due to differences in the assay as they used explant cultures instead of neurospheres. A novel finding in the current study is that blockade of HGF signaling leads to more newly generated neurons in the P14 olfactory bulb within a week after BrdU labeling. Although we cannot exclude counter-selection of neuronal precursors by selective cell death of glial precursors in the partial HGF deficiency models, we did not observe any change in cell death in our cultures. We find more BrdU-labeled cells and a greater percentage of BrdU/NeuN double-labeled cells in partial HGF deficient animals. Given that the vast majority of newly generated cells at these ages in the wild type olfactory bulb will eventually express NeuN, the most consistent interpretation is that the newborn olfactory neurons in the null animals are differentiating more rapidly. Previous findings that p27 null mice exhibit increased transit-amplifying cell numbers and decreased SVZ neuroblasts (Doetsch et al., 2002) are consistent with our *in vitro* data in that upregulation of HGF signaling slows neuronal differentiation with a resultant decrease in p27⁺ cells.

In terms of migratory effects, HGF appears to act not as a motogen but rather as a chemoattractant that restricts cells to the RMS. Decreased HGF signaling, induced by function-blocking antibody, dn-cMet or in partially HGF-deficient mice, causes SVZ cells to exit the pathway prematurely or accelerates their migration to the olfactory bulb. Recent findings using a simpler migration assay, the Boyden chamber, support our finding of a

chemoattractant effect of HGF on RMS neuroblasts. Yan and Rivkees (2002) found that HGF stimulates both the proliferation and migration of oligodendrocyte precursor cells. HGF is also a motogen for cortical interneurons (Powell et al., 2001), a mitogen for Schwann cells (Krasnoselsky et al., 1994), and promotes the proliferation and neuronal differentiation of embryonic neural stem cells (Kokuzawa et al., 2003). These studies suggest that HGF plays multiple roles in early neural development, and our findings extend the pleiotropic neural developmental functions of HGF signaling into the postnatal period.

It is unclear whether the proliferation, migration and differentiation influences of HGF on SVZ cells are interrelated. Molecules that influence both cell proliferation and migration in a coupled manner are not uncommon during development. For example, Eph/ephrin proteins, retinoid signaling and different combinations of integrin subunits have dual effects on cell proliferation and migration in the postnatal SVZ-olfactory bulb pathway (Jacques et al., 1998; Conover et al., 2000; Wang et al., 2005). The embryonic cortex also provides a good example for highly coupled cell proliferation and migration. Cortical neurons that leave cell cycle early migrate to the deepest layers of the cortical plate, whereas cells that exit the cell cycle later end up in more superficial layers (reviewed in Jessell and Sanes, 2000). Therefore, the number of cell divisions or length of active cell cycling of neural progenitors may influence their differentiation and migration.

Evidence for a link between cell migration and differentiation also is compelling in the postnatal SVZ-olfactory bulb pathway. SVZ neuroblasts continue to divide as they migrate to the olfactory bulb (Smith and Luskin, 1998). In neonatal rats, cell cycle length is 14 hours in the SVZ and 17.3 hours in the RMS (Smith and Luskin, 1998). The cell cycle length of neural progenitors undergoing neurogenic, differentiative divisions is significantly longer than those undergoing proliferative divisions (Calegari et al., 2005). HGF therefore may maintain proliferative divisions of SVZ progenitors; loss of HGF signaling, on the other hand, would cause cell cycle lengthening of SVZ/RMS cells, and their earlier neuronal differentiation and migration to the bulb. Consistent with this idea, we found more BrdU+ cells in the glomerular layer in $tPA^{-/-}$ mice than in wild-type 24 hours after BrdU labeling. Work from several laboratories has shown that cells dividing in the RMS generate more olfactory periglomerular cells than granule cells (Hack et al., 2005). At one day after BrdU injection, we propose that a more rapid cell cycle exit and earlier initiation of migration to the OB leads to more olfactory glomerular layer BrdU-labeled cells in HGF deficient mice at P8, as these cells migrate more rapidly to the OB (Hack et al., 2005). We also cannot exclude the possibility that cell death could be decreased in the OB of the HGF deficient mice, although one day would likely be too early to see a cell death effect. Therefore, cells that reach the glomerular layer one day after BrdU incorporation likely were labeled while proliferating in the RMS, and HGF deficiency may particularly influence this population.

Many factors that regulate SVZ-olfactory bulb neuroblast migration have been identified. Neuroblasts require doublecortin (Koizumi et al., 2006) and PSA-NCAM (Chazal et al., 2000) to reach the olfactory bulb, while Eph/ephrin (Conover et al., 2000) and neuregulins (Anton et al., 2004) in the SVZ-olfactory bulb pathway are permissive cues for cell migration. Extracellular signaling through $\beta 1$ integrins, lipoprotein receptors and disabled-1 are also important for maintaining SVZ neuroblasts interactions with extracellular matrix or glial tubes during olfactory bulb migration (Emsley and Hagg, 2003; Andrade et al., 2007; Belvindrah et al., 2007). The repulsive influences of Slit1 and 2 (Wu et al., 1999; Sawamoto et al., 2006) secreted by the septum and choroids plexus may push SVZ cells to migrate rostrally. Prokineticin PK2 produced by the olfactory glomerular layer can attract SVZ cells (Ng et al., 2005) and, along with Reelin (Hack et al., 2002) and Tenascin-R (Saghatelian et al., 2004), disperses chain-migrating progenitors once they reach the olfactory bulb. Retinoic acid and gamma-aminobutyric acid also appear to influence RMS neuroblast migration

(Bolteus and Bordey, 2004; Wang et al., 2005). Here we show that HGF is an attractive cue for SVZ cells *in vitro*, and this effect likely maintains neuroblasts in the RMS *in vivo*. The delicate and precise control of neuroblast migration to the OB may be accomplished by the coordinated effects of HGF and the other guidance molecules mentioned above. When HGF signaling is experimentally depleted, SVZ cells are no longer constrained to the RMS and often migrate ectopically into the surrounding striatum or further into the olfactory bulb in a disorganized manner. The abnormal migration may occur in part due to altered timing of interactions with the molecules discussed above, or may result from premature differentiation. Thus, neurogenesis in the SVZ-olfactory bulb pathway requires delicate tuning; any imbalance may significantly alter migration patterns in the postnatal forebrain. Further understanding of mechanisms underlying migration and other steps in persistent forebrain SVZ neurogenesis, moreover, offers prospects for novel brain restorative therapies using this potentially reparative system.

EXPERIMENTAL METHODS

NS cultures and electroporation

Animal experiments were approved by the Michigan University Committee on Care and Use of Animals and conform to NIH animal care standards. P15 CD-1 mice (Charles River) were anesthetized with hypothermia and the brains removed. Brains were cut into 2 mm coronal blocks, and the lateral SVZ was dissected, minced into small pieces and dissociated with trypsin. About 5×10^4 SVZ cells (pooled from 4 brains) per 60 mm dish were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 (1:1, Gibco) containing 20 ng/ml EGF (Sigma) and 10 ng/ml bFGF (Sigma). Primary NS were cultured for 6-7 days *in vitro* (DIV), mechanically dissociated and plated for differentiation (with 1% fetal bovine serum [FBS]) at 1×10^5 cells/well in 24-well poly-ornithine (Sigma)-coated plates (Corning-Costar) with vehicle or HGF added for 2-5 DIV. In other experiments, dissociated primary NS were cultured at 3×10^3 cells/well in 6-well plates or 10 cells/well in 96-well plates for 5 DIV with EGF, bFGF, and vehicle or HGF to form secondary NS. In a subset, vehicle- or HGF-treated secondary NS were dissociated and differentiated for 5 DIV. For electroporation, primary NS were cultured for 5 days, dissociated and electroporated with CMV-EGFP (2.5 μ g; a gift of D. Turner) or CMV-EGFP plus a dnMet construct (CMV-dnMet; 2.5 μ g; a gift of C. Webb) by nucleofection (Amaxa, Program A-33). Electroporated cells were cultured at 1×10^4 cells/well in 12-well plates for 5 DIV.

Reverse transcriptase polymerase chain reaction (RT-PCR)

mRNA was isolated from primary or secondary NS expanded for 6 days in mitogens using Trizol reagent (Invitrogen). RT-PCR for HGF and c-Met was performed with the Titan one-step kit (Roche Molecular) using a PCR cycler (RoboCycler Gradient 40; Stratagene). RT-PCR was carried out at 55°C (50 min) for cDNA synthesis followed by 40 cycles at 94°C (30 sec), 53°C (1 min), 72°C (40 sec) and then a final extension step at 72°C (5 min). Primer pairs were (1) HGF: forward 5'-cttgcatccacgatgttc-3', reverse 5'-tggtgctgactgcatttctca-3'; and (2) c-Met: 5'-agtgcagtagtatcgacagagt-3' and 5'-cactgactgcagcattggaa-3'. PCR products were analyzed using 1% agarose gel electrophoresis. RT-PCR for actin served as an internal control; no PCR product was detected without RT in the RNA mixture.

Slice culture and electroporation

Brains were dissected from P2 CD-1, C57BL/6J or tPA null mice (on a C57BL/6J background; Jackson Laboratories), cut into two hemispheres and the meninges removed. US2-EGFP (gift of D. Turner) or CMV-EGFP with or without CMV-dnMet plasmid (1 μ g/ μ l) was injected into the anterior lateral ventricle and electroporated into the hemisphere (85 V, 10 pulses, 50 msec/pulse, 1 sec inter-pulse interval) using a BTX830 square-wave

electroporator as described previously (Wang et al., 2005). Slices (250 μ m thick) then were prepared with a vibratome (Leica), and those containing the SVZ-olfactory bulb pathway were cultured on filter membranes (Whatman nucleopore 25mm) floating on 1 ml culture medium (Neurobasal/pen-strep/glutamax with B27 supplement). Explants were randomly assigned to treatment condition by a blinded observer. After 1 h in culture, HGF (10 ng/ml, Cell Science), anti-HGF antibody (1-3 μ l/ml, R&D), or anti-c-Met antibody (1 μ l/ml, R&D) was added. Media was replaced and fresh antibody added on day 2. Slices were cultured for 2-4 DIV (2 days for proliferation assays, and 3-4 days for migration studies). BrdU (5 μ M) was added to some cultures for 2 h before washes and fixation on day 2.

Cos7 cell transfection and Matrigel culture

Cos7 cells were cultured at 3×10^5 cells/well in 6-well plates with DMEM plus 10% FBS for 1 day and transfected with CMV-EGFP or CMV-EGFP plus CMV-HGF (1 μ g/ μ l; a gift of M. Tessier-Lavigne) using Fugene 6 (Roche). A day later, transfected cells were trypsinized and resuspended in 100% Matrigel for co-culture experiments. To obtain explants for Matrigel co-culture, P7 CD-1 mouse brains were cut coronally into 300 μ m slices, and the anterior SVZ was dissected out under a dissecting microscope (Zeiss) and minced into small pieces (100-150 μ m). Explants were cultured in 100% Matrigel on inserts placed in 12-well transwell plates (Corning-Costar) with slice culture medium (Neurobasal/pen-strep/glutamax with B27 supplement) added. A day later, aggregates of Cos7 cells transfected with either CMV-EGFP or CMV-EGFP plus CMV-HGF were placed adjacent to SVZ explants and cultured for an additional 2 days.

In vivo BrdU labeling and tissue processing

BrdU (100 mg/kg in PBS) was injected once i.p. into P7 C57BL/6J wild-type, tPA^{-/-} (Jackson Labs) or uPAR^{-/-} (C57B6/129 background, a gift of P. Carmeliet; Dewerchin et al., 1996) mice. On P8 or 14, mice were perfusion-fixed with 0.9% NaCl (10 ml) and then 4% paraformaldehyde (PFA; 10-20 ml). Brains were removed and post-fixed in 4% PFA overnight, cryoprotected with 20% sucrose and sectioned coronally with a freezing microtome at 40 μ m thickness.

Immunohistochemistry

NS or dissociated cells were fixed with 4% PFA for 1 h at room temperature (RT). Slice cultures were fixed with 4% PFA for 3 h at RT, rinsed with PBS $\times$ 3 and vibratome-resectioned at 50 μ m thickness. Matrigel cultures were fixed with 4% PFA at RT for 1 h. For immunofluorescence, sections were rinsed in tris-buffered saline (TBS, pH 7.4) and incubated in blocking buffer (10% normal goat serum, 0.05% Triton X-100 and 2% bovine serum albumin in PBS) for 1 h prior to incubation with the following primary antibodies (48 h at 4°C): rabbit anti- β -III-tubulin (1:2000; Covance); rabbit anti-calretinin (1:2000; Chemicon); rabbit anti-doublecortin (Dcx; 1:2000) (Parent et al., 2002); mouse anti-glial fibrillary acidic protein (GFAP, 1:500; Sigma); rabbit anti-Ki67 (1:500; Novocastra); mouse anti-GFP antibody (1:500; Molecular Probes); mouse anti-Nestin (1:200; Chemicon); mouse anti-NeuN (1:1000; Chemicon) or mouse anti-p27 (1:1000; BD). After washes, sections were incubated with Alexa Fluor 594- or 488-conjugated goat anti-mouse or -rabbit IgG secondary antibody (1:200; Molecular Probes) at RT for 2 h, washed, mounted on slides and coverslipped with anti-fade medium (ProLong, Molecular Probes). Immunofluorescence double labeling of frozen tissue sections (35 μ m thickness) was performed similarly except that primary antibody incubation was overnight at 4°C with goat anti-C-Met (1:150, R & D Systems) and rabbit anti-GFAP (1:500, DakoCytomation) or rabbit anti-doublecortin (1:2000, Abcom). Images were captured on a Zeiss LSM 510 confocal microscope and color merged in Adobe Photoshop.

For BrdU immunohistochemistry, sections were incubated with 2N HCl at 37°C for 30 min and then neutralized with 0.1 M sodium borate (pH 8.5; 10 min). After washes, sections were incubated with rat anti-BrdU antibody (1:200; Serotec) at 4°C for 48 h and processed as described above using Alexa 594-conjugated goat anti-rat IgG secondary antibody (1:200). For BrdU immunoperoxidase staining, sections were incubated with mouse anti-BrdU antibody (1:1000; Roche) at 4°C for 48 h, washed and incubated with biotin-conjugated horse anti-mouse IgG secondary antibody (1:200) at RT for 1 h. After washes, sections were incubated with peroxidase-conjugated avidin-biotin complex (ABC, Vector), and developed with stable diaminobenzidine (DAB; Sigma). Immunoperoxidase staining with goat anti-HGF antibody (1:200, DakoCytomation) was performed similarly on frozen brain sections (35 μ m thickness) except that incubation with H₂O₂ was done instead of the HCL and sodium borate incubations. Sections were mounted on gel-coated slides (Super-Frost Plus, Fisher), dehydrated in graded ethanols and xylenes, and coverslipped with Permount (Sigma).

Image analysis and quantification

Microscopic images were acquired with a SPOT-RT digital camera (SPOT Diagnostic Instruments). For secondary NS number, total NS/well were counted using a grid and 10-20X objective, and NS were subdivided into the following size categories by diameter: small NS = 30-80 μ m; small-medium NS=81-150 μ m; medium NS=151-200 μ m; large NS $\geq$ 200 μ m. For differentiated cell counts, labeled and total cells were counted in 5 random fields (20X objective) by an observer blinded to condition. BrdU+ cells within a defined SVZ area (0.0676 mm²) were counted in 3 resectioned slices/hemisphere (2 explants/condition; 4 replicates) using a grid-lined reticle (20X objective) without knowledge of treatment condition. Maximum migration distance of GFP+ cells was calculated as the linear distance from the anterior border of the ventricle to the most rostral GFP+ cell using NIH Image v.1.63 software (Wang et al., 2005). Data were obtained using 2 explants/condition in 4 separate experiments. For *in vivo* BrdU labeling, BrdU+ cells in the olfactory subependymal and glomerular layers (0.09 mm²) were counted from 3 mid-olfactory sections/condition. Ki67+ cells were counted in the dorsolateral SVZ (0.16 mm²) from 4 anterior SVZ sections/condition, and Dcx+ cells were counted in a predefined area of the striatum (0.48 mm²) adjacent to the anterior SVZ from 4 sections/animal. All sections were spaced at least 240 μ m apart. Migration in Matrigel co-cultures was quantified by counting cells outside the explant on the side nearest to the cell aggregate (proximal side) versus the opposite (distal) side, and data was analyzed using the ratio of proximal to distal from at least three separate experiments/condition. Statistical analyses were performed using JMP 5.1.2 software. Multiple comparisons were analyzed by one-way ANOVA with post hoc test (Fisher's PLSD); two-tailed Student's t-test was used for two-group comparisons. Results are shown as means $\pm$ s.e.m. and significance level was set at p<0.05.

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Figure 1.

HGF effects in P15 mouse striatal SVZ NS cultures. (A) RT-PCR reveals mRNA for HGF and c-Met in 1^0 and 2^0 NS expanded for 6 d. Actin (upper band in each lane) is an internal control. (B) 2^0 NS differentiated for 3 hr are immunoreactive for cMet (red), and most c-Met + cells co-express nestin (green). Insets show single labeling of the boxed region for red and green channels. After 5 d (C), many c-Met+ cells (red) co-express GFAP (green; arrows). Insets show single labeling of the boxed region for red and green channels. At 7 d (D), no β -III-tubulin+ cells (green; β -tubulin) co-express c-Met (red). (E-H) 1^0 NS were passaged and cultured with mitogens $\pm$ HGF to form 2^0 NS. In high-density cultures, HGF treatment (10 ng/ml; F) increases NS size vs. vehicle-treated cultures (E-G). (H) At clonal density, HGF (10 ng/ml) increases NS numbers. * denotes $p < 0.05$. Scale bars: 50 μ m in B-D; in E, 200 μ m for E and F.

Figure 2.

Inhibition of HGF signaling decreases SVZ NS number and size. Phase contrast (A-C) and epifluorescence (A'-C') photomicrographs of NS after electroporation of EGFP (A), or EGFP plus dnMet (B, C) constructs. Arrows denote some of the electroporated neurospheres. (A) NS electroporated with the control EGFP construct were cultured with mitogens at high-density for 5 d. (B, C) NS electroporated with EGFP and dnMet constructs were cultured with mitogens and vehicle (B) or HGF (10 ng/ml; C) at high-density. Compared to the control (A, A'), dnMet (B, B') decreases the size of 2^0 neurospheres. Adding HGF to the culture does not rescue the effect of dnMet (C, C'). Scale bar: 100 μ m.

Figure 3.

HGF stimulates cell proliferation in neonatal mouse NS and brain slice cultures. (A) Ki67-, p27- or Nestin-positive cells in differentiating 1^0 NS cultured for 2 d. HGF increases proliferating Ki67+ cells, and function-blocking antibody (anti-HGF) increases p27+ cells that have exited the cell cycle. Anti-HGF also decreases Nestin+ cells. (B-D) Dissociated 1^0 NS were cultured in differentiation media plus HGF (10 ng/ml) or vehicle for 5 d. HGF treatment leads to fewer β -III-tubulin+ neurons (red) and more GFAP+ astrocytes (green) vs. vehicle (C, D) Quantification is shown on the left side of the graph in B (Primary). 2^0 NS were treated with mitogens $\pm$ HGF (10 ng/ml) after passage, then dissociated and cultured in differentiation media without HGF for 5 d. HGF pre-treatment decreases neuronal differentiation without changing glial differentiation (B, Secondary). (E-G) P2 mouse brain slices were cultured for 2 d with vehicle (Veh, E), 10 nM HGF (F) or 1 μ g/ml anti-HGF (G), then exposed to BrdU for 2 h prior to fixation. More SVZ cells incorporated BrdU in the HGF-treated slice (F) compared to control (E); anti-HGF substantially decreases BrdU labeling (G). (H) Quantification of BrdU+ cells per fixed SVZ area. * denotes $p < 0.05$ and ** $p < 0.01$. Scale bars: 50 μ m for C, D (in C); 50 μ m for E-G (in E).

Figure 4.

HGF signaling modulates SVZ cell migration *in vitro*. Sagittal SVZ-olfactory bulb (OB, at right in all panels) slices from P2 mouse were cultured for 4 d after electroporation with EGFP (A-D, G-I) or EGFP plus dominant-negative c-Met (dnMet; E) into the SVZ. Sections were immunostained for GFP alone (green; A-C) or with calretinin (red; D, E, G-I). (A-C) Slices cultured with vehicle (Veh; A) show GFP+ cells in the SVZ, RMS and the base of the OB (arrow in A). After incubation with function-blocking antibody to HGF (anti-HGF; B) or c-Met (anti-Met; C), many GFP+ cells migrate more rostrally into the bulb (arrows in B, C). (D, E) GFP-labeled SVZ cells also migrate further into the rostral OB after co-electroporation of dnMet (E) than in controls (D). (G-I) Sagittal slices from P2 wild-type (G) or tPA^{-/-} (H, I) mouse brains were cultured with vehicle (G, H) or HGF (10 ng/ml; I) for 4 d. More GFP+ cells appear in the rostral OB in the tPA^{-/-} slice treated with vehicle (tPA-Veh; H) compared to wild-type (C57-Veh; G). Rostral migration is normalized in the tPA^{-/-} slice treated with HGF (tPA-HGF; I). Arrows point to the rostral end of the labeled migratory stream in all image panels. (F) Quantification of maximal GFP+ cell migration distance from the SVZ to the distal RMS/OB. * denotes $p < 0.05$ and ** denotes $p < 0.01$ compared to: Veh group in antibody experiments; control group in dn experiment; or to wild-type in tPA^{-/-} experiments. # denotes $p < 0.05$ compared to tPA-Veh group. LV, lateral ventricle. Scale bar: 200 μ m.

Figure 5.

HGF is a chemoattractant for SVZ neuroblasts. P7 SVZ explants (*) were cultured with control or HGF-expressing Cos7 cell aggregates (left in all image panels). Function-blocking anti-HGF antibody or control IgG was added to the media for 2 d. (A, B) Cells migrate symmetrically out of SVZ explants co-cultured with control cells in the presence of IgG (IgG-Con; A) or anti-HGF antibody (aHGF-Con; B). (C-D) HGF-expressing cell aggregates in the presence of vehicle (Veh-HGF; C) preferentially attract SVZ cells to migrate toward them (arrow), and limited migration occurs out of the explant on the side opposite the aggregate (arrowhead). This chemoattractant effect is abrogated by treatment with anti-HGF antibody (aHGF-HGF; D). (E) SVZ explants co-cultured with HGF- and GFP-expressing cells plus IgG (IgG-HGF) show more β -III-tubulin-immunoreactive neurons (red; arrow) migrating from the side nearest the GFP-labeled, HGF-expressing cell aggregate (green); some neurons even invade the aggregate (arrowheads). Bis-benzimide counterstain in blue. (F) Quantification of cell migration. Data are presented as the ratio of migrating cells on the proximal (Cos7 cell aggregate) vs. distal side of the SVZ explant. * denotes $p < 0.01$ compared to IgG-Con, and # denotes $p < 0.05$ compared to aHGF-HGF. Scale bar: 100 μ m.

Figure 6.

c-Met and HGF expression in neonatal mouse SVZ and RMS. (A-H) Confocal images through the SVZ (A-E) or RMS (F-H) of wild type mice. GFAP-immunolabeled SVZ progenitors (A; red in C) weakly co-express c-Met (B; green in C) in the SVZ. Arrows denote double-labeled cells. No c-Met expression is present in striatum (D; same confocal settings). In the most anterior aspect of the SVZ (E), cell more strongly immunoreactive for c-Met (red) co-express the neuroblast marker doublecortin (Dcx; green). Asterisk denotes the lateral ventricle. In the RMS (F-H), c-Met-positive cells (green in H) double label for Dcx (red in H) as demonstrated at higher magnification in the inset in H. (I, J) HGF expression in the RMS is decreased in tPA null mice (arrows in J) compared to wild type (I). Insets show higher magnification views of HGF+ cells which appear to have an astrocytic morphology (I). Dashed lines denote the RMS borders. Scale bars: In A, 40 μ m for A-D; in F, 75 μ m for F-H and 25 μ m for E and inset in H; in I, 100 μ m for I and J, and 25 μ m for insets.

Figure 7. SVZ cell proliferation and neurogenesis are altered by HGF deficiency. (A-C) Ki67 immunostaining of coronal sections through the striatal SVZ from wild-type (A) and tPA^{-/-} (B) mice. At P8, fewer Ki67+ cells were present in tPA^{-/-} mice (B) compared to wild-type (A). (C) Ki67+ cell numbers per fixed dorsolateral SVZ area (between arrows in A and B) in P8 animals. (D-F) Doublecortin (Dcx) immunostaining of wild-type (D) and tPA^{-/-} (E) mice at P14. More Dcx+ cells appear in the striatum (arrows in E) of mutant mice. A decrease in SVZ Dcx (arrowheads) also is apparent in tPA^{-/-} mice (E) compared to wild-type (D). (F) Counts of Dcx+ cells per fixed striatal area adjacent to the dorsolateral SVZ in wild-type and tPA^{-/-} mice. * denotes $p < 0.05$ and ** denotes $p < 0.01$. Scale bar: 200 μ m for A and B; 100 μ m for D and E.

Figure 8.

HGF deficiency influences olfactory bulb neurogenesis. P7 wild-type (A, C), tPA (B) or uPAR (D) null mice were injected with BrdU (100 mg/kg) and sacrificed at P8 (A, B) or P14 (C, D). At P8, tPA^{-/-} mice have more periglomerular BrdU+ cells in the olfactory glomerular layer (GL; arrows in B) compared to wild-type (arrows in A). By P14, many BrdU+ cells have migrated to the olfactory bulb supendymal zone (SEZ) and granular layer in wild-type mice (C), but uPAR^{-/-} mice have more BrdU+ cells in these regions (D). (E) BrdU+ cell counts per fixed GL area at P8. (F) Counts of BrdU+ cells in the olfactory SEZ (outlined in C) at P14. (G, H) Confocal images of sections double immunolabeled for NeuN (green) and BrdU (red) in the olfactory GL from P14 wild-type (G) and uPAR^{-/-} (H) mice 1 wk after BrdU injection. In wild-type mice, only infrequent cells double-labeled with NeuN and BrdU (none are present in G). In contrast, many double-labeled cells appear in the mutant GL (arrows in H). See text for quantification methods. ** denotes $p < 0.01$. Scale bar (in A): 100 μ m for (A-D); 30 μ m for (G, H).

Table

Blockade of HGF signaling decreases neurosphere number and size.

GROUP	# small NS (%)	# small-medium NS (%)	# medium NS (%)	# large	Total # 2 ⁰ NS
Control	70.8 ± 1.1	25.4 ± 1.1	3.1 ± 0.1	0.7 ± 0.1	1242 ± 68
Control+HGF	73.3 ± 0.7	23.4 ± 0.7	2.8 ± 0.3	0.5 ± 0.1	1221 ± 40
dn cMet	76.6 ± 1.7 [*]	21.3 ± 1.7 [#]	1.6 ± 0.3 ^{^+}	0.5 ± 0.1	933 ± 45 ^{**}
dn cMet+HGF	74.5 ± 1.2 [#]	23.8 ± 1.2	1.6 ± 0.1 ^{@^}	0.2 ± 0.1 ^{@%}	866 ± 26 ^{***}

All numbers are means ± SEM; small = 0.03 - 0.08 mm; small-medium = 0.081 - 0.15 mm; medium = 0.151 - 0.2 mm; large = > 0.2 mm. Separate ANOVAs were done for each neurosphere (NS) size category and for Total # NS.

^{*}, p < 0.01 vs. Control and Control+HGF;

^{**}, p < 0.001 vs. Control and Control+HGF;

^{***}, p < 0.0005 vs. Control and Control+HGF;

[#], p ≤ 0.05 vs. Control;

[@], p < 0.001 vs. Control;

[^], p < 0.005 vs. Control+HGF;

⁺, p < 0.01 vs. Control+HGF;

[%], p = 0.01 vs. Control+HGF.