Characterization of neuroprogenitor cells expressing the PDGF β-receptor within the subventricular zone of postnatal mice

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Received 25 July 2007; revised 9 November 2007; accepted 12 November 2007
Available online 3 December 2007

We report a considerable number of cells in the ventricular and the subventricular zones (SVZ) of newborn mice to stain positive for the PDGF β-receptor (PDGFRB). Many of them also stained for nestin and/or GFAP but less frequently for the neuroblast marker double-cortin and for the mitotic marker Ki-67. The SVZ of mice with nestin-Cre conditional deletion of PDGFRB expressed the receptor only on blood vessels and was devoid of any morphological abnormality. PDGFRB−/− neosopheres showed a higher rate of apoptosis without any significant decrease in proliferation. They demonstrated reduced capacities of migration and neuronal differentiation in response to not only PDGF-BB but also bFGF. Furthermore, the PDGFR kinase inhibitor STI571 blocked the effects of bFGF in control neurosphere cultures. BFGF increased the activity of the PDGFRB promoter as well as the expression and phosphorylation of PDGFRB. These results suggest the presence of the signaling convergence between PDGF and FGF. PDGFRB is needed for survival, and the effects of bFGF in migration and neuronal differentiation of the cells may be potentiated by induction of PDGFRB.

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Keywords: PDGFR-receptor; FGF; Neural stem cells; Neurosphere

Introduction

Neural stem cells have been shown to play important roles for repair and regeneration following ischemic injuries, and also in experimental neurodegenerative diseases (Park et al., 2006; Snyder and Macklis, 1995). The expression of growth factors, such as PDGF and its receptors, increases in such conditions (Funa et al., 1996; Hermanson et al., 1995; Sjoborg et al., 1998). PDGF has been shown to stimulate proliferation (Erlandsson et al., 2001), neurogenesis (Richards et al., 2006), and survival (Lobsiger et al., 2000) of various types of neuroprogenitors. Furthermore, activation of the PDGF signals has been frequently shown to be associated with tumorigenesis of malignant glioma (Hermanson et al., 1996; Lokker et al., 2002; Ma et al., 2005). Adult neuroprogenitors have been considered to be the cells that could give rise to glioma upon acquiring genetic and epigenetic alterations. These somatic gene alterations have been hypothesized to occur upon cellular stress and injuries, as well as during the repair processes when progenitors are stimulated for proliferation (Manuelidis, 1994). A recent report on the presence of PDGFRA-expressing adult neural stem cells has demonstrated the importance of PDGF in neurogenesis, and also suggesting the identity of such cells as glioma progenitors (Fomchenko and Holland, 2007; Jackson et al., 2006). However, the knowledge about the role of PDGFRB in postnatal neurogenesis still remains to be clarified.

PDGFRA knockout is lethal to mice, exhibiting incomplete cephalic closure similar to that observed in a subset of Patch mutants (Soriano, 1997). In these mice, increased apoptosis was observed on the pathway of migrating neural crest cells. In contrast, the conventional PDGFRB knockout mice have not shown any obvious abnormality in the nervous system (NS) (Betscholtz, 1995). However, since PDGFRB knockout mice die at birth, it is difficult to study dysfunctions in the postnatal NS. Recently, neural cell-specific PDGFRB knockout mice were produced by crossing PDGFRBFL/FL mice with mice expressing Cre recombinase under regulation of the nestin promoter (Ishii et al., 2006). These mice showed no gross anatomical abnormalities or functional deficits in the NS. However, the deletion of PDGFRB resulted in markedly reduced neuronal cell survival after cryogenic and NMDA injuries to the adult mice. Since neural stem cells are known to migrate to the site of damage (Felling and Levison, 2003), such cells lacking PDGFR might explain the reason of the poor cell survival and ineffective healing in PDGFRB−/− mice.
In order to test these hypotheses, we have examined the SVZ of nestin promoter PDGFRB$^{-/-}$ mice to see the presence of neural stem cells and their phenotypes in comparison with the PDGFRB$^{FL/FL}$ control mice. We further attempted to isolate and characterize neuroprogenitors from mice with the conditional depletion of PDGFRB. However, depletion of the nestin-specific PDGFRB does not occur in 100% of neuroprogenitors. Consequently, a minute fraction of PDGFB possessing cells will be enriched after serial passages of such cultures. Therefore, we explanted postnatal day 1 (P1) SVZ cells from the PDGFRB$^{FL/FL}$ mice and stably transfected

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Fig. 1. Coronal sections of the P1 SVZ in control mouse (PDGFRB$^{FL/FL}$; A, B, E–T) and nestin-Cre conditional PDGFRB$^{-/-}$ mouse (C, D) stained for PDGFRB (green except for I–L where PDGFRB is red) identified with the confocal microscope. Note that many PDGFRB-expressing cells are observed in the SVZ of PDGFRB$^{FL/FL}$ mouse (A, B), whereas the immunoreactivity is completely abolished in cells in the SVZ except for the blood vessels (arrows in panels B, D) in conditional nestin-Cre PDGFRB$^{-/-}$ mouse (C, D). (E, F) Co-immunostaining for PDGFRB and GFAP (red). Arrows for PDGFRB-expressing blood vessels and arrowheads for co-immunostained SVZ cells. (I–L) Only a few cells express both PDGFRB (red) and Dcx (green). (M–P) PDGFRB and nestin (red) are coexpressed by a majority of SVZ cells (arrowheads) and blood vessels (arrows). (Q–T) PDGFRB and Ki-67 (red). Arrowheads point to co-expressing cells. Counterstained with DAPI (blue). CP, choroid plexus; LV, lateral ventricle; ST, striatum. Scale bars = 200 μm (A and C), 75 μm (B, D, E, I, M, and Q), and 25 μm (F–H, J–L, N–P, R–T).
Cre recombinase into these cells. The obtained PDGFRB\(^{-/-}\) SVZ cells have been cultured in neurospheres and characterized for their capacities in proliferation, migration, differentiation, and survival. They were compared with control neurospheres from the wild type (WT) mice, which were stably transfected with Cre recombinase. Furthermore, we prepared primary-cultured neurosphere from the WT or PDGFRB\(^{FL/FL}\) mice in order to analyze potential crosstalk between PDGF and bFGF signals, thus validating the data obtained from the Cre-transfected PDGFRB\(^{-/-}\) neurospheres.

**Results**

Expression of PDGFRB in newborn mice SVZ compared with the expression in nestin-Cre conditional PDGFRB\(^{-/-}\) mice

First, we compared the SVZ of the forebrain of P1 PDGFRB\(^{FL/FL}\) mice with the expression in the conditional PDGFRB\(^{-/-}\) mice. A majority of PDGFRB-positive cells were detected in the VZ and the SVZ of PDGFRB\(^{FL/FL}\) mice where radial glia cell bodies and neural stem cells have been shown to reside (Figs. 1A, B) (Lois and Alvarez-Buylla, 1993; Merkle et al., 2004). In contrast, the nestin-Cre conditional PDGFRB\(^{-/-}\) mice exhibited no PDGFRB-positive cells in these zones, except pericytes-like cells in the blood vessels in the choroidal plexus and those located several cell layers away from the ventricle (Figs. 1C, D) (Hellstrom et al., 1999). However, the PDGFRB-stained structures on and around blood vessels were clearly larger in the control SVZ, suggesting cells around vascular cells are also stained.

We characterized the cells expressing PDGFRB in PDGFRB\(^{FL/FL}\) mice more in detail by using markers for various stages of neuroprogenitors (Figs. 1E–T). In P1 mice, GFAP was expressed strongly on thick radial fibers extended from the ependymal layer inwards to several cell layers away from the ventricle as well as on the SVZ astrocytes (Figs. 1E–H). Both GFAP-positive and -negative cells stained positive for PDGFRB, yielding many structures that are double positive.

In order to identify neuroblasts, we used Dcx that are expressed by postmitotic but not yet specified neurons. As seen in Figs. 1I–L, the Dcx cells are detected slightly distant from the lumen compared to those expressing PDGFRB. There are a few double positive cells, but a majority of Dcx-positive cells does not express the receptor. Cells known as transit amplifying neuroprogenitors have been shown to lack GFAP, but some of those cells continue to express nestin (Doetsch et al., 1997). The adjacent section was therefore stained for PDGFRB and nestin. The expression patterns of these two markers are very similar including around the capillaries as shown in the panels in Figs. 1M–P. The double-stained vessel structure might represent end-feet of progenitors around the vessels and possibly nestin-positive microvascular progenitors (Dore-Duffy et al., 2006; Mokry et al., 2004).

We used the mitotic marker Ki-67 to determine the distribution and the fraction of dividing cells that are also PDGFRB positive (Figs. 1Q–T). These double positive cells were found mostly in a few layers beneath the ependymal layer. Further away from the ventricle, there are several solitary cells that stained positive for Ki-67 but negative for PDGFRB. It is thus clear that PDGFRB is expressed also in a part of amplifying cells. Taken together, PDGFRB seems to be expressed in both quiescent and amplifying neural stem cells, and also in a very small fraction of neuroblasts.

**Phenotypic characterization of the PDGFRB\(^{-/-}\) and control neurospheres**

Neuroprogenitors were obtained from dissociated SVZ cells of the forebrains of P1 WT or PDGFRB\(^{FL/FL}\) mice. PDGFRB\(^{-/-}\) stable neurospheres were made by stable transfection of Cre expressing plasmid (pCrePac) to the PDGFRB\(^{FL/FL}\) neurospheres at soon as they were established. Three stable PDGFRB\(^{-/-}\) neurosphere cultures were examined in comparison with pCrePac transfected neurospheres obtained from WT mice. Possible cross-reactivity and non-specific binding were excluded by using control cell lines expressing only PDGFRA or RB as well as omission of primary or secondary antibody. Control as well as PDGFRB\(^{-/-}\) neurospheres were dispersed gently in growth media and attached onto object
glasses for immunofluorescence staining. Both of them stained positive for PDGF-BB and PDGFRA (Fig. 2). However, in PDGFRB knockout cells, the expression of PDGF-BB was reduced and PDGFRA was enhanced. As expected, control cells, but not PDGFRB knockout cells, stained positive for PDGFRB. Both cell types are positive for GFAP and completely negative for MAP2 and β-tublin in growth media; however, these markers could be induced already 1 day after withdrawal of bFGF (data not shown).

Fig. 3. (A) The diameter of control and PDGFRB knockout neurospheres. Graph shows means±SEM. There is no significance between control and PDGFRB knockout neurospheres (p=0.42, two-way ANOVA, followed by Ryan’s methods). (B) The percentage of BrdU incorporation of control and PDGFRB knockout cells dissociated from neurospheres is not significant (p=0.16). (C) The number of apoptotic cell death, stained with Hoechst, is significantly increased in PDGFRB knockout cells dissociated from neurospheres (p<0.0001). Mean percentages±SEM are shown for the three separate experiments (B and C). One-way ANOVA, followed by Fisher’s PLSD test, is used for statistical analysis, with p-values <0.05 (*) and <0.01 (**) considered significant. (D and E) Representative photographs of cell nuclei after Hoechst staining of control (D) and PDGFRB knockout (E) dissociated from neurospheres. Apoptotic bodies are indicated by arrowheads. Scale bars=50 μm.
PDGFRB signaling is not absolutely required for neural stem cell maintenance but essential for cell survival

PDGFR signaling is reported to be involved in neural stem cell maintenance (Jackson et al., 2006). Here, we examined whether PDGFRB signaling also contributes to the maintenance of neural stem cells. Mutant neurospheres generated many secondary neurospheres, indicating self-renewal capacity to be preserved in PDGFRB−/− cells (data not shown). We analyzed the size of neurospheres, proliferation capacity, and survival. In these analyses, we used pCrePac-transfected WT neurospheres as controls. In comparison with the control neurosphere populations, the mutant populations display a slightly higher percentage of small spheres (less than 60 μm in diameter), but the difference did not reach any significance (Fig. 3A). In order to estimate the rate of proliferating neuroprogenitors, we used BrdU incorporation assay. No significant reduction in the number of BrdU-positive cells was found when compared with the controls (Fig. 3B). However, inspection of the mutant neurosphere cultures demonstrated clearly increased numbers of apoptotic bodies (Figs. 3D, E). Dispersed progenitors undergoing programmed cell death were identified by the morphology of nuclei stained with Hoechst. The percentage of apoptotic cells in the mutant populations was significantly higher than those in controls (Fig. 3C).

Migration of neurosphere-derived cells is significantly decreased

PDGFRB−/− neurospheres generate significantly decreased population of neurons in response to not only PDGF, but also bFGF

PDGF and its receptors have shown to be expressed in the developing and mature brain. We demonstrate here that the SVZ stem cells express PDGFRB. In order to identify which roles PDGF play for these cells, we examined the abilities of PDGF-AA, PDGF-BB, and bFGF to generate neurons and glial cells from control and PDGFRB−/− neurospheres. We plated intact neurospheres on chamber slides with and without growth factors for 5 days. Control neurospheres transfected with pCrePac plasmid cultured without any of the factors resulted in generation of up to 20% of total cells to MAP2-positive neurons, significantly higher than those seen in PDGFRB−/− neurospheres (Figs. 5A, B, K). In control neurospheres, addition of growth factors significantly increased the number of neurons (Figs. 5C, E, G, K). Particularly, bFGF caused almost 70% of cells to become MAP2 positive (Figs. 5G, K). The same yield with PDGF-BB was 52% and with PDGF-AA 33% (Figs. 5C, E, K). However, none of the growth factors could increase the neuronal population of PDGFRB−/− neurospheres (Figs. 5D, F, H, K). Addition of bFGF or PDGF-BB, but also to a smaller extent, PDGF-AA, decreased the number of GFAP-positive cells in control neurospheres (Figs. 5C, E, G, K). In contrast, the percentage of GFAP-positive cells was increased in PDGFRB−/− neurospheres by PDGF-BB or bFGF (Figs. 5F, H, K).

The marked reduction of MAP2-positive neurons generated by PDGFRB−/− neurospheres in response to bFGF was striking when compared with the controls. We assumed that the intact PDGFRB is necessary for the normal bFGF signal to occur. In order to test this hypothesis, we added the PDGFR inhibitor STI571 in the control cultures with intact PDGFRB with or without bFGF. STI571 alone decreased the neuronal population and increased the GFAP-positive cell population compared with those cultured without any growth factor (Figs. 5I, J, K). Furthermore, an addition of bFGF could not increase the neuronal population in the presence of STI571 (Figs. 5J, K). These results confirm that the defective response to bFGF has not been caused by a phenotypic change occurred during the establishment of the PDGFRB−/− neurospheres.

FGF upregulates the PDGFRB promoter activity

The reason why the bFGF-induced neural phenotypes were not induced in the PDGFRB−/− neuroprogenitor cells might be that PDGFRB signals are required for the effects of bFGF. We used a PDGFRB-promoter reporter assay in order to see whether PDGFRB could be transcriptionally upregulated by bFGF. Primary neurospheres from PDGFRBfl/fl mice were dispersed and cultured without bFGF for 24 h. Luciferase activity was determined 48 h after addition of bFGF. This promoter construct has an additional PDGFRB enhancer that has been shown to be active for both neuroblastoma (Kaneko et al., 2006) and neuroprogenitor cells. Stimulation of pCrePac-transfected control neurosphere cells with bFGF induced activation of the PDGFRB promoter (Fig. 6). PDGFRB promoter became also activated but did not reach to significance (data not shown).

Phosphorylation of PDGF-receptors and their downstream signaling molecules activated by PDGF-BB or bFGF

We next examined intracellular signaling kinases, activated by PDGF-BB and bFGF, in primary neurospheres of WT mice after
5 days of differentiation. An addition of PDGF-BB strongly increased the amounts of both total and phosphorylated PDGFRB, in contrast to the weak expression of total PDGFRB protein detected in control culture without any growth factor (Fig. 7). We used the antibody that recognizes phosphoY1021, the phosphophasry vs Cret binding site. As expected, bFGF also induced a similarly strong total expression of PDGFRB, as well as a slightly weaker but almost similar expression level of phosphorylated PDGFRB. PDGF-BB, which is known also to bind PDGFR, induced a larger expression level of total PDGFR than that of PDGFRB. However, a similarly increased phosphorylation level of PDGFR was seen in cultures stimulated by PDGF-BB or bFGF, as well as the control, suggesting the presence of an autocrine stimulation via this receptor in the primary neurospheres.

Extracellular signal-regulated kinase (ERK) is known to stimulate cell proliferation and differentiation such as neurite outgrowth (Sun et al., 2006), whereas Akt/PKB has been shown to promote growth factor-mediated cell survival, to block apoptosis, and to stimulate migration (Eves et al., 1998). Stimulation of 20 ng/ml bFGF or PDGF-BB induced a large amount of phosphorylated ERK expression. PDGF-BB also induced a high level of phosphorylated Akt expression, whereas bFGF resulted in only a weakly phosphorylated Akt. Interestingly, PDGF-BB also induced a higher level of phosphorylation of Glycogen synthase kinase-3β (GSK3β) when compared with bFGF. GSK3β has been shown to decrease the stability of microtubules in growing axons, leading to axon growth and increased filopodia dynamics in growth cones (Goold and Gordon-Weeks, 2004; Owen and Gordon-Weeks, 2003). This might be yet another explanation for the neural differentiation effect of the PDGFR pathway. Together, these data show that PDGF-BB is able to activate pathways that induce neurite outgrowth and promote survival of neuroprogenitor cells.

Discussion

PDGFRB expression was clearly detected in the GFAP-expressing SVZ cells of newborn control PDGFRB+/−/− mice, in contrast to that of the conditional PDGFRB−/− mice where no expression was found in neuronal cells. SVZ astrocytes were previously reported not to express PDGFRB (Jackson et al., 2006). The reason for the discrepancy might depend on the difference of antibodies. The antibody we used did not efficiently detect PDGFRB on frozen tissues. In fact, the staining patterns of neuroprogenitors for PDGFRB and nestin are similar in the control tissues. Nestin is expressed in quiescent neural stem cells and also in some amplifying progenitor cells, which can undergo cell division upon stimulation by various factors, typically EGF (Sun et al., 2005) and PDGF-AA (Jackson et al., 2006).

In addition, there are blood vessels that stained positive for both PDGFRB and nestin in the choroidal plexus and in the SVZ. These cells might represent end-feet of progenitors around the vessels because the stained vessels structures are somewhat larger in controls than in the PDGFR−/− brain. Alternatively, they might be nestin-positive microvascular progenitors (Dore-Duffy et al., 2006; Mokry et al., 2004), in particular capillaries in the choroidal plexus. Early endothelial progenitors have also been shown to express PDGFRB (Magnusson et al., 2007).

The majority of Dcx-positive neuroblasts in the SVZ of PDGFRB−/−/− mice did not express the receptor. However, the loss of PDGFRB in neurons does not considerably affect the structure and the size of the stem cell population in the conditional PDGFRB−/− mice, in spite of the existence of Ki-positive PDGFRB cells in controls. One of the obvious reasons might be the presence of the remaining PDGFR, binding most of the PDGF ligands to maintain the proliferation capacity of the SVZ astrocytes.

Further in vitro characterization of the PDGFR−/− progenitors has provided us with an important insight in the role of this receptor in the NS. First, loss of the receptor leads to increased apoptotic cells of the progenitors. Second, chemotactic responses of the progenitors became considerably reduced. Third, a marked decrease was detected in the response of the progenitors, not only to PDGF, but also to bFGF. Even though these defects did not significantly affect the developmental process of the NS, it could cause severe problems upon stress and injuries that adult NS cells are exposed to. It is well recognized that adult resident stem cells become activated in such conditions. These are extraordinarily mobile cells that sense the injured site and migrate toward it. PDGFRB is known to be one of the most potent receptors that stimulate chemotaxis of cells, especially during inflammation.

Although we could not find any significant differences in cell proliferation, the distribution of the size of individual neurospheres was skewed to the left. This could be due to the observed increase in the number of apoptotic cells. The decreased Akt signal observed in the PDGFR−/− cells might be responsible for this phenotype (Gao et al., 2005). The strong Akt phosphorylation was indeed detected in the PDGFR-BB stimulated primary WT neurospheres.

The induction of chemotaxis has been known to be one of the major differences between the signaling downstream of the receptors α and β (Ronnstrand and Heldin, 2001). The strong chemotaxis mediated by PDGFRB involves activation of PI3K and PLCγ and their downstream targets Rac and PKC. On the contrary, PDGFRB can inhibit migration in several cell types (Eriksson et al., 1992; Koyama et al., 1994), whereas stimulate it in other cells (Laurent et al., 2003). These differences could explain the observed lack of migratory responses to both PDGF-AA and PDGF-BB in the PDGFR−/− cells, in which the activity of PDGFRB is increased. Chemotaxis is important during development in order to secure orientation dependency of the organ structure in the nervous system. PDGFRB has been shown to be an important chemotactic factor for neuroprogenitors (Forsberg-Nilsson et al., 1998). In healthy conditions, it seems to be fully compensated in the brains of the neuron-specific PDGFRB−/− mice (Ishii et al., 2006), which do not exhibit any phenotypic deficits. Presence in the developing NS of other factors such as NGF, GDNF, and neurturin (Ishii et al., 2006; Ming et al., 1997), might have helped avoid a deficiency.

The PDGFR−/− neurospheres have reduced capacities to differentiate to neurons as compared with the controls. However, it has been shown that neurosphere cells generated from the SVZ...
vascular cells (Nissen et al., 2007). They also collaborate in the demonstrated previously that PDGF and FGF2 synergize in been distinctly observed in control neurospheres. It has been respond to bFGF by migration or neural differentiation, which had study implies the Akt signaling to be important for the might be important for the robust neural induction observed by optic nerves (Noble et al., 1989) as well as PDGFR-responding neurospheres from the conditional PDGFRB−/− mice, which will be summarized elsewhere.

It is striking that the PDGFRB−/− neurospheres were unable to respond to bFGF by migration or neural differentiation, which had been distinctly observed in control neurospheres. It has been demonstrated previously that PDGF and FGF2 synergize in promoting angiogenesis and cell proliferation (Cao et al., 2003; Kano et al., 2005). Reciprocal transcriptional induction of their receptors has been shown to be involved in the synergism in vascular cells (Nissen et al., 2007). They also collaborate in the continued renewal of oligodendrocyte progenitors derived from the optic nerves (Noble et al., 1989) as well as PDGFR-responding progenitors isolated from embryonic forebrain (Chojnacki and Weiss, 2004). In primary WT neurosphere cells, both FGF and PDGF induced a similar level of ERK2 activation. However, PDGF induced the stronger AKT phosphorylation and also a high level of GSK3β phosphorylation. It is possible that these signals might be important for the robust neural induction observed by FGF by way of synergism with the induced PDGFRB. A recent study implies the Akt signaling to be important for the differentiation of neural stem cells (Otacig, 2006).

PDGF-AA that binds only PDGFRB induced neural differentiation in PDGFRB−/− cells, indicating that both of the PDGFRs can activate the neural differentiation signal upon ligand stimulation. In spite of this, the presence of PDGFRA alone in PDGFRB−/− cells could not rescue their lack of response to bFGF, suggesting that the PDGFRB signal is indispensable for the FGF signal. However, since the primary progenitors appear to express constitutively activated PDGFRA, it is difficult to rule out whether PDGFRA is also required for the effect of FGF in addition to PDGFRB.

In contrast to the constitutively activated PDGFRA that is important in maintaining the self-renewing progenitor pools (Erlandsson et al., 2006; Jackson et al., 2006), PDGFRB is likely to be activated by the ligand in these progenitors. The expression of PDGFRA and its specific ligands, A and C, is abundant in the developing CNS, whereas the PDGFRB expression and its specific ligands, B and D, becomes more evident in the postnatal CNS (Hamada et al., 2002). These reported expression patterns support our results and emphasizes the notion that the PDGFRB signal plays important roles in the maintenance of postnatal neurogenesis. These studies clearly demonstrate PDGF to play a versatile role, both in maintaining the progenitor pools and in inducing differentiation of various cell types in the NS through cross-talk between both of the PDGF receptors as well as with FGF.

**Experimental methods**

**Animals and genotyping**

The generation of PDGFRB-floxed mice has been described previously (Gao et al., 2005). The PDGFRBFL/− mice on a C57Bl/6 background were used for isolation of forebrain neural stem cells. Genotypes were determined by holding out PCR on genomic DNA. For the conditional deletion of PDGFRB, we crossed the PDGFRB floxed mice and the Cre recombinase-expressing mice under the control of the nestin promoter and enhancer (nestin-Cre+ mouse, The Jackson Laboratory, Bar Harbor, ME). These mice with PDGFRBFL/− and nestin-Cre+ are referred as nestin-Cre conditional PDGFRB−/− mice. All animal experiments were performed in accordance with the recommendations of the animal Ethics Committee at Göteborg University.

**Tissue sampling and immunohistochemistry for the deparaffinized sections**

Mice were perfused with cold phosphate buffered saline (PBS) under anesthesia with sodium pentobarbital (intraperitoneal injection, 50 mg/kg body weight). For the staining of the paraffin section, after further immersion in paraffin, and 10-μm-thick tissue coronal sections were glass-mounted. The staining method was described previously (Ishii et al., 2006). Antibodies used were goat anti-PDGFRB (1:100; R&D Systems, Minneapolis, MN), rabbit anti-GFAP (1:500; Dako, Glostrup, Denmark), mouse anti-microtubule-associated protein 2 antibody (MAP2; 1:1000; Sigma, St. Louis, MO), guinea pig anti-doublecortin (Dcx; 1:500; Chemicon, Temecula, CA), mouse anti-nestin (1:100; Chemicon), and rat anti-Ki-67 (1:1000; Dako). The sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and observed with a confocal microscope (TCS-SP5, Leica).

**Neurosphere cell culture**

Neural precursors were obtained from dissociated forebrains of P1 WT or PDGFRBFL/− mice using a fire-polished Pasteur pipette. Cells were plated at...
20 ng/ml bFGF or PDGF-BB. GAPDH is used as an internal standard.

neurospheres after 5 days differentiation without growth factors and with PDGFRB FL/FL mice (PDGFRB FL/FL). These cells showed almost identical transfected with pCrePac (control) as well as neuroprogenitors isolated from primary antibody, sections were washed in PBS. As secondary antibodies, mouse anti-O4 (1:50; Chemicon) were used. After incubation with the primary antibodies, a rabbit anti-GFAP (1:500; Dako), and a mouse anti-MAP2ab (1:500; Sigma), a rabbit anti-GSK3 β mouse anti-PDGFRA (1:1000; eBioscience, San Diego, CA), a mouse anti-PDGFRB (1:200; Upstate Biotechnology, Lake Placid, NY), a rabbit anti-PDGFRB (1:100; ebioscience, San Diego, CA), a mouse anti-PDGFRB-BB (provided by Mochida Co.), a mouse anti-β-tubulin (1:500; Sigma), a mouse anti-MAP2ab (1:500; Sigma), a rabbit anti-GFAP (1:500; Dako), and a mouse anti-O4 (1:50; Chemicon) were used. After incubation with the primary antibody, sections were washed in PBS. As secondary antibodies, three different cell lines were used in all experiments; however, they were not derived from a single clone but from mixtures of several clones. They showed concordant results in all the experiments we performed.

PCR

Genotyping of the recombinant allele of PDGFRB of the each colony was performed by PCR-based analyses previously described (Gao et al., 2005). The primers for genomic PCR of PDGFRB allele were as follows: primer 1, 5'-TAGCCATGGAGTCATCTCTCTTCGACCCCTAAA-3'; primer 2, 5'-CCTGCAATCAAGTAGCTACAACAGGTGCTGTA-3'; and primer 5, 5'-AGCAAGTCGCGCAAGGATGAAACGC-3'.

Characterization of neurospheres

For measuring the size of neurospheres, the diameter was calculated from the cross-section of neurospheres. In total, 1000 neurospheres (500 control and 500 mutant spheres) were measured.

BrdU incorporation assay

For determination of the cellular composition of neurospheres, 8-day-old spheres were dissociated into single cells, plated on poly-l-lysine and laminin (Invitrogen)-coated chamber slides in neurobasal medium supplemented with B27 and l-glutamine (Invitrogen) for 24 h. BrdU (10 μM) was added to the medium for 24 h prior to fixation. Cells were fixed in 4% PFA for 10 min at room temperature, incubated in 2 M HCl at 37 °C, and stained as described above using a mouse anti-BrdU antibody (1:100 dilution, Immunotech, Luminy, France).

Differentiation assay

Neurospheres were differentiated onto precoated chamber slides as described above in the presence or absence of 20 ng of bFGF, PDGF-AA and PDGF-BB (provided by Dr. Heldin), or 1 μM STI571 (Novartis, Basel, Switzerland) for 5 days.

Migration assay

Neurospheres were plated and treated in the same manner as described above. Migration was quantified by measuring the extent of migration of cells from neurospheres after 5 days. The average of the four largest distances per neurosphere was divided by the core diameter of the neurosphere, thereby compensating for the different sizes of the individual neurospheres. In each experiment, at least 15 neurospheres were analyzed.

Immunohistochemistry for cultured cells

Neurospheres of WT as well as PDGFRB−/− cells were rinsed and dispersed gently in growth media and attached onto object glasses with the aid of a cytocentrifuge for immunofluorescence staining. Both dispersed and non-dispersed neurospheres were stained in a similar procedure at room temperature. Cells were fixed in 4% PFA/PBS for 10 min, permeabilized in 0.3% Triton X-100 containing 10% goat serum for 30 min, and incubated overnight with primary antibodies. As primary antibodies, a rabbit anti-PDGFRB (1:200; Upstate Biotechnology, Lake Placid, NY), a rabbit anti-phospho-PDGFRB (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), a rat anti-PDGFRB (1:100; ebioscience, San Diego, CA), a mouse anti-PDGFRB-BB (provided by Mochida Co.), a mouse anti-β-tubulin (1:500; Sigma), a mouse anti-MAP2ab (1:500; Sigma), a rabbit anti-GFAP (1:500; Dako), and a mouse anti-O4 (1:50; Chemicon) were used. After incubation with the primary antibody, sections were washed in PBS. As secondary antibodies, we use pCrePac plasmid (provided by Dr. T Yagi, Osaka University, Japan) expressing Cre recombinase to generate PDGFRB-depleted neuroprogenitor cells (PDGFRB−/−). For controls, we used WT neuroprogenitors transfected with pCrePac (control) as well as neuroprogenitors isolated from PDGFRB FL/FL mice (PDGFRB FL/FL). These cells showed almost identical patterns in the analyses we performed. Primary neurospheres obtained from WT and PDGFRB FL/FL mice were dissociated and plated at 5000 cells/ml. Cells were transfected with pCrePac plasmid using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. At 72 h after transfection, 1 μg/ml puromycin was added to the cultures. Fresh medium was added to the cultures at 6 days after transfection, and the medium was changed after another 8 days. Colony formation was monitored over a 4-week period. Several colonies were expanded and screened by PCR. Two to four colonies were expanded from each well. Some colonies showed correct genotypes, whereas others showed partial deletions.

Transfection

We use pCrePac plasmid (provided by Dr. T Yagi, Osaka University, Japan) expressing Cre recombinase to generate PDGFRB-depleted neuroprogenitor cells (PDGFRB−/−). For controls, we used WT neuroprogenitors transfected with pCrePac (control) as well as neuroprogenitors isolated from PDGFRB FL/FL mice (PDGFRB FL/FL). These cells showed almost identical patterns in the analyses we performed. Primary neurospheres obtained from WT and PDGFRB FL/FL mice were dissociated and plated at 5000 cells/ml. Cells were transfected with pCrePac plasmid using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. At 72 h after transfection, 1 μg/ml puromycin was added to the cultures. Fresh medium was added to the cultures at 6 days after transfection, and the medium was changed after another 8 days. Colony formation was monitored over a 4-week period. Several colonies were expanded and screened by PCR. Two to
Alexa-conjugated IgG (anti-rabbit, mouse, or rat) or anti-mouse IgM (1:500; Invitrogen) were used. They were mounted and observed by using a Zeiss fluorescence microscope. As specificity controls, we used oligodendrocyte progenitors and a mouse neuroblastoma cell line expressing only PDGFRα or PDGFRβ and a PDGFR-negative aorta endothelial cell line. Omission of either primary or secondary antibody was performed to exclude non-specific reaction.

Promoter reporter assay

Primary control neurospheres from PDGFRβ+/− mice were dissociated and cells were seeded as above in 24-well plates at a density of 2 × 10^4 cells/well without any growth factor. On the following day, the cells associated and cells were seeded as above in 24-well plates at a density of 2 × 10^4 cells/well. As specificity controls, we used oligodendrocyte progenitors and a mouse neuroblastoma cell line expressing only PDGFRα or PDGFRβ and a PDGFR-negative aorta endothelial cell line. Omission of either primary or secondary antibody was performed to exclude non-specific reaction.

Western blot

Primary neurospheres from wild type mice were lysed and separated by SDS–PAGE using 5–20% of gradient gel, electrobotted onto polyvinylidene difluoride membrane, and incubated in a blocking buffer as described elsewhere (Gao et al., 2005). The membranes were then probed with the respective antibodies: a rabbit anti-PDGFRα (1:1000; Upstate Biotechnology), a rabbit anti-PDGFRα (1:1000; Santa Cruz), a rabbit anti-phospho-PDGFRα (1:1000; Santa Cruz), a rabbit anti-phospho-PDGFRα (1:500; Novus Biologicals, Littleton, CO), a mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000; Chemicon), a mouse anti-Akt (1:300; Santa Cruz), a rabbit anti-phospho-Thr308Akt (1:300; Cell Signaling Technology, Beverly, MA), a rabbit anti-phospho-Ser473Akt (1:300; Cell Signaling), a rabbit anti-phospho-ERK1/2 (1:300; Cell Signaling), a rabbit anti-phospho-Thr202/Tyr204 ERK1/2 (1:300; Cell Signaling), a rabbit anti-GSK3β (1:10000; Cell Signaling), and a rabbit anti-phospho-Ser9GSK3β (1:5000; Cell Signaling) for 24 h at 4 °C. After the membranes had been washed in the buffer without dry milk, the blots were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK). The bands were compared with those of the GAPDH protein, after re-probing of each membrane by the use of a Blot Restore Membrane Rejuvenation Kit (Chemicon).

Statistical analysis

Quantitative data were expressed as means±SEM, and each experiment was repeated at least three times. One-way ANOVA followed by Fisher’s PLSD test and two-way ANOVA followed by Ryan’s method were used for statistical analysis, with p values less than 0.05 considered significant.

Acknowledgments

We thank Dr. Yagi for the pCrepac plasmid, Dr. Heldin for the PDGF ligands, Novartis Pharma for STI157, and Mochida Co. for the PDGF antibody. This work was supported by grants from the Swedish Science Council, the Swedish Cancer Society, the Swedish Children’s Cancer Society, the Hjalmar Svensson Foundation, and the Grants-in-Aid for Scientific Research 17590338 and 16390114 from the Ministry of Education, Science, and Culture of Japan. It was also supported in part by CREST, Japan Science and Technology Agency and by the Scandinavia-Japan Sasakawa Foundation.

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