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Retroviral manipulation of the expression of bone morphogenetic protein receptor Ia by SVZa progenitor cells leads to changes in their p19^{INK4d} expression but not in their neuronal commitment

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Abstract

Bone morphogenetic proteins (BMPs), a group of cytokines in the TGF- β superfamily, have complex regulatory roles in the control of neural proliferation and cell fate decision. In this study, we analyzed the potential role(s) of BMP signaling on the regulation of the proliferation and differentiation of the unique progenitor cells of the neonatal anterior subventricular zone (SVZa). Unlike other progenitor cells of the brain, SVZa progenitor cells have the capacity to divide even though they express a neuronal phenotype. In order to augment or inhibit endogenous BMP signaling, we injected into the neonatal rat SVZa replication-deficient retroviruses encoding for either the wild-type BMP receptor subtype Ia (wt-BMPR-Ia) or a mutated dominant-negative version of BMPR-Ia (dn-BMPR-Ia) in conjunction with a reporter gene, human alkaline phosphatase (AP) and perfused the pups 1, 4 and 7 days post injection. We analyzed whether changing the expression of BMPR-Ia has an effect on the spatial-temporal expression pattern of the cyclin dependent kinase inhibitor, p19^{INK4d}, or on the phenotype of SVZa derived cells. The results of our study confirmed and extended our previous findings that in control (non injected) animals, the rostral migratory stream (RMS), traversed by the SVZa-derived cells en route to the olfactory bulb, exhibits an anterior^{high}-posterior^{low} gradient of p19^{INK4d} expression; p19^{INK4d} expression is essentially absent in the SVZa and highest in the subependymal zone in the middle of the olfactory bulb. However, SVZa progenitor cells encoding the wt-BMPR-Ia gene express p19^{INK4d} within the SVZa, suggesting that the BMPs induce SVZa cells to ectopically undergo cell cycle exit within the SVZa. Furthermore, unlike striatal SVZ progenitor cells, which acquire an astrocytic phenotype when exposed to BMPs, SVZa progenitor cells retain their neuronal commitment under augmented BMP signaling.

Keywords

Bone morphogenetic proteins; Cell cycle; p19^{INK4d}; Progenitor cells; Retrovirus; Rostral migratory stream; Subventricular zone

1. Introduction

Progenitor cells that are located in a discrete region of the anterior part of the neonatal subventricular zone (SVZa; Luskin, 1993) differ in their proliferation and migration characteristics from the rest of the progenitor cells of the CNS. SVZa-derived cells proliferate and migrate to the olfactory bulb along a highly restricted pathway called the rostral migratory stream (RMS), while expressing markers associated with postmitotic neurons (e.g., neuron-specific β -tubulin, MAP-2 and PSA-NCAM) (Menezes et al., 1995). This is in distinct contrast to immature neurons arising from the telencephalic ventricular zone, which become postmitotic before expressing neuronal cell-type specific markers and migrating to their final destinations (Brand and Rakic, 1979; Menezes and Luskin, 1994; Takahashi et al., 1995; Bittman et al., 1997; Kornack and Rakic, 1998). In essence, proliferation and differentiation are concurrent in SVZa-derived cells, whereas proliferation precedes differentiation in the immature neurons of the developing cerebral cortex.

Recent studies in our laboratory have indicated that the unusual proliferation and differentiation characteristics of the SVZa-derived cells can be, in part, attributed to the cyclin dependent kinase inhibitor, p19^{INK4d}, a member of the INK4 family (Coskun and Luskin, 2001). These proteins prevent the phosphorylation of the retinoblastoma (Rb) protein by negatively regulating the cyclin dependent kinases (CDKs), a group of proteins that phosphorylate Rb (Elledge and Harper, 1994; Hirai et al., 1995; Sherr, 1996; Sherr and Roberts, 1999). The hypophosphorylated form of Rb inhibits the cell cycle at the G1 phase, by remaining bound to the transcription factor E2F and preventing cell cycle progression to the S phase (Kato et al., 1993; Lukas et al., 1995; Weinberg, 1995). Unbound E2F is required to activate a set of genes prior to entry into S phase. Withdrawal from the cell cycle at the G1 phase is an obligatory step for a cell to become postmitotic. Despite the overlapping structure and functions of the INK4 family members, there are differences in their spatiotemporal patterns of expression. p18^{INK4c} and p19^{INK4d}, in particular, are expressed during neurogenesis (Zindy et al., 1997, 1997b) and have putative roles in both cerebral cortical and cerebellar development (Watanabe et al., 1998; Zindy et al., 1999). Our recent data has demonstrated that p19^{INK4d} expression is essentially absent in the SVZa, but upregulated as the cells complete their migration to the olfactory bulb (Coskun and Luskin, 2001). Accordingly, nearly all SVZa-derived cells express p19^{INK4d} in the subependymal zone in the middle of the olfactory bulb. It stands to reason, therefore, that increased expression of p19^{INK4d} by the SVZa cells and their progeny leads to cell cycle arrest at the G1 phase in the olfactory bulb.

The extrinsic factors inducing increased p19^{INK4d} expression and cell cycle arrest at G1 by the SVZa-derived cells in the olfactory bulb are unknown. Candidate molecules are the bone morphogenetic proteins (BMPs), a group of cytokines in the TGF- β superfamily, which are expressed in the neonatal RMS (Coskun et al., 2000; Venkatraman et al., 2000). These

proteins and their receptors have complex regulatory roles in neurogenesis. For example, BMP4 is the major dorsalizing signal during neural tube formation, whereas both BMP2 and 4 regulate the fate decisions of neural crest cells (Anderson et al., 1997). BMPs also lead to the cessation of cell proliferation and induction of astrocyte differentiation in cultured progenitor cells from the SVZ surrounding the lateral ventricle adjacent to the striatum (Zhu et al., 1999; Gross et al., 1996). In cell culture systems BMPs have been shown to act directly on members of the CDKIs, such as p21^{CIP1} (Yamato et al., 2000) to induce apoptosis or cell cycle exit. Thus, BMPs play a pivotal role in regulating the cell cycle in various progenitor cell populations.

In order to determine whether BMPs regulate p19^{INK4d} expression by SVZa progenitor cells, we injected into the neonatal SVZa replication-deficient retroviruses encoding for either the wild-type BMP receptor subtype Ia (wt-BMPR-Ia) or a mutated dominant-negative version of BMPR-Ia (dn-BMPR-Ia). Progeny of cells infected with the wt-BMPR-Ia construct express extra copies of the receptor, which augments endogenous BMP signaling. Conversely, cells expressing the dn-BMPR-Ia gene are immune to endogenous BMP signals. Our results, showing that SVZa progenitor cells expressing the wt-BMPR-Ia gene express p19^{INK4d} within the SVZa itself, suggest that the BMPs induce cell cycle exit at the G1 stage. In contrast to the induction of the astrocytic phenotype by the striatal SVZ progenitors following BMP exposure, augmented BMP signaling by SVZa progenitors leads to exclusive expression of neuronal cell-type specific markers. Thus, our findings substantiate the notion that the neonatal SVZa is a unique region in the forebrain containing unipotential progenitors committed to a neuronal lineage.

2. Materials and methods

2.1. Retrovirus injections

In order to obtain neonatal pups of specific gestational ages, Sprague–Dawley rats were mated overnight in our colony and checked for a vaginal plug the following morning. The day of conception was considered to be embryonic day 0 (E0); birth usually occurred on the 21st or 22nd day of gestation. By convention, E22 was considered equivalent to post-natal day 0 (P0) to normalize the ages of the experimental animals.

To determine the effects of BMP signaling on the cell cycle and phenotype of SVZa-derived cells in vivo, two bicistronic recombinant retroviruses encoding the wild-type (wt) or dominant-negative (dn) form of the BMP receptor Ia (BMPR-Ia) along with human alkaline phosphatase (AP) were injected into the SVZa of P1 animals. The bicistronic construct allows for the progeny of the infected cells to be identified by their AP expression.

The injection protocol followed was a modification of the procedure described previously (Luskin, 1993; Zigova et al., 1996; Yang et al., 2000). Each rat received a 2 µl injection of either the wt-BMPR-Ia ($n=12$) or dn-BMPR-Ia ($n=10$). Rat pups were allowed to survive for 1, 4 or 7 days following retrovirus injections and perfused as described below. As a control, litter-mates to those injected with one of the BMPR constructs were injected with a retrovirus encoding for human alkaline phosphatase ($n=5$).

2.2. Tissue processing and immunocytochemistry

At 1, 4 and 7 days following retroviral injection, the rat pups were perfused and processed for immunocytochemical studies to determine the distribution and phenotype of the AP(+) cells. The animals were anesthetized with ether and then perfused transcardially with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The perfused brains were removed, postfixed overnight at 4°C in the same fixative and cryoprotected with 20% sucrose in 0.1 M PBS for 24 h. All brains were embedded in OCT (Miles Inc., IN), frozen with liquid nitrogen, cut in the sagittal plane on a cryostat at 10 µm and mounted on Superfrost Plus slides (Fisher Scientific, PA).

We modified a previously described procedure (Menezes et al., 1995) in order to detect AP (to identify wt- and dn-BMPR-Ia expressing cells) in combination with either neuron-specific type III β -tubulin (to identify neurons), GFAP (to identify astrocytes) and p19^{INK4d}. Sections were rinsed in PBS and kept in blocking serum (1.5% normal goat serum (NGS) and 0.01% Triton X-100 in 0.1 M PBS) for 1 h. All antibodies were diluted with blocking serum at the following dilutions: anti-AP, 1:250; anti β -tubulin, 1:400; anti-GFAP, 1:400; anti-p19^{INK4d}, 1:100. One series of sections were incubated in only anti-AP to clearly visualize the progeny of infected cells. Another set of adjacent sections were double-labeled overnight with anti-AP in combination with TuJ1 (Promega, MI), anti-GFAP (Dako, CA) and anti-p19^{INK4d} (Santa Cruz Research Laboratories, CA). The following day, the sections were rinsed with PBS and incubated in fluorescein- or rhodamine-conjugated species-specific secondary antibodies (Jackson ImmunoResearch Labs, PA) for 1 h at room temperature, each diluted to a final concentration of 1:200 in blocking serum. For consistency, the anti-AP was always visualized with the rhodamine-conjugated secondary antibody. Subsequently, the sections were washed with PBS and coverslipped with Vectashield (Jackson ImmunoResearch Labs).

2.3. Image analysis

Fluorescently labeled sections were examined using a conventional fluorescence microscope (Zeiss Axioscope) or a confocal microscope (Zeiss Axioplan, LSM 510). Confocal images were obtained from a single optical section with a thickness of 2.5 µm by sequential laser scanning at 488 nm (for fluorescein) and 543 nm (for rhodamine). The captured images were processed using Adobe Photoshop software.

3. Results

In order to determine the effects of BMP signaling on neuronal progenitor cells of the SVZa, we injected one of two bicistronic retroviral constructs encoding for either the wild-type (wt) or a dominant-negative (dn) variant of BMP receptor Ia (BMPR-Ia) along with the reporter gene, human alkaline phosphatase (AP), thus allowing immunohistochemical identification of the progeny of infected cells. The phenotype of the wt-BMPR-Ia or dn-BMPR-Ia expressing SVZa-derived cells was determined by double-label immunohistochemistry using anti-AP in conjunction with antibodies to neuronal and astrocyte cell-type specific markers. To ascertain the cell cycle state of the wt- and dn-BMPR-Ia(+) cells, we used an antibody against the cyclin dependent kinase inhibitor p19^{INK4d}, which acts as a G1 phase

checkpoint protein. We recently demonstrated that p19^{INK4d} is expressed in a spatiotemporal gradient by cells of the RMS (Coskun and Luskin, 2001).

The SVZa, a specialized region located at the anterodorsal tip of the lateral ventricle in the forebrain of the neonatal rodent, contains exclusively neuronal progenitor cells (Fig. 1). The RMS serves as a conduit for SVZa-derived cells en route to the olfactory bulb. The progenitor cells of the SVZa are true neuroblasts; they incorporate the cell proliferation marker BrdU while expressing cell-type specific markers associated with postmitotic neurons (Luskin, 1993; Luskin et al., 1997). The density of proliferating cells gradually declines from the SVZa to the subependymal zone. The pattern of expression of p19^{INK4d} along the RMS is the inverse of the gradient of actively dividing cells. To determine whether BMPs provide an extracellular signal that modulates the proliferation and differentiation of the SVZa-derived cells, we analyzed the effects of amplified and inhibited BMP signaling on the proliferation state and the phenotype of the SVZa-derived cells.

3.1. Amplified and inhibited BMP signaling induces opposite effects on p19^{INK4d} expression by SVZa-derived cells in the RMS

Previous studies have demonstrated that there is a gradient of p19^{INK4d} expression along the rostrocaudal extent of the neonatal rodent RMS (Coskun and Luskin, 2001). In this study, we confirmed the findings that there is negligible expression in the SVZa adjacent to the lateral ventricle, whereas virtually all the SVZa-derived cells in the subependymal zone are p19^{INK4d}(+) (Fig. 2A,B). To determine the proliferative state of SVZa progenitor cells transduced with the wt- and dn-BMPR-Ia retrovirus (both of which also encode AP), we examined whether and where AP(+) cells express p19^{INK4d} along the rostrocaudal extent of the RMS. We observed that the wt-BMPR-Ia(+) SVZa progenitor cells express p19^{INK4d} ectopically in the SVZa. This finding was true 1 day (Fig. 2C) as well as 7 days (Fig. 2D) after the injection. In contrast, the dn-BMPR-Ia(+) SVZa progenitor cells do not express p19^{INK4d} 1 day after retroviral injection (Fig. 2E) in the SVZa. Seven days post retroviral injection, the vast majority of the dn-BMPR-Ia(+) cells had migrated to the subependymal zone olfactory bulb. These AP(+) cells, however, were still p19^{INK4d}(-) in the subependymal zone (Fig. 2F), a region where endogenous SVZa-derived cells are uniformly p19^{INK4d}(+). Taken together, our data indicates that BMPs directly regulate the cell cycle checkpoint gene p19^{INK4d}; upregulation of the BMP signals leads to ectopic p19^{INK4d} expression and, conversely, inhibition of BMP signaling eliminates p19^{INK4d} expression.

3.2. SVZa-derived cells expressing either exogenous wt-BMPR-Ia or dn-BMPR-Ia retain their neuronal phenotype throughout the rostrocaudal extent of the RMS

As mentioned previously, when progenitor cells from the neonatal and adult striatal SVZ are exposed to BMPs, astrocytic differentiation is induced (Zhu et al., 1999; Gross et al., 1996). To determine whether BMPs alter the neuronal commitment of the SVZa progenitor cells, we used antibodies against the cell-type specific markers neuron-specific β -tubulin and GFAP along with anti-AP to identify the progeny of the infected cells. In response to either amplified or diminished BMP signaling, the cells encoding wt-BMPR-Ia and the dn-BMPR-Ia universally express neuron-specific type III β -tubulin (Fig. 3). Irrespective of whether we examined the tissue 1 (data not shown) 4 (Fig. 3A–D) or 7 (Fig. 3E–H) days after

retroviral injection, the findings were similar. Moreover, GFAP was not expressed by either wt- or dn-BMPR-Ia(+) SVZa progenitor cells 4 or 7 days after retrovirus injection (Fig. 4A–D). However, there were endogenous GFAP(+) cells in the RMS at these time points (Law et al., 1999). Taken together, our data suggests that the SVZa progenitor cells retain their commitment to the neuronal lineage irrespective of BMP signaling. Furthermore, the neonatal SVZa has a markedly different response to BMPs when compared to progenitor cells from the striatal SVZ.

4. Discussion

The forebrain subventricular zone (SVZ), a layer of cells surrounding the lateral ventricles, emerges in the rodent during late embryogenesis and persists postnatally. In the late embryonic and early postnatal stages, the SVZ progenitors generate primarily glia (Levison and Goldman, 1993; Brock et al., 1998). Many studies, however, have recently demonstrated that the postnatal SVZ is neither exclusively a gliogenic nor a homogeneous structure. Specifically, the progenitor cells in the SVZa, a localized region within the anterior part of the SVZ, give rise exclusively to neurons destined for the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1993; Betarbet et al., 1996). In contrast, the portion of the postnatal SVZ adjacent to the striatum contains multipotent progenitors (Reynolds and Weiss, 1992). Several authors have investigated the actions of cytokines, including BMPs on progenitor cells from the striatal portion of the postnatal SVZ in vitro. BMPs, in particular, have been shown to induce astrocyte differentiation and downregulate the differentiation of neurons and oligodendrocytes (Zhu et al., 1999, 1999b). BMPs also promote astrocyte differentiation and down-regulate oligodendrocyte formation by bipotent O2A cells (Mabie et al., 1997). In contrast to the stated actions of BMPs on progenitors from the postnatal striatal SVZ, our results indicate that progenitors from the neonatal SVZa remain committed to a neuronal lineage (Figs. 3 and 4). Similar to the reported anti-proliferative effects of the BMPs on postnatal striatal SVZ progenitors, SVZa progenitors also respond to BMP signaling by exiting the cell cycle, evidenced by their expression of the CDKI, p19^{INK4d} (Fig. 2).

We took advantage of retroviral-mediated gene transfer to deliver wild-type or dominant-negative BMP receptor constructs in vivo, to augment and diminish endogenous BMP signaling, respectively. We then examined the phenotype of the SVZa progenitor cells expressing the retroviral gene products. Results from this study demonstrate that SVZa progenitor cells retain a neuronal phenotype in response to BMP signaling. Our data indicate that (1) progenitor cells in the neonatal SVZa are committed to a neuronal lineage and remain so irrespective of the level of BMP signaling, and (2) the SVZ is a mosaic in which the SVZa is a unique subdivision. Findings in this study concur with reported actions of BMPs on neural crest progenitor cells that give rise exclusively to neurons. During neural crest development, BMPs have been shown to induce progenitor cells to give rise to postmitotic, noradrenergic neurons (Anderson et al., 1997; Lo et al., 1997, 1998; Reissmann et al., 1996). Conversely, BMPs induce astrocyte differentiation of multipotent or glial restricted precursors from the postnatal SVZ, as well as the embryonic spinal cord. Moreover, neuronally committed progenitor cells, such as the SVZa or the recently described spinal cord NRP cells (Kalyani et al., 1998), respond to BMP signaling by

retaining their neuronal phenotype, but exiting the cell cycle. Therefore, the actions of BMPs in the developing nervous system are varied and depend on the differentiation state of the progenitor cells they act upon (see Mehler et al., 2000).

During the development of both the central and peripheral nervous systems, proliferation usually precedes differentiation. However, SVZa-derived cells constitute a notable exception to this sequence of events. Previous studies from our laboratory have indicated that proliferation and differentiation occur concurrently in the RMS (Menezes et al., 1995). Initially, we demonstrated that SVZa-derived cells in the RMS incorporate the cell proliferation marker BrdU, even though they express a neuronal phenotype. More recently, we have shown that there is an anterior^{high}-posterior^{low} gradient of the CKDI p19^{INK4d} expression along the RMS (Fig. 2A,B). The distribution of p19^{INK4d} immunoreactivity is the reverse of BrdU incorporation patterns; p19^{INK4d} expression is highest and BrdU incorporation is minimal in the subependymal zone of the olfactory bulb, suggesting that an increasing proportion of SVZa-derived cells withdraw from the cell cycle as they approach the olfactory bulb. In order to determine whether BMPs govern this unusual pattern of proliferation and differentiation characterizing the cells of the SVZa, we examined the effects of amplified and diminished BMP signaling on the expression of p19^{INK4d} using wt-BMPRIa and dn-BMPRIa retroviruses, respectively. Unlike the endogenous cells, the SVZa cells transfected with the wt-BMPRIa gene uniformly express p19^{INK4d} (Fig. 2C,D). Conversely, p19^{INK4d} is virtually absent from dn-BMPRIa(+) SVZa progenitor cells even in the subependymal zone of the olfactory bulb, where a majority of the endogenous SVZa-derived cells are p19^{INK4d}(+). Our results therefore demonstrate that BMP signaling leads to the cessation of proliferation by SVZa progenitor cells and this effect is mediated by the CDKI protein p19^{INK4d}. We previously concluded that SVZa progenitor cells repeatedly downregulate and re-express p19^{INK4d} while traversing the pathway enroute to the olfactory bulb. Findings from the current study showing ectopic expression of p19^{INK4d} by wt-BMPRIa SVZa cells does not entirely exclude the possibility that these cells do not downregulate p19^{INK4d} expression prior to a subsequent cell division. However, this seems unlikely because p19^{INK4d} expression was also evident in the SVZa 7 days after wt-BMPRIa retrovirus injection. Moreover, since the dn-BMPRIa SVZa-derived cells do not express p19^{INK4d} anywhere in the pathway, endogenous BMP signaling may be involved in the cyclic downregulation and re-expression of p19^{INK4d} observed in the pathway. Future studies are designed to resolve this issue.

Many studies have shown that TGF- β proteins, a superfamily to which the BMPs belong, have direct effects on the CIP/KIP as well as INK4 family of proteins in vitro, leading to cell cycle arrest and/or apoptosis (Hannon and Beach, 1994; Florenes et al., 1996; Sandhu et al., 1997; Kamesaki et al., 1998; Suzuki et al., 1999). Recently, BMPs themselves have been shown to upregulate p21^{CIP1} expression in vitro (Yamato et al., 2000), thereby inducing cell cycle arrest. Ours is the first study to demonstrate the direct effect of BMPs on a cell cycle regulatory protein during neurogenesis in vivo. Although the underlying molecular mechanisms responsible for p19^{INK4d} upregulation have not been elucidated in this study, we will investigate them in the future.

As mentioned above, BMPs have diverse and seemingly conflicting roles during neurogenesis. This is evident even within a restricted and well-defined region, such as the postnatal SVZ, where BMPs have been shown to promote acquisition of both neuronal and astrocytic phenotypes. Further studies are underway to characterize the phenotype and migratory patterns of the wt-BMPR-Ia and dn-BMPR-Ia expressing SVZa progenitor cells to better understand the role BMPs play in the proliferation, differentiation and migration of SVZa-derived cells.

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References

- Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*. 1993; 11:173–189. [PubMed: 8338665]
- Menezes JR, Smith CM, Nelson KC, Luskin MB. The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol Cell Neurosci*. 1995; 6:496–508. [PubMed: 8742267]
- Brand S, Rakic P. Genesis of the primate neostriatum: [3H]thymidine autoradiographic analysis of the time of neuron origin in the rhesus monkey. *Neuroscience*. 1979; 4:767–778. [PubMed: 113693]
- Menezes JR, Luskin MB. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. *J Neurosci*. 1994; 14:5399–5416. [PubMed: 8083744]
- Takahashi T, Nowakowski RS, Caviness VS Jr. The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J Neurosci*. 1995; 15:6046–6057. [PubMed: 7666188]
- Bittman K, Owens DF, Kriegstein AR, LoTurco JJ. Cell coupling and uncoupling in the ventricular zone of developing neocortex. *J Neurosci*. 1997; 17:7037–7044. [PubMed: 9278539]
- Kornack DR, Rakic P. Changes in cell-cycle kinetics during the development and evolution of primate neocortex. *Proc Natl Acad Sci USA*. 1998; 95:1242–1246. [PubMed: 9448316]
- Coskun V, Luskin MB. The expression pattern of the cell cycle inhibitor p¹⁹INK4d in the rat embryonic telencephalon compared to the neonatal anterior subventricular zone. *J Soc Neurosci Abst*. 2001 in press.
- Elledge SJ, Harper JW. Cdk inhibitors: on the threshold of checkpoints and development. *Curr Opin Cell Biol*. 1994; 6:847–852. [PubMed: 7880532]
- Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol*. 1995; 15:2672–2681. [PubMed: 7739547]
- Sherr CJ. Cancer cell cycles. *Science*. 1996; 274:1672–1677. [PubMed: 8939849]
- Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*. 1999; 13:1501–1512. [PubMed: 10385618]
- Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev*. 1993; 7:331–342. [PubMed: 8449399]
- Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, Peters G, Bartek J. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature*. 1995; 375:503–506. [PubMed: 7777060]
- Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995; 81:323–330. [PubMed: 7736585]

- Zindy F, Soares H, Herzog KH, Morgan J, Sherr CJ, Roussel MF. Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development. *Cell Growth Differ.* 1997; 8:1139–1150. [PubMed: 9372237]
- Zindy F, Quelle DE, Roussel MF, Sherr CJ. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene.* 1997b; 15:203–211. [PubMed: 9244355]
- Watanabe G, Pena P, Shambaugh GE III, Haines GK III, Pestell RG. Regulation of cyclin dependent kinase inhibitor proteins during neonatal cerebella development. *Brain Res Dev Brain Res.* 1998; 108:77–87.
- Zindy F, Cunningham JJ, Sherr CJ, Jugal S, Smeyne RJ, Roussel MF. Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc Natl Acad Sci USA.* 1999; 96:13462–13467. [PubMed: 10557343]
- Coskun V, Venkatraman G, Noyes CP, Luskin MB. Bone morphogenetic proteins regulate the proliferation SVZa progenitor cells by modulating the expression of p19INK4d. *Soc Neurosci Abst.* 2000; 26:1092.
- Venkatraman G, Coskun V, Wu Y, Ciombor KJ, Rao MS, Luskin MB. SVZa progenitor cells retain their commitment to the neuronal lineage in response to amplified BMP signaling. *Soc Neurosci Abst.* 2000; 26:1092.
- Anderson D, Groves A, Lo L, Ma Q, Rao M, Shah NM, Sommer L. Cell lineage determination and the control of neuronal identity in the neural crest. *Cold Spring Harbour Symposium Quant Biol.* 1997; 62:493–504.
- Zhu G, Mehler MF, Zhao J, Yu Yung S, Kessler JA. Sonic hedgehog and BMP2 exert opposing actions on proliferation and differentiation of embryonic neural progenitor cells. *Dev Biol.* 1999; 215:118–129. [PubMed: 10525354]
- Gross R, Mehler M, Mabie P, Zang Z, Santschi L, Kessler J. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron.* 1996; 17:595–606. [PubMed: 8893018]
- Yamato K, Hashimoto S, Okahashi N, Ishisaki A, Nonaka K, Koseki T, Kizaki M, Ikeda Y, Nishihara T. Dissociation of bone morphogenetic protein-mediated growth arrest and apoptosis of mouse B cells by HPV-16 E6/E7. *Exp Cell Res.* 2000; 257:198–205. [PubMed: 10854068]
- Zigova T, Betarbet R, Soteres BJ, Brock S, Bakay RA, Luskin MB. A comparison of the patterns of migration and the destinations of homotopically transplanted neonatal sub-ventricular zone cells and heterotopically transplanted telencephalic ventricular zone cells. *Dev Biol.* 1996; 173:459–474. [PubMed: 8606005]
- Yang H, Mujtaba T, Venkatraman G, Wu YY, Rao MS, Luskin MB. Region-specific differentiation of neural tube-derived neuronal restricted progenitor cells after heterotopic transplantation. *Proc Natl Acad Sci USA.* 2000; 97:13366–13371. [PubMed: 11087876]
- Luskin MB, Zigova T, Soteres BJ, Stewart RR. Neuronal progenitor cells derived from the anterior subventricular zone of the neonatal rat forebrain continue to proliferate in vitro and express a neuronal phenotype. *Mol Cell Neurosci.* 1997; 8:351–366. [PubMed: 9073397]
- Law AK, Pencez V, Buck CR, Luskin MB. Neurogenesis and neuronal migration in the neonatal rat forebrain anterior subventricular zone do not require GFAP-positive astrocytes. *Dev Biol.* 1999; 216:622–34. [PubMed: 10642797]
- Levison SW, Goldman JE. Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. *Neuron.* 1993; 10:201–212. [PubMed: 8439409]
- Brock SC, Bonsall J, Luskin MB. The neuronal progenitor cells of the forebrain subventricular zone: intrinsic properties in vitro and following transplantation. *Methods.* 1998; 16:268–281. [PubMed: 10071066]
- Lois C, Alvarez-Buylla A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci USA.* 1993; 90:2074–2077. [PubMed: 8446631]
- Betarbet R, Zigova T, Bakay RA, Luskin MB. Dopaminergic and GABAergic interneurons of the olfactory bulb are derived from the neonatal subventricular zone. *Int J Dev Neurosci.* 1996; 14:921–930. [PubMed: 9010735]

- Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. 1992; 255:1707–1710. [PubMed: 1553558]
- Zhu G, Mehler MF, Mabie PC, Kessler JA. Developmental changes in progenitor cell responsiveness to cytokines. *J Neurosci Res*. 1999b; 56:131–145. [PubMed: 10494102]
- Mabie PC, Mehler MF, Marmur R, Papavasiliou A, Song Q, Kessler JA. Bone morphogenetic proteins induce astroglial differentiation of oligodendroglial-astroglial progenitor cells. *J Neurosci*. 1997; 17:4112–4120. [PubMed: 9151728]
- Lo L, Sommer L, Anderson D. Mash-1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. *Curr Biol*. 1997; 7:440–450. [PubMed: 9197246]
- Lo L, Tiveron MC, Anderson D. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. *Development*. 1998; 125:609–620. [PubMed: 9435282]
- Reissmann E, Ernsberger U, Francis-West P, Reuger D, Brickell P, Rohrer H. Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development*. 1996; 122:2079–2088. [PubMed: 8681789]
- Kalyani AJ, Piper D, Mujtaba T, Lucero MT, Rao MS. Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. *J Neurosci*. 1998; 18:7856–7868. [PubMed: 9742154]
- Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA. Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. *Dev Neurosci*. 2000; 22:74–85. [PubMed: 10657700]
- Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature*. 1994; 371:257–261. [PubMed: 8078588]
- Florenes VA, Bhattacharya N, Bani MR, Ben-David Y, Kerbel RS, Slingerland JM. TGF-beta mediated G1 arrest in a human melanoma cell line lacking p15INK4B: evidence for cooperation between p21Cip1/WAF1 and p27Kip1. *Oncogene*. 1996; 13:2447–2457. [PubMed: 8957087]
- Sandhu C, Garbe J, Bhattacharya N, Daksis J, Pan CH, Yaswen P, Koh J, Slingerland JM, Stampfer MR. Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. *Mol Cell Biol*. 1997; 17:2458–2467. [PubMed: 9111314]
- Kamesaki H, Nishizawa K, Michaud GY, Cossman J, Kiyono T. TGF-beta 1 induces the cyclin-dependent kinase inhibitor p27Kip1 mRNA and protein in murine B cells. *J Immunol*. 1998; 160:770–777. [PubMed: 9551912]
- Suzuki T, Narita T, Uchida-Toita M, Yoshida M. Down-regulation of the INK4 family of cyclin-dependent kinase inhibitors by tax protein of HTLV-1 through two distinct mechanisms. *Virology*. 1999; 259:384–391. [PubMed: 10388662]

Fig. 1.

Schematic representation of a parasagittal view of the neonatal rat forebrain depicting the pathway traversed by the SVZa-derived cells. Neuronal progenitor cells, which are located within a distinct region of the anterior part of the postnatal subventricular zone (SVZa, solid black circle), proliferate and migrate along a highly restricted pathway, the rostral migratory stream (RMS, shaded region rostral to open arrowheads), to the olfactory bulb. After the SVZa-derived cells reach the subependymal zone in the middle of the olfactory bulb, they migrate radially to their final destinations in the overlying granule cell and glomerular layers, where they differentiate into interneurons. The border between the SVZa and the thinner gliogenic posterior portion of the SVZ is indicated by open arrowheads and the caudal border of the olfactory bulb is denoted by closed arrowheads. Abbreviations: A, anterior; AOB, accessory olfactory bulb; CC, corpus callosum; CTX, cerebral cortex; D, dorsal; epl, external plexiform layer; gcl, granule cell layer; gl, glomerular layer; hl, horizontal limb of the RMS; LV, lateral ventricle; mcl, mitral cell layer; OB, olfactory bulb; onl, olfactory nerve layer; sez, subependymal zone; SVZa, anterior part of the neonatal subventricular zone; vl, vertical limb of the RMS.

Fig. 2. p19^{INK4d} expression by the cells of the rostral migratory stream is differentially regulated by amplified and inhibited BMP signaling. (A,B) Representative confocal images of the P2 SVZa (A) and subependymal zone (B) stained with an antibody to p19^{INK4d}, recognized by a fluorescein-conjugated secondary antibody (green). Only negligible amounts of the CDK inhibitor p19^{INK4d} are expressed by the SVZa progenitor cells at their site of generation. Note that the cells of the ependymal layer exhibit intense p19^{INK4d} immunoreactivity, consistent with their very low rate of proliferation. Almost all the cells in the subependymal zone of the olfactory bulb express p19^{INK4d} unlike the progenitor cells of the SVZa. (C–F) Representative confocal images obtained from P2 (C,D) and P8 (E,F) rostral migratory stream of pups that were injected with a retrovirus encoding either the wt-BMPR-Ia (C,E) or dn-BMPR-Ia (D,F). The sections were stained with antibodies against alkaline phosphatase (AP), recognized by a rhodamine-conjugated secondary antibody (red) and p19^{INK4d}, recognized by a fluorescein-conjugated secondary antibody (green) to identify the progeny of virally transduced SVZa progenitor cells and their cell cycle state, respectively. In contrast to the expression pattern of p19^{INK4d} in control animals (A,B), SVZa-derived cells encoding the wt-BMPR-Ia express p19^{INK4d} in the SVZa 1 day post retrovirus injection (C). Furthermore, there is persistent expression of p19^{INK4d} in the SVZa even 7 days after the injection of retrovirus (E). Arrows point to representative p19^{INK4d}(+)/AP(+) cells. Conversely, cells in the SVZa encoding the dn-BMPR-Ia gene do not express p19^{INK4d} in the SVZa 1 day post injection (D). Arrowheads point to the AP(+)/p19^{INK4d}(–) cells. Seven days after dn-BMPR-Ia retrovirus injection, AP(+)/p19^{INK4d}(–), SVZa-derived cells were

found mainly in the subependymal zone adjacent to uninfected p19^{INK4d}(+)/AP(−) cells in the subependymal zone (F). However, a few AP(+)/p19^{INK4d}(+) cells were detected in the subependymal zone (arrows). Dashed lines demarcate the border between the SVZa and the overlying corpus callosum. Abbreviations: CC, corpus callosum; dn, dominant-negative BMPR; ep, ependymal layer; LV, lateral ventricle; sez, subependymal zone; SVZa, anterior part of the neonatal subventricular zone; wt, wild-type BMPR. Scale bar = 20 μm.

Fig. 3.

Throughout the rostrocaudal extent of the RMS, SVZa-derived cells encoding either the wt-BMPR-Ia or dn-BMPR-Ia gene retain their neuronal phenotype. (A–H). Representative confocal photomicrographs from the P5 (A–D) and P8 (E–H) rat forebrain double-labeled with antibodies to AP recognized by a rhodamine-conjugated secondary antibody, (red) and with the neuron-specific antibody TuJ1 recognized by a fluorescein-conjugated secondary antibody, (green). (A,B) and (E,F), respectively show identical sections from the SVZa individually visualized with a rhodamine (A,E) or a fluorescein (B,F) filter demonstrate SVZa progenitors expressing the wt-BMPR-Ia gene (arrows) at both 4 (A,B) and 7 days (E,F) following the retrovirus injections at P1. Note that all of the AP(+) SVZa-derived cells are also TuJ1(+) (corresponding arrows). (C,D) and (G,H), respectively show dn-BMPR-Ia expressing SVZa-derived cells located in the hIRMS (C,D) and subependymal zone (G,H) 4 and 7 days after the injections at P1. Note that the AP(+) dn-BMPR-Ia expressing SVZa-derived cells are also TuJ1(+) (corresponding arrows). Collectively, regardless of whether SVZa-derived cells express the wt- or dn-BMPR-Ia, they retained their neuronal commitment, as indicated by the AP(+)/TuJ1(+) cells found throughout the rostrocaudal extent of the RMS (arrows). As expected, the uninfected TuJ1(+) SVZa-derived cells along the RMS were AP(–) (arrowheads). Abbreviations: CC, corpus callosum; dn, dominant-negative BMPR-Ia; hIRMS, horizontal limb of the RMS; sez, subependymal zone; SVZa,

anterior part of the neonatal subventricular zone; wt, wild-type BMPR-Ia. Scale bar = 20 μ m (A–D), 40 μ m (E–F).

Fig. 4.

SVZa-derived cells transduced with either the wt-BMPR-Ia or dn-BMPR-Ia retrovirus do not express glial fibrillary acidic protein (GFAP). (A–D) Representative confocal photomicrographs of sections from the P8 SVZa (A,B) and subependymal zone (C,D) stained with antibodies to alkaline phosphatase (AP), recognized with a rhodamine-conjugated secondary antibody (red) to identify the transduced cells and with an antibody against GFAP, recognized with a fluorescein-conjugated secondary antibody (green). Identical sections visualized with a rhodamine (A,C) or a fluorescein (B,D) filter show that the SVZa-derived cells expressing the wt-BMPR gene (A,B) or the dn-BMPR gene (C,D) do not co-localize the astrocytic marker GFAP. This labeling pattern indicates that amplification or inhibition of BMP signaling does not alter the neuronal commitment of SVZa-derived cells. In (A,C) arrows designate AP(+) SVZa-derived cells, whereas in (B,D) arrowheads point to uninfected GFAP(+) glial cells in the corpus callosum and subependymal zone, respectively. The dashed line indicates the border between the SVZa and the overlying corpus callosum. The inset in C shows a representative AP(+) SVZa-derived cell with a leading and trailing process. Abbreviations: CC, corpus callosum; dn, dominant-negative BMPR; sez, subependymal zone; SVZa, anterior part of the neonatal subventricular zone; wt, wild-type BMPR. Scale bar = 20 μm.