The Cerebrospinal Fluid Provides a Proliferative Niche for Neural Progenitor Cells

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SUMMARY

Cortical development depends on the active integration of cell-autonomous and extrinsic cues, but the coordination of these processes is poorly understood. Here, we show that the apical complex protein Pals1 and Pten have opposing roles in localizing the Igf1R to the apical, ventricular domain of cerebral cortical progenitor cells. We found that the cerebrospinal fluid (CSF), which contacts this apical domain, has an age-dependent effect on proliferation, much of which is attributable to Igf2, but that CSF contains other signaling activities as well. CSF samples from patients with glioblastoma multiforme show elevated Igf2 and stimulate stem cell proliferation in an Igf2-dependent manner. Together, our findings demonstrate that the apical complex couples intrinsic and extrinsic signaling, enabling progenitors to sense and respond appropriately to diffusible CSF-borne signals distributed widely throughout the brain. The temporal control of CSF composition may have critical relevance to normal development and pathological conditions.

INTRODUCTION

Neural development involves a dynamic interplay between cell autonomous and diffusible extracellular signals that regulate symmetric and asymmetric division of progenitor cells (Johansson et al., 2010). In mammalian neural progenitors, homologs of C. elegans and Drosophila polarity proteins, including Par3 (partitioning defective protein 3) and Pals1 (protein associated with Lin 7), assemble as apical complexes that play essential roles in regulating self-renewal and cell fate (Margolis and Borg, 2005). The unequal distribution of apical surface components during mitosis is a key determinant of daughter cell fate in C. elegans and Drosophila (Fishell and Kriegstein, 2003; Kempphues, 2000; Siller and Doe, 2005; Wodarz, 2005). Recently, mammalian Par3 was shown to promote asymmetric cell division by specifying differential Notch signaling in radial glial daughter cells (Bultje et al., 2009), suggesting that the inheritance of the apical complex guides progenitor responses to proliferative signals as well.

Secreted signals can act at a distance to guide decisions governing progenitor proliferation and cell fate (Johansson et al., 2010), but little is known of how secreted signals interact with cell-autonomous ones. Insulin-like growth factor 1 (Igf1) promotes progenitor proliferation (Hodge et al., 2004; Popken et al., 2004). Insulin/Igf1 signaling is regulated by E-cadherin in keratinocytes (Vasioukhin et al., 2001) and β-catenin in oligodendrocyte progenitors (Ye et al., 2010), suggesting that cell polarity proteins govern cellular responses to extrinsic cues.

Direct interactions between Par3 and Pten (phosphatase and tensin homolog) (Feng et al., 2008; Pinal et al., 2006; von Stein et al., 2005; Wu et al., 2007) suggest that the apical complex interacts with growth factor signaling pathways. Indeed, disrupting the apical complex via Pals1 leads to attenuated pS6 signaling, premature cell cycle exit, and rapid cell death, resulting in the absence of nearly the entire cerebral cortex (Kim et al., 2010). In turn, Pals1-deficiency can be partially rescued by concomitant activation of mTOR (mammalian target of rapamycin) (Kim et al., 2010), a downstream effector of growth factor signaling. Growth factor signaling, in particular via the type 1 Igf receptor (Igf1R), mediates powerful, age-dependent effects on the development and maintenance of many organ systems.
including the brain through the regulation of progenitor cell division (Baker et al., 1993; Hodge et al., 2004; Liu et al., 2009; Popken et al., 2004; Randhawa and Cohen, 2005). Nevertheless, the mechanisms coordinating the availability of Igf ligands to cortical progenitor cells have remained unclear.

Though vascular sources of secreted proliferative signals are well characterized (Palmer et al., 2000; Shen et al., 2004, 2008; Tavazoie et al., 2008), the apical surfaces of early cortical precursors and their primary cilia do not approximate blood vessels but instead directly contact the cerebrospinal fluid (CSF) (Fuchs and Schwark, 2004; Kim et al., 2010), suggesting that secreted factors may interact with progenitor cells at this interface. The CSF proteome shows a complex and dynamic pattern of protein expression (Dziegielewksa et al., 1981; Parada et al., 2005; Zappaterra et al., 2007), suggesting important roles beyond provision of a fluid cushion for the central nervous system and maintenance of extracellular ionic balance. The CSF has recently been implicated in carrying secreted proteins in several contexts, including Fgf2 to midbrain progenitors (Martin et al., 2006), Sonic hedgehog to cerebellar progenitors (Huang et al., 2010) and Slit guidance of neuroblasts in adult brain (Sawamoto et al., 2006). Regulation of cerebral cortical progenitor cells by growth factors distributed in the lateral ventricular CSF would provide potentially global control over cerebral cortical neurogenesis, but this hypothesis has not been examined.

Here, we show that the apical complex couples autonomous regulation of progenitor proliferation to CSF-borne signals in the developing cerebral cortex. Pals1 and Pten interact genetically to regulate cerebral cortical size and progenitor proliferation and have opposing roles in localizing the Igf1R to the apical domain of cortical progenitors. Apically localized Igf1Rs respond to CSF-borne Igf ligands, particularly Fgf2, and CSF regulates cortical progenitor proliferation in an Fgf2-dependent fashion. Finally, CSF Fgf2 concentration is elevated in patients with malignant glioblastoma, suggesting that CSF proteins may regulate CNS tumorigenesis. Our findings suggest that the apical complex couples autonomous and extrinsic signaling in cerebral cortical progenitors, enabling these cells to respond appropriately to diffusible CSF-borne signals that regulate cortical neural stem cells during development and disease.

RESULTS

Genetic Interactions of Pals1 and Pten at the Apical Surface Region

Since Pals1 loss disrupts growth factor signaling and cortical development (Kim et al., 2010), we looked for potential interactions of Pals1 with other regulators of growth factor signaling and found genetic interactions between Pals1 and Pten (Groszer et al., 2001). Cerebral cortex-specific deletion of Pals1 was achieved by crossing mice with a conditional Pals1 allele (Pals1floxtloxP) (Kim et al., 2010) with mice carrying Emx1-promoter-driven Cre recombinase (Emx1Cre+/−) (Gorski et al., 2002). Pals1floxtloxP/Emx1Cre+/− mice lacked nearly the entire cortical structure due to premature cell cycle exit and cell death (Kim et al., 2010), with heterozygotes having an intermediate phenotype (Figure 1A). In contrast, Pten deficiency, obtained by crossing PtenloxP/loxP mice (Groszer et al., 2001) with either Emx1Cre+/− or NestinCre+/− mice, resulted in cortical hyperplasia arising from excessive and extended proliferation of apical progenitors (Figure 1A; see Figures S1A–S1E available online; Groszer et al., 2001). While the broadest groupings of cells were preserved in Pten mutants, the cortical plate was disorganized across its entire radial extent (Figures S1A–S1C). No phenotypic abnormalities were observed in either heterozygous PtenloxP/+/NestinCre−/− mice or in PtenloxP/loxP/NestinCre−/− littermate controls (Figure S1A and data not shown). Conditional deletion of Pten in the Pals1loxP/+, Emx1Cre+/− mice resulted in an almost normal cortical size (Figure 1A). Histological analyses of Pals1loxP/+/Emx1Cre+/− mice or PtenloxP/+/Pals1loxP+/−/Emx1Cre+/− mice revealed a severely disrupted laminar organization of the dorsomedial cortex (Figure 1B; Kim et al., 2010). Double mutants showed a relatively normal organization of the marginal zone (Figure 1B), consistent with a genetic interaction between the apical complex and Pten. The expression of apical complex components, especially Cdc42, were abnormal in Pten cortex (Figure S1F and data not shown). The proportion of proliferative progenitor cells marked by Ki67-positive staining cells was greater in the double mutant cortex compared to conditional Pals1 heterozygotes (Figure 1C) and brain size was also more normal by embryonic day (E) 15.5 (Figures S1G and S1H). Proportions of early-born neurons marked by Tbr1 and Ctip2 were also more normal in the PtenloxP/+, Pals1loxP+/−/Emx1Cre+/− mice than in either Pals1 or Pten mutants alone (Figure 1D and data not shown). However, cells in the double mutant brain appeared irregular in size and lamination (Figure 1D), a finding consistent with roles for Pten in the regulation of cell size and polarity (Figure S1C; Challoub et al., 2009; Groszer et al., 2001) and with a role for Pten downstream of the apical complex.

The genetic interaction between Pals1 and Pten and the decreased proliferation of progenitors and prominent cell death in Pals1 mutants (Kim et al., 2010) prompted us to test whether the apical complex interacts with Igf1R signaling, since Igfs play a prominent role in cell cycle kinetics of cortical progenitors, cell survival, and brain size (Hodge et al., 2004; Liu et al., 2009; Popken et al., 2004; Schubert et al., 2003). The Igf1R, which binds both Igf1 and Igf2, mediates the proliferative response to Igf signaling (Weber et al., 1992). Surprisingly, Igf1R was enriched in cortical progenitors at the apical, ventricular surface, interdigitating with b-catenin (Figures 2A–2D), suggesting the apical region as the likely site for binding of Igf1R ligand. Apical Igf1R expression was strikingly decreased in Pals1loxP/loxP/Emx1Cre+/− mice (Figure 2E). By contrast in the absence of Pten, Igf1R immunoreactivity demonstrated a considerable basal-lateral spread in clusters of radial glia (Figure 2F and data not shown). Analyses of downstream signaling events, using a specific antibody against the phosphorylated form of Rsk substrate S6 ribosomal protein (phospho-S6rp), revealed an apical pattern of activity within control brains (Figure 2G). In contrast in Pten mutants, phospho-S6rp showed a broad distribution across the cortical tissue, with many robust phospho-S6rp-positive cells extending basally away from the lateral ventricle (Figure 2G). While the majority of cells positive for Igf1R were clearly apical progenitors, some upregulation of Igf1R in basal progenitors is possible. Though we cannot rule
out that Pals1 and Pten could function independently to regulate Igf signaling and cortical growth, we interpret our data to suggest that within the cortical ventricular zone, Pals1 and Pten spatially restrict IgfR expression and Igf signaling to the apical membrane domain.

Loss and gain of Igf signaling in mutant mice produced phenotypes similar to those seen when apical complex signaling is disrupted. Mice with Igf1R deficiency limited to neural precursors (Igf1R$^{loxP/loxP}$.NestinCre$^{+/−}$) were microcephalic (Figure 2H–2J; Kappeler et al., 2008; Liu et al., 2009) and had a reduced frequency of phospho-Histone H3 (PH3, a marker of cell division) proliferative progenitors in the ventricular zone (PH3-positive cells/100 μm VZ ± SEM at E16.5: control, 2.9 ± 0.3; Igf1R$^{loxP/loxP}$.NestinCre$^{+/−}$, 1.7 ± 0.1; unpaired t test, p < 0.01; n = 4 and n = 3, respectively). We did not observe differences in progenitor cell survival at the ventricular zone in these mice as assessed by cleaved caspase 3 (CC3) immunoreactivity (data not shown). Conversely, mice with increased Igf activity (Igf1 expressed from the human GFAP promoter) were macrocephalic (data not shown; Ye et al., 2004) and had increased proliferative progenitors at the ventricular surface (PH3-positive cells/100 μm VZ ± SEM at E18.5: control, 0.9 ± 0.08; Igf1.Tg, 1.2 ± 0.07; unpaired t test, p < 0.05, n = 3 and n = 4, respectively). Together with published work demonstrating that Insulin receptor substrate 2 (Irs2) deletion leads to microcephaly (Schubert et al., 2003), these data suggest that Igf signaling in cortical progenitors, facilitated at the apical surface via Pals1 and an intact apical complex, regulates cortical development.

**CSF-Borne Igf Signaling**

The normal apical localization of the Igf1R, and the fact that we did not observe Igf1 or Igf2 mRNA in neural progenitor cells by in situ hybridization (Figures 3A, 3B, and data not shown; Ayer-le Lievre et al., 1991), suggested that progenitor cells may be exposed to Igfs derived from the lateral ventricle CSF. We confirmed the presence of Igf2 in an unbiased tandem mass
spectrometry (LC-MS/MS) analysis of CSF (Table S1; Binoux et al., 1986) and detected Igf1 in CSF by ELISA (E14 CSF [Igf1], 72.2 ng/ml, n = 2; E17 CSF [Igf1], 69.6 ng/ml; adult CSF [Igf1], 68.8 ng/ml, n = 3). Igf1 expression in the CSF remained stable across the ages sampled (see above). In contrast, expression of Igf2 in rat CSF was temporally dynamic; it peaked during periods of neurogenesis and declined in adulthood (Figure 3C). High levels of Igf2 mRNA expression by the choroid plexus suggested this as a source of CSF Igf2 (Figure 3B), and quantitative PCR revealed that rat choroid plexus expressed 10.7-fold more Igf2 than its cortical counterpart at E17 (data not shown). We confirmed that Igf2 mRNA was also expressed in vascular endothelial cells, and leptomeninges in the rat embryo at E14 and E17 as well as pericytes at E17 (Figures 3A, 3B, and data not shown; Bondy et al., 1992; Dugas et al., 2008; Stylianopoulou et al., 1988), suggesting that extrachoroidal sources of Igf2 may contribute to CSF-Igf2 content as well. Immunogold labeling revealed Igf2 binding to progenitors along the apical, ventricular surface (Figure 3D). Moreover, Igf2 binding to progenitors was highly enriched along primary cilia (Figure 3E; Cohen et al., 1988). We did not observe enriched Igf2 binding beyond the apical surface of ventricular zone progenitor cells (data not shown). Thus, the robust expression of Igf2 by the choroid plexus and the apical binding of Igf2 to progenitors along the ventricular zone strongly suggest that the CSF distributes choroid plexus secreted Igf2 to cortical progenitor cells.

Purified rat E17 CSF directly stimulated Igf1R mediated signaling activity, reflected by Igf1Rβ phosphorylation as well as phosphorylation of Akt and MAPK (Figure 3G), two downstream targets of Igf signaling as well as other growth factors that may be present in CSF. Igf2 treatment by itself induced Igf
signaling similar to embryonic CSF (Figure 3G). Igf2 binding to progenitors, the localization of the Igf1R, its phosphorylation, as well as the phosphorylation of its downstream targets Akt and MAPK in response to CSF, strongly suggest that the CSF is a primary source of Igf ligands for cerebral cortical neuroepithelial cells, although additional sources cannot be completely excluded.

We next tested whether Igf2 supports progenitor proliferation in a cerebral cortical explant system. In this system, rat embryonic cortex dissected from the lateral pallium is placed on polycarbonate membranes and floated on defined media (Figure 3H). We found that Igf2 added to neurobasal medium (NBM) with 20% artificial CSF (ACSF) stimulated the proliferation of progenitor cells marked by phospho-Vimentin 4A4 in rat cortical explants (Figure 3I; Noctor et al., 2002). In addition, Igf2 treatment alone maintained GLAST-positive neurospheres, an in vitro model of neural stem cells, even in the absence of Fgf2 (fibroblast growth factor 2) and Egf (epidermal growth factor) (Figure 3J; Vescovi et al., 1993). Finally, pharmacologic activation of the signaling pathway with insulin demonstrated that activation of Igf signaling by ligands other than Igf2 is sufficient to stimulate proliferation (PH3-positive cells/100 μm VZ ± SEM in E16 rat explant: control mean, 5.6 ± 0.7; insulin (10 μg/ml) mean, 11.2 ± 0.4; Mann-Whitney, p < 0.05; n = 6). Therefore, Igf signaling modulates proliferation of isolated cortical precursors or those maintained in their pallial environment in vitro.

**CSF Promotes Proliferation of Progenitor Cells in an Age-Dependent Manner**

Since the CSF is a complex fluid containing many factors including Igf binding proteins that may modulate Igf2 bioavailability and signaling (Figures 4A and 4B; Table S1; Clemmons, 1997; Zappaterra et al., 2007), we tested whether native CSF alone could support cortical tissue growth. We used a heterochronic “mix-and-match” approach for exposing cortical tissue to CSF collected at different ages. E16 rat cortical explants with intact meninges and vasculature cultured with 100% E17 rat CSF for 24 hr, without any additional exogenous media or factors, retained remarkable tissue architecture, cell viability, and proliferation, approximating in vivo E17 rat cortex (Figure 4C). In contrast, E16 explants cultured with 100% artificial CSF failed to thrive, had decreased mitotic activity, disorganized neuronal morphology, and increased cell death (Figures 4C, S2A, and S2B). Filtration analysis of E17 CSF showed that the sizes of CSF factors that support stem cells likely range from 10 kDa–100 kDa, suggesting that they are proteins (Table S2 and data not shown). Thus, the embryonic CSF proteome provides essential growth and survival factors for the developing cortex.

**Figure 3. Igf2 Is Expressed in Cerebrospinal Fluid and Stimulates Progenitor Proliferation**

(A and B) Igf2 in situ hybridization of rat E14 and E17 cortex. Arrow points to choroid plexus.
(C) Transient Igf2 expression in rat CSF.
(D) Immunogold labeling of endogenous Igf2 in E17 rat brain. Left panel: no primary control. Right panel: Igf2 binding to ventricular surface of cortical progenitors. Scale bar represents 500 nm.
(E) Igf2 binding to primary cilium of cortical progenitor cell. Arrow points to ciliary basal body. Scale bar represents 500 nm.
(F) Scanning EM of mouse ventricular surface at E12.5. Arrowheads point to primary cilia projecting into the ventricular space. Scale bar represents 2 μm.
(G) Lysates of cortical cells deprived of growth factors for 6 hr and treated with ACSF, E17 CSF, or Igf2 for 5 min were immunoblotted with antibodies to P-Igf1R, P-Akt, Akt, P-ERK1/2, and ERK1/2.
(H) Schematic of cortical explant dissections: explant placed on membrane with ventricular side down contacting CSF and notch making medial-caudal side.
(I) Left panel: E16 explants cultured with NBM plus 20% ACSF (control) or with supplemental Igf2 immunostained with anti-Vimentin 4A4 and Hoechst represented as mean ± SEM (Igf2 mean, 36.7 ± 2.1; control mean, 20.4 ± 4.46; n = 8; Mann-Whitney; p < 0.005). Vimentin 4A4-positive cells increased in explants cultured with Igf2 compared to control. Right panels: representative images of explants quantified in left panels.
(J) Single cells dissociated from primary neurospheres cultured in control media or control media containing Igf2 (20 ng/ml). Igf2 stimulated secondary sphere formation after 10 DIV (Igf2 mean, 39.3 ± 4.1; control mean, 2.2 ± 0.75; n = 3; t test; p < 0.005).

See also Table S1.
By comparing rat CSF from several ages, we determined that the effects of CSF on survival and proliferation are strikingly age dependent and mimicked the temporal profile of CSF-Igf2 expression (Figure 3C). E17 CSF (near the middle of neurogenesis) maintained the healthiest explants and produced the maximal increase in the frequency of PH3-labeled proliferating cells in E16 cortical explants compared to explants cultured with E13 (early in neurogenesis), P6, or adult CSF (Figures 4D, 4E, S2C, and data not shown). Many mitotic cells were identified as proliferating neuroepithelial progenitor cells by their immunoreactivity for phospho-Vimentin (4A4; Figures 4F and S2C). In contrast, no differences were seen in Tbr2-positive basal progenitors, which do not contact the CSF directly (data not shown). Together, these data suggest that age-dependent differences in CSF signals are both supportive and instructive for neuroepithelial precursor proliferation in the developing cortex. The CSF effects may be specific to neuroepithelial progenitors, which contact the ventricle through the apical
complex, without affecting the intermediate progenitors of the SVZ.

We tested directly whether CSF-borne Igf2 was necessary to explain the effects of age-specific CSF on rat cortical explants. The frequency of proliferating cells declined in explants grown in E17 CSF in the presence of Igf2 neutralizing antibodies (Igf2 NAb; Figure 4G). Igf2 neutralization with Igf2 NAB did not interfere with Igf1 levels in CSF compared to control as assayed by ELISA (data not shown). While Igf signaling is known to promote neuronal survival (Popken et al., 2004), we did not observe differences in ventricular progenitor cell survival in these explant experiments (data not shown), suggesting that Igf actions on neural cell survival likely depends on the cell type, developmental stage, and microenvironment. These data confirm the important role for CSF borne Igf2 in regulating cerebral cortical progenitor cells but do not rule out roles of other CSF borne factors as well.

CSF Influence on Isolated Neural Stem Cells Requires Igf Signaling

Neural stem cells cultured as neurospheres confirmed the age-dependent capacity of CSF to maintain neural stem cells (Reynolds and Weiss, 1996) and provided additional evidence suggesting that Igf2-mediated signaling is an essential determinant of CSF activity on neural stem cells. CSF from any age supported the proliferation and maintenance of isolated cortical stem cells cultured as primary or secondary neurospheres (Figure 4H and data not shown; Vescovi et al., 1993). However, E17 CSF was maximally effective in generating increased numbers of neurospheres, larger neurospheres, and maintained neurospheres even in long-term cultures for up to 44 days in vitro (Figures 4H, S2D–S2G, and data not shown). Neurospheres grown in CSF retained responsiveness to Fgf2 and Egf, indicating that the CSF maintains stem cells in an uncommitted fate (Figure S2H). CSF generated neurospheres from adult SVZ precursors as well (Figure 4I). Consistent with these observations and our explant studies, the Igf1R inhibitor picropodophyllin blocked the formation of spheres in the presence of E17 CSF (data not shown). Our data suggest that the choroid plexus is the most prominent source of Igf2 in CSF (Figures 3 and S3A). Accordingly, media conditioned with E17 choroid plexus provided enhanced support for neurosphere formation compared to media conditioned with embryonic cortex, adult choroid plexus, or adult brain (Table S3), demonstrating that one or more factors actively secreted from the embryonic choroid plexus, including potentially Igf2, is sufficient for stem cell growth and maintenance. Thus, distinct factors secreted by the choroid plexus into the embryonic CSF, including Igf2, confer E17 CSF with an age-associated advantage to stimulate and maintain neural stem cell proliferation, and Igf signaling is likely one pathway that promotes this process.

Genetic Inactivation of Igf Signaling Impairs Brain Development

Mouse explant experiments confirmed a requirement for Igf signaling in the proliferation of progenitor cells. Mouse embryonic CSF supported the survival and proliferation of mouse cortical progenitors (C57BL/6 explants: 20% ACSF in NBM mean, 7.4 ± 0.2; 20% E16.5 CSF in NBM mean, 14.1 ± 1.4; Mann-Whitney; p < 0.01; n = 3), and purified Igf2 in 20% ACSF in NBM stimulated cortical progenitor proliferation (Figure 5A). When the Igf1R was genetically inactivated in cortical progenitors (Igf1RloxP/loxP/NestinCre+/−) (Liu et al., 2009), wild-type CSF no longer stimulated cortical progenitor proliferation (ACSF, 17.6 ± 2.9; E16.5 CSF, 16.4 ± 3.0; Mann-Whitney; N.S.; n = 3). Importantly, CSF obtained from Igf2−/− mice failed to stimulate progenitor proliferation in wild-type explants compared to controls (Figure 5B), suggesting that Igf2 in its native CSF environment stimulates proliferation of progenitor cells during cerebral cortical development.

As expected for the roles we have shown for Igf2 in regulating proliferation, we found that Igf2-deficiency reduced brain size (Figure 5C). Igf2−/− brain weight decreased by 24% at P8 compared to controls (Figure 5D). Accordingly, the overall cortical perimeter and surface area were reduced in Igf2−/− brains compared to controls as well (Figures 5E–5G). Profound defects in somatic size couple to brain size (Purves, 1988). As previously reported (DeChiara et al., 1991; Baker et al., 1993), Igf2−/− body weight was reduced compared to control (mean body weight (g) at P8: Igf2+/+, 5.6 ± 0.01; Igf2−/−, 2.8 ± 0.1; Mann-Whitney; p < 0.001; n = 11), suggesting that Igf2 may be a secreted factor that scales brain size to body size. Consistent with the mouse CSF Igf2 expression pattern that is significantly increased during later embryonic development (Figure S3B), blunting Igf2 expression markedly reduced the proliferating progenitor cells at E16.5 compared to controls (PH3-positive cells/100 μm VZ ± SEM at E16.5: Igf2+/+, 5.6 ± 0.3; Igf2−/−, 1.7 ± 0.1; Mann-Whitney; p < 0.05; n = 5). NeuN- and late-born Cux1-staining neurons were reduced in Igf2−/− mice (Figure 5H and data not shown), confirming that Igf2 contributes to cortical progenitor proliferation and to late stages of neurogenesis. Taken together, our genetic experiments support a model in which the apical complex localizes Igf signaling in progenitors by ensuring the apical, ventricular localization of the Igf1R. In this manner, the apical complex couples cell autonomous and extracellular signals to the regulation of cortical development.

Glioblastoma CSF Expresses High Igf2

Our data, together with recent findings implicating Igf signaling in the maintenance of adult neural stem cells (Llorens-Martín et al., 2010), raised the possibility that abnormalities of the CSF may be relevant to conditions showing abnormal proliferation, including in glioblastoma multiforme (GBM), a malignant astrocytic brain tumor. Igf-PI3K-Akt signaling has been implicated as a key regulator of gliomagenesis (Louis, 2006; Soroceanu et al., 2007), and mutations in PTEN are commonly found in patients with GBM (Louis, 2006). We analyzed Igf2 concentration in a panel of 56 human GBM patient CSF samples collected from 21 individuals representing the full range of disease progression and 8 disease-free controls and found that CSF from GBM patients contained significantly more Igf2 than CSF from disease-free controls (Igf2 concentration expressed as mean ± SEM for GBM patients, 340.4 ± 12.9 ng/ml; n = 56; disease-free controls, 222.9 ± 41.5 ng/ml; n = 8; Mann-Whitney, p < 0.01). Three GBM samples containing the highest Igf2 concentrations (605.8 ng/ml,
Whereas our studies suggest an important role for Igf2 in controlling proliferation in late stages of neurogenesis and potentially postnatally, they do not rule out the presence of other secreted factors that may act at long ranges via the CSF, and so we performed functional screening tests for several other families of factors. The CSF contained Wnt signaling activity (Zhou et al., 2006), based upon phosphorylation of LRP6, a Wnt coreceptor in response to CSF exposure (Figure 7A). Several Wnt ligands were expressed along the ventricular surface and in the choroid plexus (Figure 7B and data not shown; Grove et al., 1998). Frizzled (Fz) receptors, which bind LR6 to transduce Wnt signals, showed enhanced expression in ventricular progenitors (Figure 7B and data not shown; Zhou et al., 2006), suggesting that CSF may distribute Wnt ligands to precursors throughout the ventricular surface. Additional signaling activities that influence cortical development were also found in the CSF, with responsive cells seen broadly in the ventricular zone. There were dynamic levels of bone morphogenetic protein (Bmp) activity in the CSF during different stages of cortical development (Figure 7C). Using a luciferase-based assay in which overall Bmp activity can be quantified between 0.1 and 100 ng/ml (data not shown), we found that Bmp activity in the CSF decreased during embryogenesis and peaked in adulthood (Figure 7C). CSF-borne Bmp activity may be responsible for stimulating progenitors widely throughout the cortical ventricular zone in vivo, based on

502.8 ng/ml, and 468.7 ng/ml) came from patients with advanced disease (Figure 6A and Table 1). By contrast, the three patients with the lowest levels of Igf2 (142.1 ng/ml, 145.4 ng/ml, and 153.9 ng/ml) all had early or stable glioma (Figure 6A and Table 1). Similar to rodent ventricular CSF, human lumbar CSF stimulated cortical progenitor cell proliferation in our explant assay, with CSF from GBM patients causing greater proliferation than CSF from disease-free controls (Figure 6B). Moreover, human GBM patient CSF neutralized with Igf2 antibodies failed to stimulate the proliferation of progenitor cells (Figure 6B; Igf2 concentration following NAb absorption, GBM1(PBS): 605.8 ng/ml; GBM1 (NAb), 45.6 ng/ml; GBM2(PBS), 502.8 ng/ml; GBM2(NAb), 218.3 ng/ml; GBM3(PBS), 468.7 ng/ml; GBM3(NAb), 248.8 ng/ml). Taken together, these data suggest that beyond embryonic brain development, CSF-Igf2, in particular, is a potential mediator of GBM pathology and that the CSF mechanisms that normally regulate neural stem cells are misregulated in GBM.

**CSF-Mediated Long-Range Distribution of Additional Secreted Factors**

Whereas our studies suggest an important role for Igf2 in controlling proliferation in late stages of neurogenesis and potentially postnatally, they do not rule out the presence of other secreted factors that may act at long ranges via the CSF, and so we performed functional screening tests for several other families of factors. The CSF contained Wnt signaling activity (Zhou et al., 2006), based upon phosphorylation of LR6, a Wnt coreceptor in response to CSF exposure (Figure 7A). Several Wnt ligands were expressed along the ventricular surface and in the choroid plexus (Figure 7B and data not shown; Grove et al., 1998). Frizzled (Fz) receptors, which bind LR6 to transduce Wnt signals, showed enhanced expression in ventricular progenitors (Figure 7B and data not shown; Zhou et al., 2006), suggesting that CSF may distribute Wnts to precursors throughout the ventricular surface. Additional signaling activities that influence cortical development were also found in the CSF, with responsive cells seen broadly in the ventricular zone. There were dynamic levels of bone morphogenetic protein (Bmp) activity in the CSF during different stages of cortical development (Figure 7C). Using a luciferase-based assay in which overall Bmp activity can be quantified between 0.1 and 100 ng/ml (data not shown), we found that Bmp activity in the CSF decreased during embryogenesis and peaked in adulthood (Figure 7C). CSF-borne Bmp activity may be responsible for stimulating progenitors widely throughout the cortical ventricular zone in vivo, based on

**Figure 5. CSF Igf2 Regulates Progenitor Proliferation and Brain Size**

(A) Left panels: E15.5 C57BL/6 explants cultured in NBM supplemented with 20% ACSF or ACSF/Igf2. Igf2 stimulated the proliferation of Ph3-positive cortical progenitor cells (C57BL/6 explants: ACSF mean, 7.4 ± 0.2; Igf2 mean, 11.2 ± 0.3; Mann-Whitney, p < 0.05; n = 3). Right panels: representative images of explants quantified in left panels. (B) E15.5 C57BL/6 explants cultured in NBM supplemented with 20% E16.5 wild-type or Igf2−/− CSF. Igf2-deficient CSF failed to stimulate progenitor cell proliferation compared to control (Igf2+/+, 17.9 ± 0.8; Igf2−/−: CSF, 11.4 ± 1.0; Mann-Whitney, p < 0.05; n = 3 and n = 4, respectively). (C) Representative images of P6 Igf2−/− and control brains. (D) Igf2 deficiency reduced P8 brain weight (Igf2+/+, 0.34 g ± 0.008; Igf2−/−, 0.26 g ± 0.004; Mann-Whitney, p < 0.0001, n = 11). (E) Igf2 deficiency reduced P8 cortical perimeter (Igf2−/−, 30.9 mm ± 0.01; Igf2+/+, 26.4 mm ± 0.1; Mann-Whitney, p < 0.0001, n = 11). (F) Igf2 deficiency reduced P8 cortical surface area (Igf2−/−, 13.0 mm² ± 0.1; Igf2+/+, 9.4 mm² ± 0.1; Mann-Whitney, p < 0.0001, n = 11). (G) H&E staining of Igf2−/− and control brains at P8. (H) Left panels: Igf2−/− brains have reduced numbers of upper layer neurons marked by Cux1 (total Cux1-positive staining cells in equally sized cortical columns expressed as mean ± SEM: Igf2+/+, 161 ± 5.1; Igf2−/−, 131 ± 3.3; t test, p < 0.005, n = 3). Right panels: representative images of Igf2−/− and control brains quantified in left panels. See also Figure 7.
widespread labeling for nuclear phospho-SMAD1/5/8 (Figure 7D) in the absence of any known Bmp ligands localizing to the ventricular zone (Shimogori et al., 2004), whereas Bmps 2, 4, 5, and 7 are expressed in embryonic and adult choroid plexus (Figure 7E; Hébert et al., 2002; Shimogori et al., 2004). Moreover, growth and differentiation factors 3 and 8 (GDF3 and GDF8), both members of the TGF-β superfamily of proteins that can influence Bmp signaling (Levine and Brivanlou, 2006) were found in our MS analyses of CSF (data not shown), though we do not consider our MS analysis to have recovered all potential smaller ligands in the CSF. Retinoic acid (RA) (Haskell and LaMantia, 2005; Siegenthaler et al., 2009) activity in CSF also varied over the course of cortical development (Figure 7F). A luciferase-based assay that quantifies RA activity ranging between 10⁻⁹ and 10⁻⁶M (data not shown) revealed that RA activity in CSF peaked early and decreased in adulthood (Figure 7F). In parallel, RA responsive cortical progenitors localized to the developing ventricular zone (Figure 7G). Similar to Wnts and Bmps, RA is most likely released into CSF since RA synthetic and catabolic enzymes were expressed in the choroid plexus (Figure 7H) and meninges (data not shown). Thus, CSF shows bioavailability of a wide range of activities known to regulate neurogenesis, patterning, and neuronal survival in the cerebral cortex and throughout the CNS.

DISCUSSION

We show that the CSF plays an essential, active role in distributing signals in the central nervous system. The key findings of our study are (1) the apical complex is essential for the apical localization of Igf1R; (2) Pten deficiency in the Pals1 background results in an almost normally sized brain; (3) CSF Igf2 binds to the apical domain of cortical progenitor cells, stimulating their proliferation in an age-dependent manner; (4) Igf2 is upregulated in GBM patient CSF, contributing to the range of proliferative activities of GBM patient CSF; and (5) the CSF provides an adaptive library of secreted factors throughout life. The dynamic regulation of several potent modulators of neural stem cells reinforces the central relationship between local signaling at the apical surface via ligands delivered by the CSF during cortical neurogenesis.

Asymmetric Growth Factor-Based Signaling

It has been suggested that asymmetry of signaling at the apical versus basolateral aspect of cortical progenitors regulates progenitor progress through the cell cycle (Bultje et al., 2009; Sun et al., 2005). The basolateral expansion of the Igf1R signaling domain we report in Pten mutants suggests potential links between asymmetric growth factor signaling and proliferation. Although asymmetric localization of the Egfr in cortical progenitors has previously been reported (Sun et al., 2005), the ventricular enrichment of the Igf1R was not known and raises the possibility that the apical enrichment of the Igf1R along with other apical proteins confers a differential responsiveness to mitogenic signals, akin to Notch signaling (Bultje et al., 2009). Since IGFs are potent mitogens for cortical progenitors (Hodge et al., 2004; Popken et al., 2004), one model might suggest that inheritance of the apical complex promotes progenitor fate by differentially concentrating Igf1R and its
downstream signaling proteins into cells that retain their perikarya or at least a process (likely a cilium) in the ventricular zone, causing these cells to remain in the cycling pool. The presence of proliferation-inducing factors in the CSF suggests that withdrawal of the progenitor’s apical ventricular process may be an important step in neuronal differentiation (Cappello et al., 2006), by insulating progenitor cells from proliferative signals in CSF, with vascular niches potentially supplying sources of secreted factors for stem cells at other stages (Palmer et al., 2000; Shen et al., 2004, 2008; Tavazoei et al., 2008).

Our data provides a new perspective on the production and provision of Igf ligands, which are known to regulate stem cell populations in the brain and other proliferative epithelia (Bendall et al., 2007; Hodge et al., 2004; Liu et al., 2009; Popken et al., 2004; Ye et al., 2004; Zhang and Lodish, 2004). In the E17 rat brain, the choroid plexus was the strongest source of Igf2, though we cannot discount a contribution by the vasculature or other cellular sources of Igf2 that may percolate into the CSF. Indeed, both pericytes and endothelial cells express Igf2 (Dugas et al., 2008), and IGFs from vascular tissue may have local effects beyond apically mediated Igf1R signaling shown here. Thus, locally derived Igf2 may play distinct roles at different developmental time points and in different cellular contexts, and Igf signaling may also be influenced by CSF Igf1 and insulin. Although Igf2 availability decreased in adult CSF (Figures 3C and S3B), Igf2 continued to be expressed in adult choroid plexus (data not shown) and maintained adult neurospheres (Figure 4I), suggesting that low levels of CSF Igf2 contribute to the maintenance of adult neural stem cells. The aberrant increase in Igf2 in advanced GBM patients reinforces the hypothesis that Igf signaling has an influence on proliferation of cortical precursors. Our identification of Igf2 regulation of neurogenesis and brain size complements a literature in which Igf signaling is well known to influence body and brain size (Baker et al., 1993; DeChiara et al., 1991; Purves, 1988), raising the intriguing possibility that Igf2 represents a secreted factor that may scale brain size to body size.

Fluid-Based Signaling in the CNS and Beyond

The activity of growth promoting factors in the CSF and their action on progenitors across the apical surface may be a model for other epithelia including lung, gut, and vascular endothelia that develop in relation to extracellular fluids (Bendall et al., 2007; Scadden, 2006). Extracellular fluid apparently regulates the microenvironment of hematopoietic stem cells, where Igf signaling regulates progenitor proliferation (Orkin and Zon, 2008; Zhang and Lodish, 2004). The differential capacity of Igf signaling to confer a proliferative advantage to stem cells may be regulated in part by Igf’s interactions with binding proteins or other secreted factors in the environment (Clemmons, 1997). Our experiments focused on the age-associated effects of CSF on survival and proliferation across the cortical ventricular zone. However, the distribution of CSF resident proteins, as well as the flow of the CSF, may also influence ciliary orientation and maturing ependymal cell polarity (Mirzadeh et al., 2010), which create activity gradients as has been shown for Slt (Sawamoto et al., 2006).

If a major component of the stem cell niche reflects secreted factors acting at long distances from their sources, modulation of the proteomic composition of extracellular fluids may also provide unexpected ways to regulate stem cell behavior in health and disease. For example, while Igf2 activity peaked in embryonic CSF, some CSF-borne Igf persisted in adulthood (Figures 3, S3B, and data not shown). Igf2 and Igf1 in adult CSF may contribute to the retention of neural stem cell properties in the adult SVZ (Doetsch et al., 1999). Importantly, the regulation of CSF growth factors may also extend to pathologic states. Igf2 and other diffusible growth factors that drive neural progenitor proliferation during development are upregulated in some GBM patients (Louis, 2006; Soroceanu et al., 2007), and GBM patients have elevated Igf2 levels in their CSF. CSF Aþh1.42 and phosphorylated Tau levels were recently shown to assist in Alzheimer’s disease diagnosis (De Meyer et al., 2010). Thus, modulation of the proteomic composition of extracellular fluids together with the integration of cell autonomous determinants of self-renewal by the apical complex may ultimately provide unexpected ways to regulate stem cell behavior in health and disease.

### Table 1. Clinical Presentation of GBM Patients with Lowest and Highest CSF Igf2 Concentrations

<table>
<thead>
<tr>
<th>Patient</th>
<th>[Igf2] ng/ml</th>
<th>Tumor size: T1-Gad (cm³)</th>
<th>Tumor size: FLAIR (cm³)</th>
<th>Life Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low CSF Igf2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>142.1</td>
<td>7.14</td>
<td>6.46</td>
<td>Stable disease at follow-up; 3 weeks post-CSF collection</td>
</tr>
<tr>
<td>L2</td>
<td>145.4</td>
<td>8.50</td>
<td>54.12</td>
<td>Stable disease at follow-up; 3 weeks post-CSF collection</td>
</tr>
<tr>
<td>L3</td>
<td>153.9</td>
<td>5.94</td>
<td>20.5</td>
<td>Stable disease at follow-up; 5 weeks post-CSF collection</td>
</tr>
<tr>
<td>High CSF Igf2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>605.8</td>
<td>47.31</td>
<td>102.83</td>
<td>Deceased at 1 week post-CSF collection</td>
</tr>
<tr>
<td>H2</td>
<td>502.8</td>
<td>13.69</td>
<td>53.90</td>
<td>Deceased at 52 weeks post-CSF collection</td>
</tr>
<tr>
<td>H3</td>
<td>468.7</td>
<td>23.94</td>
<td>36.48</td>
<td>Deceased at 30 weeks post-CSF collection</td>
</tr>
</tbody>
</table>

Patients with the lowest CSF Igf2 concentrations (L1–L3) had early or stable GBM disease state, while patients with the highest CSF Igf2 concentrations (H1–H3) had advanced disease and aggressive tumor progression at time of CSF collection. Tumor size was determined by Macdonald’s criteria, where T1-Gad MRI sequence delineated the contrast-enhanced portion of tumor, and FLAIR images include areas of nonvascularized and invasive tumor (Macdonald et al., 1990). High-CSF Igf2 patients had larger T1-Gad tumor sizes compared to low-CSF Igf2 patients (Mann-Whitney; p < 0.05; n=3).

**EXPERIMENTAL PROCEDURES**

**Animals**

Time pregnant Sprague-Dawley, C57BL/6, and Swiss Webster dams were purchased from Charles River Laboratories and Taconic. Pals1loxP/loxP/NestinCreERT2, Pals1loxP/loxP/Emx1CreERT2, Igf1RloxP/loxP/NestinCreERT2, and...
GFAP-Igf1Tg mice were obtained from heterozygous breedings, and PtenloxPlox/Pals1loxPlox/Emx1Cre+/− mice were obtained from homozygous knockouts (DeChiara et al., 1991) and control P8 brains and fixed (60% methanol, 30% chloroform, and 10% acetic acid; 10 min). Explants were pulsed with BrdU for 30 min and subjected to immunoblotting with the P-LRP6 or LRP6 antibodies. Bmp activity was measured in E14, E17, and adult rat CSF as luciferase signal in a clonally derived Bmp-sensitive cell line. Responses were compared to linear responses generated in the same cell line by pure ligand (Bmp4; data not shown). Bmp activity levels varied with age and were statistically significant between E17 and adult (ANOVA, p < 0.001; n = 4).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.01.023.

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Cortical Explants

The telencephalic wall was dissected onto polycarbonate membranes (Whatman; 13 mm, 8.0 μm) and cultured for 24 hr as described in text. Artificial CSF (NaCl 119 mM, KCl 2.5 mM, NaHCO3 26 mM, NaH2PO4 1 mM, glucose 11 mM, MgCl2 2 mM, CaCl2 2.8 mM) was supplemented with Igf2 (2 ng/ml; US Biologicals) as indicated. Igf2 NAb antibody was incubated with E17 CSF for 1 hr at 4°C. Explants were pulsed with BrdU for 30 min and fixed (60% methanol, 30% chloroform, and 10% acetic acid; 10 min). For in vivo BrdU labeling, pregnant dams were administered a 3 hr BrdU (60 mg/kg) pulse. Tissue was paraffin sectioned (5 μm).

Figure 7. The CSF Proteome Coordinates Multiple Signaling Pathways that Regulate Brain Development

(A) Lysates of cortical cells were left untreated or treated with 20% ACSF or E17 CSF and 10% Wnt3a conditioned medium or its control medium for 2 hr and subjected to immunoblotting with the P-LRP6 or LR6i antibodies.

(B) In situ hybridization for Wnt5a and Fz1 in mouse E14.5 cortex.

(C) Bmp activity was measured in E14, E17, and adult rat CSF as luciferase signal in a clonally derived Bmp-sensitive cell line. Responses were compared to linear responses generated in the same cell line by pure ligand (Bmp4; data not shown). Bmp activity levels varied with age and were statistically significant between E17 and adult (ANOVA, p < 0.001; n = 4).

(D) Top panel: expression and nuclear localization of phospho-Smad (P-Smad) 1/5/8 in E14 rat cortical ventricular cells. Bottom panel: arrow points to expression and nuclear localization of P-Smad1/5/8 in E16.5 mouse cortical ventricular cells.

(E) qPCR measurement of Bmp2, 4, 5, and 7 in the E16, E18, P0, and adult rat choroid plexus (CP).

(F) Quantification of RA activity in E14, E17, and adult rat CSF. RA activity declined, based on comparison of CSF activation of an RA responsive, clonally derived cell line with response to RA at known concentrations, from midgestation through adulthood (ANOVA, p = 0.07; n = 4).

(G) RA responsive progenitor cells at the cortical ventricular zone from an E16.5 D5S-RARE transgenic mouse (LaMantia et al., 1993). (H) qPCR of Radh1, 2, 3, and Rdh10, in rat CP.
REFERENCES


