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Akt1 interacts with epidermal growth factor receptors and Hedgehog signaling to increase stem/transit amplifying cells in the embryonic mouse cortex

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Abstract

A subset of precursors in the embryonic mouse cortex and in neurospheres expresses a higher level of the serine/threonine kinase Akt1 than neighboring precursors. We reported previously that the functional significance of high Akt1 expression was enhanced Akt1 activity, resulting in an increase in survival, proliferation, and self-renewal of multipotent stem/transit amplifying cells. Akt1 can interact with a number of signaling pathways, but the extrinsic factors that are required for specific effects of elevated Akt1 expression have not been identified. In the present study we addressed the contributions of signaling via epidermal growth factor (EGF) and hedgehog (Hh) receptors. In EGF receptor-null precursors or following transient inhibition of EGF receptor tyrosine kinase activity, elevating Akt1 by retroviral transduction could still increase survival and proliferation but could not increase self-renewal. We also found that elevated Akt1 expression induced the expression of EGF receptors (EGFRs) in wild-type precursors. Several extrinsic factors, including Shh, can induce EGFR expression by cortical precursors, and we found that elevating Akt1 allowed them to respond to a sub-threshold concentration of Shh to induce EGFRs. In precursors that lack the Hh receptor smoothened, however, elevating Akt1 did not increase EGFR expression or self-renewal, though it could still stimulate proliferation. These findings suggest that a subset of precursors in the embryonic cortex that express an elevated level of Akt1 can respond to lower concentrations of Shh than neighboring precursors, resulting in an increase in their expression of EGFRs. Signaling via EGFRs is required for their self-renewal.

Keywords

Akt1; cerebral cortex; EGF receptor; stem cell; hedgehog

INTRODUCTION

The development of the central nervous system (CNS) is controlled by extrinsic factors that can elicit different cellular responses at specific times and locations. Several strategies are employed to generate appropriate responses to these pleiotropic signals. For example, distinct responses can be elicited at specific levels of signaling (reviewed in Freeman and Gurdon, 2002). One way to achieve differences in signaling level is to vary the concentration of ligands or inhibitors in the extracellular environment, but cell-intrinsic differences have also been identified that allow neighboring cells to respond differently to the same concentration of an extrinsic factor (reviewed in Freeman and Gurdon, 2002).

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The regulation of stem cells is thought to involve specialized niches where extrinsic factors necessary for their maintenance are localized (reviewed in Watt and Hogan, 2000; Morrison and Spradling, 2008). This strategy might be more relevant for stem cell regulation in the adult CNS, where the distribution of stem cells is relatively restricted, than in the developing CNS, where stem cells appear to be more widely distributed (reviewed in Temple, 2001; Doetsch, 2003; Alvarez Buyla and Lim, 2004; Miller and Gauthier-Fisher, 2009). In the developing cerebral cortex, the “stem cell niche” is thought to be generated by other cortical precursors (Zhang et al., 2010) and by factors present in the cerebrospinal fluid, which is accessible to all of the precursors at the ventricular surface (reviewed in Johansson et al., 2010). If extrinsic factors necessary for stem cell maintenance are less restricted spatially, other strategies that depend on intrinsic differences might be more important for regulating stem cells in the developing cortex.

In a previous study we described an intrinsic difference among precursors in the E13.5 and E16.5 mouse cerebral cortex: the level of expression of the serine/threonine kinase Akt1 (Sinor and Lillien, 2004). Fewer than 5% of the cells in the embryonic cortex express a high level of Akt1 protein (Sinor and Lillien, 2004). The Akt1^{high} cells co-express markers of neural precursors including nestin and are located in the proliferative layer of the cortex (Sinor and Lillien, 2004). Akt1^{high} cells are also enriched in cortical “neurospheres”, which are enriched in stem and transit amplifying (TA) cells (Reynolds and Weiss, 1992). Akt1 mediates responses to extrinsic factors that activate PI3K (reviewed in Franke et al., 1997, Vanhaesebroeck and Alessi, 2000; Brazil and Hemmings, 2001) and its activity is negatively regulated by Pten (Stambolic et al., 1998). We reported that the functional consequence of increased expression of Akt1 protein was an increase in Akt1 activity (Sinor and Lillien, 2004). Akt1 activity is also increased in cortical precursors from Pten-null mutants, and loss of Pten has been reported to increase the self-renewal of multipotent stem /TA cells (Groszer et al., 2001). As in Pten-null mutants, we found that elevated expression of Akt1 changed the behavior of cortical precursors, increasing their survival, proliferation, and self-renewal as multipotent precursors (Sinor and Lillien, 2004).

One mechanism underlying differences in the behavior of cortical precursors that exhibit higher Akt activity may be a shift in their sensitivity to extrinsic factors. For example, loss of Pten in cortical precursors increases their responsiveness to FGF2, enhancing self-renewal at lower concentrations of FGF2 (Groszer et al., 2006). We did not identify the extrinsic factor(s) that acted upstream of elevated Akt1 to elicit changes in precursor behavior. In the present study, we assess the contributions of two candidate pathways: signaling mediated by EGF receptors and the Hh receptor smoothened.

EGF receptors are expressed by a subset of embryonic cortical precursors at a high level (Eagleson et al., 1996; Kornblum et al., 1997). Activation of EGF receptors can elicit multiple responses in cortical precursors: proliferation, migration, self-renewal as multipotent stem/TA cells, or differentiation into astrocytes rather than neurons or oligodendrocytes, with response choice dependent in part on ligand concentration (Burrows et al., 1997; Caric et al., 2001; Reynolds and Weiss, 1992; Reynolds et al., 1992; Sun et al., 2005). Up-regulation of EGF receptor expression occurs between E13 and E16 in the mouse cortex, beginning at the pallial-subpallial boundary (PSB) and proceeding medially (Caric et al., 2001). Several EGF family ligands are expressed in the embryonic cortex. For example, TGF α is expressed at the PSB (Kornblum et al., 1997; Assimacopoulos et al., 2003). Following ligand binding, EGF receptors can activate several intracellular signal transduction pathways, including the PI3K/Akt pathway (Okano et al., 2000).

Up-regulation of EGFR expression by telencephalic precursors has been reported to occur in response to at least two extrinsic factors: FGF2 (Lillien and Raphael, 2000; Ciccolini and

Svendson, 1998) and Shh (Viti et al., 2003; Palma and Ruiz y Altaba, 2004). Both of these factors can elicit multiple responses in neural precursors, with response choice regulated in part by a concentration-dependent mechanism (Roelink et al., 1995; Qian et al., 1997; Danesin et al., 2006; Chan et al., 2009; Xu et al., 2010). For example, the concentration of either Shh or FGF2 required to induce EGFR expression is higher than the concentration needed to stimulate proliferation (Lillien and Raphael, 2000; Viti et al., 2003). Although some responses to FGF2 appear to depend on Hh signaling (Gabay et al., 2003; Kessaris et al., 2004), the effect of FGF2 on EGFR expression is Hh-independent (Viti et al., 2003). Examination of mice that lack the Hh receptor *smoothed* revealed a dramatic reduction in EGF receptor expression in the late embryonic cortex (Gulacsi and Lillien, 2006), suggesting that Hh signaling may be more relevant physiologically than FGF2 as a regulator of EGFR expression in cortical precursors, at least during embryonic development.

An association between Akt1 and Hh signaling has been reported (Riobo et al., 2006a,b), but is not as well characterized as the association between Akt1 and EGFR signaling. Hh acts via two receptors: it binds to patched, which de-represses a second receptor, *smoothed*; as a consequence, expression of the activator forms of transcription factors *gli1* and *gli2* is induced (reviewed in Ingham and McMahon, 2001; Ruiz y Altaba et al., 2003). The transcriptional activity of *Gli2* is negatively regulated by protein kinase A (PKA), but Akt can promote Hh signaling by modulating PKA-induced inactivation of *Gli2* (Riobo et al., 2006a). Activation of Akt can also increase the nuclear localization and transcriptional activity of *Gli1* in glioma cells (Stecca et al., 2007). Moreover, stimulation of the PI3-kinase – Akt pathway potentiates the action of low concentrations of Shh (Riobo et al., 2006a).

In the embryonic cortex, loss of the Hh receptor *smoothed* results in reduced proliferation at E13.5, indicating that the pathway is functional even in early cortical precursors (Komada et al., 2008). The source(s) of Hh ligand for cortical precursors has been difficult to identify. Recent reports indicate that Shh is present in cerebrospinal fluid (CSF) of the E12.5–E15.5 mouse (Huang et al., 2010), making it available to precursors at the ventricular surface, some of which express a high level of Akt1 (Sinor and Lillien, 2004). Ligand and precursor responses to Hh signaling in cortical precursors are therefore in place when Akt^{high} precursors are present but before the increase in EGFR expression occurs.

Given their functions and relationship to Akt signaling, we assessed the contributions of EGF receptor and Hh signaling to the responses of embryonic cortical precursors elicited by elevated Akt1 expression.

MATERIAL AND METHODS

Mice

CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). EGFR-mutant (Threadgill et al. 1995), conventional *smoothed* mutant (*smo*^{+/-}; Zhang et al., 2001), conditional (floxed) *smo*-mutant (Long et al., 2001), and *Emx1*-cre recombinase mice (Gorski et al., 2002) were obtained from Jackson Laboratory (Bar Harbor, MA). EGFR-mutant mice were maintained on a CD1 background and genotyped by PCR. Mice lacking one or both copies of the Hh receptor *smoothed* in cortical precursors were generated by crossing *smo*^{flox/flox}, *smo*^{+/-}, and *Emx1* cre recombinase mice, resulting in embryos with the following genotypes: *Emx1*^{cre/+}; *smo*^{+/+} (*smoothed* wild type), *Emx1*^{cre/+}; *smo*^{flox/+} (*smoothed* heterozygous), *Emx1*^{cre/+}; *smo*^{flox/-} (*smoothed* null). The genetic background of the conditional *smoothed* mutants was mixed (129X1/SvJ;C57BL/6). Embryos were genotyped by PCR. The University of Pittsburgh IACUC approved all animal studies.

Retroviruses

The Akt-1 coding sequence was subcloned into pLig-ns or pLie for co-expression of β -geo (β -galactosidase + neomycin-phosphotransferase), or green fluorescent protein (GFP), respectively (Sinor and Lillien, 2004). Viruses with titers of $1-2 \times 10^7$ cfu/ml were selected for experiments. For controls, viruses expressing β -geo or GFP alone were used. We showed previously that the level of virally transduced Akt1 protein approximates that expressed normally by a subset of precursors in the embryonic mouse cortex, as determined by anti-Akt fluorescence intensity measurements (Sinor and Lillien, 2004).

Explant cultures

Explants of embryonic cortex were prepared from the dorso-lateral region including the PSB. Embryos aged E11–E13.5 were used for experiments, assigning age by crown-rump length and external features (Theiler, 1972). The cortex was dissected and placed ventricular surface down on a nucleopore filter (13 mm, 0.2 μ m, Corning, Fisher Scientific, Pittsburgh, PA) floating in serum-free medium containing 25 μ g/ml insulin (Sigma, St. Louis, MO). 20–30 μ l of medium containing retrovirus was added to the explants 30–60 minutes later. These viruses infect dividing cells at the ventricular surface (Burrows et al., 1997). For experiments involving mutant tissue, one hemisphere of an embryo was infected with control virus, the other with Akt1 virus. After 4–5 days in culture, explants were dissociated and either stained with antibodies or cultured in EGF or FGF2 in a clonogenic “neurosphere” assay for self-renewal (see below). For experiments involving exogenous Shh, explants were infected with control or Akt1 viruses then treated with Shh (0.5 μ g; R&D Systems, Minneapolis, MN) for 3 days, beginning 1 day after infection. For experiments addressing the role of EGFR tyrosine kinase activity, tyrphostin AG1478 (100 nM; Calbiochem, San Diego, CA) or DMSO (Sigma) were added to explant cultures daily for 3 days, beginning 1 day after infection. AG1478 was not present in subsequent neurosphere cultures. To confirm that AG1478 inhibited EGF receptor signaling, cortical cells treated with AG1478 were exposed to EGF for 30 minutes then stained with phospho-EGFR (Tyr-1068) antibody (Cell Signaling Technology, Beverly, MA). To assess restoration of EGFR signaling during the neurosphere assays, after removal of AG1478 the cultures were grown for two days in FGF2 (10ng/ml) and insulin (25 μ g/ml) then stimulated with EGF for 30 minutes and stained with phospho-EGFR (Tyr-1068) antibody.

Self-renewal assay

Dissociated cells were seeded at 5×10^4 cells per well in a 24-well plate (Fisher Scientific) and cultured in serum-free medium containing 25 μ g/ml insulin (Sigma) and either 1–10 ng/ml of EGF (R&D Systems) or 10ng/ml of FGF2 (R&D Systems). After 10 days in these culture conditions, individual infected cells divide to generate clonal colonies of several hundred progeny, referred to as “neospheres” (Reynolds and Weiss, 1992). The initial number of infected cells seeded was determined by X-gal histochemistry or GFP antibody staining. Approximately 1/1000–1/500 cells in the explants were infected initially. After ten days, the number of neurospheres per well was counted. The proportion of infected cells that generated neurospheres was calculated by dividing the number of neurospheres derived from infected cells (GFP+ or β -gal+ neurospheres) by the initial number of GFP+ or β -gal+ cells per well. The majority of cells in a neurosphere derived from an infected precursor expressed the virally transduced marker gene. To assay self-renewal, primary colonies (neurospheres) were passaged by incubation in 0.05 % Trypsin – 0.53 mM EDTA (Gibco-BRL) for 5–10 minutes. Egg white trypsin inhibitor and DNase I (Sigma) were added and cells were triturated. Cells were seeded at $2-4 \times 10^3$ cells per well in a 24-well plate (Fisher) with either EGF (1–10 ng/ml) or FGF2 (10 ng/ml). Every four days, 200 μ l of fresh medium with growth factor and insulin was added to each well. After ten days, the percentage of infected cells that generated secondary colonies (neurospheres) was

determined as described above. Sister cultures (control versus Akt-1 virus infected) were used for serial passaging.

Immunocytochemistry

Sections of wild-type E15 cortex (fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at 4 degrees C overnight, frozen and cut at 20 μ m) were stained overnight at 4 degree C with anti-EGFR (Upstate Biotechnology, Lake Placid, NY) and anti-phospho-Akt (pAkt) substrate (Cell Signaling Technology; catalog number 9611) or anti-EGFR and anti-phospho-Akt (Ser473) (Cell Signaling; catalog number 9277) antibodies. We counted the proportions of pAkt substrate⁺/EGFR⁺ (121 cells) or pAkt(Ser473)⁺/EGFR⁺ (163 cells) cells in coronal sections from two brains at the pallial/subpallial boundary. To assess Akt pathway activity in the smoothened-null cortex, coronal sections of E16 wild-type and smoothened-null cortex were stained with pAkt substrate antibody. The number of labeled cells in an area 150 μ m wide and 100 μ m deep that included the ventricular and subventricular zones near the pallial-subpallial boundary was counted in captured images of stained sections from the mid-anterior/posterior level of the cortex (n= two brains per genotype). Dissociated cells were plated on poly-D-lysine (Sigma) coated slides for 1 hour (activated caspase, PCNA, Ki67) or 3–3.5 hours (EGF receptor) then fixed for 10 minutes with 4 % paraformaldehyde in 0.1 M phosphate buffer or 10% neutral buffered formalin (for EGFR; Sigma) at room temperature. Cells were rinsed in PBS, incubated for 10 minutes in block (10 % FBS and 0.1 % triton-X-100 in PBS), then incubated in primary antibody diluted in block for 1 hr at room temperature or overnight at 4 degree C. Primary antibodies included: rabbit anti- β -gal (Cortex Biochem Inc., San Leandro, CA), mouse anti- β -galactosidase (Promega, Madison, WI), rabbit anti-GFP (Invitrogen, Carlsbad, CA), mouse anti-Ki67 (BD Biosciences, San Jose, CA), mouse anti-PCNA (Sigma), rabbit anti-activated caspase 3 (Biovision, Mountain View, CA), sheep anti-EGFR (Upstate Biotechnology, Lake Placid, NY); rabbit anti-phospho-EGFR (Tyr-1068, Cell Signaling Technology). Cells were rinsed in PBS then incubated for 30 minutes in donkey anti-mouse Cy2 or Cy3, donkey anti-rabbit Cy2 or Cy3, donkey anti-sheep Cy2 or Cy3 (Jackson ImmunoResearch, West Grove, PA). Cells were counted with a Leica DMR microscope using fluorescence optics. For each experiment at least 50 cells were counted per condition, unless otherwise noted. Data represent mean $\pm$ SEM from at least three independent experiments. Significance of differences was determined by t-tests, with $p < 0.05$ considered significant.

RESULTS

Akt signaling in EGFR^{high} cells in the embryonic cerebral cortex

A subset of precursors in the mouse cortex expresses a high level of EGF receptors beginning at approximately E15 in precursors near the PSB (Caric et al., 2001). TGF α , a ligand for the EGFR, is also expressed at the PSB (Assimacopoulos et al., 2003). At E15, $58.9 \pm 2.1\%$ (n= 2 brains) of the EGFR^{high} cells near the PSB can be labeled with an antibody that recognizes phosphorylated substrates of Akt (Fig 1; pAkt substrate). $10.3 \pm 3.2\%$ of the EGFR^{high} cells were also labeled with pAkt(Ser473) antibody, another indicator of activated Akt. These observations suggest that EGFR signaling via Akt is active in some of the EGFR^{high} cells in vivo at this age. The larger proportion of pAkt substrate-labeled cells could reflect differences in the latency/duration of substrate versus Akt phosphorylation, and/or a difference in signal detection, since pAkt substrate staining includes many more antigenic targets than pAkt.

We reported previously that EGF receptor-mediated self-renewal depends on Akt signaling, since reducing Akt signaling in precursors with a dominant-negative form of Akt reduced self-renewal stimulated by EGF (Sinor and Lillien, 2004). In the present study we asked

whether EGFRs and EGFR tyrosine kinase activity are necessary for responses associated with elevated Akt-1 expression.

Elevated Akt1 depends on EGFRs for self-renewal, but not for survival or proliferation

Cortical precursors induced to express a higher level of Akt1 exhibited increased Akt activity when stimulated with a growth factor cocktail that included EGF (Sinor and Lillien, 2004). Increasing Akt1 expression also enhanced survival, proliferation, and the self-renewal of multipotent precursors. To determine whether EGFR-mediated signaling is necessary for generating these responses, we examined responses to elevated Akt1 expression in precursors from EGFR receptor mutant mice (Threadgill et al., 1995). For these experiments, precursors in explants of E13–13.5 EGFR wt (+/+), heterozygous (+/-), or null (-/-) dorso-lateral cortex were infected with control or Akt-1 retroviruses and cultured for 4 days. Cell death was assessed by staining with an antibody to activated caspase 3, while proliferation was analyzed with an antibody to PCNA. Elevated Akt1 expression could still reduce cell death (Fig 1 A) and stimulate proliferation in EGFR-null precursors, although the magnitude of the increase in proliferation was smaller than that observed wild-type cells (Fig 2 B).

Capacity for self-renewal was determined by culturing EGFR^{+/+} or EGFR^{+/-} cells in EGF, or by culturing EGFR^{+/+}, EGFR^{+/-}, or EGFR^{-/-} cells in FGF2 for 10 days to generate primary neurospheres; these were dissociated and cultured for an additional 10 days to generate secondary neurospheres. In EGFR^{+/-} precursors, elevating Akt1 expression did not increase the generation of primary (Fig 2C) or secondary (Fig 2E) neurospheres in response to EGF, compared to EGFR^{+/+} precursors. When tested with FGF2, the generation of primary (Fig 2D) or secondary (Fig 2 F) neurospheres by control virus-infected EGFR^{-/-} and EGFR^{+/-} precursors was reduced compared to EGFR^{+/+} cells. Elevating the level of Akt1 expression could not compensate for this deficiency.

These results indicate that expression of a sufficient level of EGFRs is critical for self-renewal, but not for proliferation in general. Moreover, the ability of elevated Akt1 to increase self-renewal depends on EGFRs. The increase in survival observed after elevating Akt1 does not require EGFRs, suggesting that survival effects of elevated Akt1 reflect the action of a different extrinsic signaling pathway(s).

It should be noted that the reduction in primary and secondary neurospheres generated in response to FGF2 that we observed among control virus-infected EGFR^{+/-} and EGFR^{-/-} precursors differs from results reported by others (Tropepe et al., 1999). The reduction in response to FGF2 we observed was not restricted to virus-infected precursors, because we also saw a reduction of FGF2-induced neurospheres among uninfected EGFR^{-/-} precursors. For example, EGFR^{-/-} cortex generated 115.6 ± 13.6 primary neurospheres, per 50,000 cells, compared to 181.1 ± 20.9 neurospheres per 50,000 cells from wild-type cortex ($N = 3$, $p = 0.02$). It is possible that differences in genetic backgrounds of EGFR-mutant mice account for differences in FGF-responsiveness (CD1 in the present study, 129/B6 in Tropepe et al. (1998). It is also not clear whether tissue studied in Tropepe et al. (1998) included ventral telencephalic precursors, rather than just cortical precursors as in the present study.

In the assays described above, EGFR signaling was reduced or lost in EGFR^{+/-} or EGFR^{-/-} precursors, respectively, during the assays for self-renewal. To inhibit EGFR signaling reversibly, we used a pharmacological inhibitor of EGFR tyrosine kinase activity, AG 1478 (100 nM), in explants of wild-type cortex; DMSO was used as a control. The AG1478 and DMSO were removed during the assays for self-renewal. Efficacy of EGFR activity block and recovery of EGFR activity were confirmed by staining for phospho-EGFR (Tyr-1068)

antibody, as described in Material and Methods (data not shown). As shown in Fig 3, elevated Akt1 still enhanced survival (Fig 3A) and proliferation (Fig 3B) when EGFR tyrosine kinase activity was inhibited with AG1478. We observed a lasting reduction in the ability of elevated Akt1 expression to enhance self-renewal in response to either EGF or FGF2 (Fig 3 C–F), even though the inhibitor of EGFR activity was removed during the assays of self-renewal. These findings are comparable to results obtained with EGFR mutant cells (Fig 2).

Elevated Akt1 induces expression of EGF receptors

In addition to its effects on survival, proliferation, and self-renewal in wild-type cells, we found that elevated Akt1 increased the expression of EGF receptors, assayed 4 days after infection of E13–13.5 precursors (Fig 4). Inhibition of EGF receptor tyrosine kinase activity did not reduce the proportion of EGFR^{high} precursors after infection with either control or Akt1 viruses. These results have several implications. First, the reduction of self-renewal following transient exposure to AG1478 (Fig 3 C–F) is not due to the loss of EGFR^{high} cells, but to a reduction in their capacity for self-renewal. Second, the result suggests that an extrinsic factor other than an EGF family member acts upstream of elevated Akt1 to induce EGFR expression. Up-regulation of EGFR expression can be induced in cortical precursors by FGF2 (Lillien and Raphael, 2000) or Shh (Viti et al., 2003a), making them candidates for the factors that act via elevated Akt1 to increase EGFR expression.

Akt1 sensitizes precursors to a sub-threshold concentration of Shh

Pten is a negative regulator of Akt1 activity, and it has been reported that loss of Pten expression can sensitize precursors to lower concentrations of FGF2 (Groszer et al., 2006). The expression of EGF receptors by embryonic cortical precursors can be induced by either FGF2 or Shh, but this response requires a higher concentration of ligand than proliferation (Lillien and Raphael, 2000; Viti et al., 2003). Examination of mice that lack the Hh receptor smoothened revealed a striking reduction in EGFR expression at E17–18 (Gulacsi and Lillien, 2006), suggesting that during late embryonic stages of development, Hh signaling is the more physiologically relevant inducer of EGFR expression in cortical precursors than FGF2.

To determine whether expression of elevated Akt1 can sensitize precursors to a concentration of Shh that is not normally sufficient to induce EGFR expression, we infected precursors in explants of E11–12 CD1 dorsolateral cortex with control or Akt1 viruses and exposed them to a concentration of Shh 10-fold lower than that required to up-regulate EGF receptor expression (Viti et al., 2003). Precursors expressing elevated Akt1 were able to respond to this sub-optimal concentration of Shh as though it were 10-fold higher to induce premature expression of EGFRs (Fig 5). Note that the explants used for these experiments were 1–2 days younger (E11–12 + 4 days, equivalent to E15–16) than the explants used in Fig 4 (E13.5 + 4 days, equivalent to E17–18). In the older explants, an increase in EGF receptor expression was observed in Akt1-infected precursors even in the absence of exogenous Shh (Fig 4). In previous studies, we compared the time interval needed for EGFR^{high} cells to develop in explants prepared from cortex at different embryonic ages and noted that a longer period of time was required for precursors in younger (E12) compared to older (E15) tissue (Burrows et al., 1997; Lillien and Raphael, 2000).

Hh signaling is required for elevated Akt1 to increase expression of EGF receptors and self-renewal

To determine whether Hh signaling is required for responses associated with elevated Akt1 expression, we used precursors from conditional mutants of the Hh receptor smoothened. This approach was chosen over the use of a pharmacological inhibitor of Hh signaling

because recent reports indicate that these inhibitors have off-target effects (Yauch et al., 2008). To generate smoothened wild-type, heterozygous, and null cortical precursors, *Emx1-cre;smo^{+/+}* or *Emx1-cre;smo^{+/-}* mice were bred with *smo^{flox/flox}* mice, resulting in the deletion of smoothened in cortical precursors by embryonic day 12 (Gorski et al., 2002). Explants of E12.5–13.5 cortex from smoothened wild-type, heterozygous, or null embryos were infected with control or Akt1 retroviruses and the ability of elevated Akt1 to stimulate proliferation, induce EGFR expression, and increase self-renewal were assayed as described above.

We found that elevated Akt1 was still able to increase proliferation in smoothened-null precursors ($1 \pm 0.5\%$ of control-infected cells were Ki67+ compared to $6 \pm 0.5\%$ of Akt1-infected cells; $N=8$, $p<0.0001$), but elevated Akt1 could not increase their expression of EGFRs (Fig 6 A) or enhance their self-renewal in response to EGF (Fig 6 B). The failure of elevated Akt1 to increase EGFR expression and self-renewal was also observed in smoothened- heterozygous precursors. This could reflect a failure to achieve a sufficiently high level of Hh signaling to activate a high threshold response such as EGFR expression, even when Akt1 was also elevated.

It has been reported that Hh signaling can activate the PI3K/Akt pathway (for example, Peltier et al., 2007; Kanda et al., 2003), though this appears to reflect an indirect mechanism involving Hh-regulated expression of components of signaling pathways known to activate the PI3K/Akt pathway, such as PDGF receptors (Xie, et al., 2001) or IGF2 (Hahn et al., 2000). To assess Akt signaling in smoothened-null precursors, we stained sections of wild-type and smoothened-null E16 cortex with pAkt substrate antibody (Fig 6, C), and counted the number of labeled precursors (VZ + SVZ) in the lateral cortex near the PSB in an area 150 μm wide (in the lateral/medial plane) and 100 μm deep. No significant reduction in the number of pAkt substrate⁺ precursors was observed in the smoothened-null cortex (35.9 ± 8.9 pAkt substrate⁺ cells in smoothened-null cortex, compared to 46.9 ± 1.6 in wild-type cortex; $n=2$ brains per genotype; $p=0.35$). These observations suggest that loss of Hh signaling does not reduce Akt signaling generally in embryonic cortical precursors.

DISCUSSION

Precursors in the embryonic mouse cortex represent a heterogeneous population of cells that can be distinguished by a number of molecular features that control their ability to divide and produce specific types of progeny. Some of these differences reflect their exposure to distinct kinds, concentrations, and/or combinations of factors in their environment. Cell intrinsic differences in precursors also influence whether and how they respond to environmental factors. In the present study, we focused on a difference in a subset of precursors that influences the way they respond to Hh. Our results suggest that this sets up an additional difference in precursor expression of EGFRs, which alters their responses to EGF family ligands. The subset of precursors that expresses a higher level of EGF receptors includes multipotent stem/TA cells. The expression of EGFRs by stem cells is thought to reflect an “activated” state (Pastrana et al., 2009) in which their numbers expand and their progression to a TA state occurs, but stimulation of TA cells with EGF has also been reported to induce TA cells to behave like stem cells (Doetsch et al., 2002). Our results link high Akt1 expression, Hh signaling and EGFR signaling to the maintenance or acquisition of stem cell properties by these precursor subsets (Fig 7).

Akt1 protein is expressed at a high level in a small subset of precursors in the cortical ventricular zone at E13.5 (Sinor and Lillien, 2004). It is not clear what controls their elevated expression of Akt1, but β -catenin (Dihlmann et al., 2005) and NF-kappa B (Meng and D’Mello, 2003) have been reported to promote Akt1 transcription in other types of cells.

In cortical precursors, elevated Akt1 expression results in increased Akt kinase activity (Sinor and Lillien, 2004), though the mechanism responsible for this change in Akt activity has not yet been defined. In the present study we showed that elevated Akt1 expression sensitized precursors to a sub-threshold concentration of Shh. Akt activity is also increased in precursors from Pten-null cortex (Groszer et al., 2001), and this is associated with their sensitization to lower concentrations of FGF2 (Groszer et al., 2006).

FGF2 signaling can activate PI3K signaling upstream of Akt1 (Eswarakumar et al., 2005), but the mechanism of interaction between Hh signaling and Akt activity is less well characterized. Studies in other types of cells have shown that Akt1 kinase activity can modify responses to Hh signaling in several ways. For example, Akt can promote Hh signaling by modulating PKA-induced inactivation of the Hh mediator Gli2 (Riobo et al., 2006a). Elevated Akt could thereby enhance the efficacy of low concentrations of Shh in mid-embryonic cortical precursors to elicit responses that would otherwise require a higher concentration of Shh. This explanation for the effect of elevated Akt1 on Hh signaling raises several issues. It assumes that Akt is activated prior to Hh signal initiation, implying that another factor that acts via PI3K precedes Hh signaling. There are many candidates for such a factor in the developing cortex, including FGF2 (Qian et al., 1997) and IGF-1 (Mairet-Coello et al., 2009). These factors can act on cortical precursors in a Hh- and EGFR-independent manner, making them candidate mediators of responses to elevated Akt that did not depend on Hh signaling or EGFRs, such as proliferation.

The mechanism by which elevated Hh signaling can increase the expression of EGF receptors is not clear. The Hh mediator Gli2 has been reported to activate transcription of Sox2 in telencephalic precursors (Takanaga et al. 2009), and Sox2 has been reported to up-regulate transcription of EGF receptors in embryonic cortical precursors (Hu et al., 2010), raising the possibility that Sox2 might serve as an intermediate in the regulation of EGFR expression by Hh/Gli2 signaling. Gli2 can also act downstream of non-Hh factors, including FGF2 (Brewster et al., 2000). Gli2 might therefore represent the point of integration for two of the extrinsic factors that can up-regulate EGFRs in cortical precursors. Consistent with this idea, Gli2 mutants exhibit a reduction in EGF receptor expression by cortical precursors (Palma and Ruiz y Altabla, 2004).

The roles of Hh and EGF receptor signaling in the regulation of stem cell self-renewal have been the subject of a number of studies. Sox2 is critical for self-renewal in many types of stem and TA cells (Avilion et al., 2003), including those in the embryonic cortex (Graham et al., 2003). It is expressed following activation of EGFR (Hu et al., 2010) and Hh signaling (Takanaga et al., 2009), suggesting that either pathway alone could act via Sox2 to increase self-renewal. Synergistic interactions between Hh and EGFR pathways have also been reported (Palma et al., 2004; Kasper et al., 2008), suggesting that the two pathways might also co-operate to increase self-renewal.

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Fig 1.

Akt signaling is active in EGFR^{high} precursors at the PSB. Cells in sections of E15 mouse cortex were stained with anti-EGFR (A) and anti-phospho-Akt (pAkt) substrate (B) antibodies. Arrows point to cells that are double-labeled for EGFR and pAkt substrate. 58.9±2.1% of the EGFR^{high} cells at the PSB were double-labeled with pAkt-substrate antibody (n=2 brains). Sections from the same brains were double-labeled with pAkt(Ser473) and EGFR antibodies. 10.3±3.2% of the EGFR^{high} cells at the PSB were also pAkt(Ser473)⁺. Bar = 50 μ m.

Fig 2.

Elevating Akt1 expression in EGF receptor mutant cortical precursors increased survival (A, activated caspase), and proliferation (B, PCNA) but could not maintain stem/transit amplifying cells in EGF (C, primary neurospheres, E secondary neurospheres) or FGF2 (D, primary neurospheres; F, secondary neurospheres). Precursors in explants of E13.5 EGFR wild-type (+/+), heterozygous (+/-) or null (-/-) cortex were infected with control or Akt1 viruses and assayed 4 days later. N=4 experiments for panels A and B; N= 5 for panel C; N=8 for panel D; N=3 for panels E and F. * $p < 0.04$; ** $p \leq 0.009$.

Fig 3.

Elevating Akt1 expression in cortical precursors treated transiently with AG1478 (100 nM) to inhibit EGFR tyrosine kinase activity still increased survival (A, activated caspase) and proliferation (B, PCNA), but did not maintain stem/transit amplifying cells in EGF (C, primary neurospheres; E secondary neurospheres) or FGF2 (D primary neurospheres; F secondary neurospheres) even when the inhibitor was removed during the neurosphere assays. Precursors in explants of E13.5 CD-1 cortex were infected with control or Akt1 viruses and assayed 4 days later. N= 3 experiments for panel A; N= 4 for panel B; N=8 for panels C and D; N=7 for panel E; N=6 for panel F. * $p < 0.03$; ** $p \leq 0.008$, *** $p \leq 0.0007$.

Fig 4.

Elevated Akt1 induces expression of EGFRs, even when EGFR tyrosine kinase activity is inhibited with AG1478 (100nM). Precursors in explants of E13.5 CD-1 cortex were infected with control or Akt1 viruses and assayed 4 days later. N= 3 experiments. * $p \leq 0.0001$.

Fig 5.

Elevated Akt1 sensitizes precursors to a low concentration Shh. Explants of E11–12 CD-1 dorso-lateral cortex were treated with a 10-fold lower concentration of Shh than required to induce EGFR expression (Viti et al., 2003). In contrast to control-infected cells, Akt1-infected cells could respond to a low concentration of Shh by increasing their expression of EGF receptors. (N=4 experiments). * $p \leq 0.003$.

Fig 6.

Elevated Akt1 does not increase EGFR expression (A) or generation of primary neurospheres (B) by precursors from smoothened mutant cortex. Loss of these responses was observed even in smoothened heterozygous cells. Explants of E12.5–E13.5 smoothened wild type ($Emx1^{cre/+}; smo^{+/+}$), heterozygous ($Emx1^{cre/+}; smo^{flox/+}$), and null ($Emx1^{cre/+}; smo^{flox/-}$) cortex were infected with control or Akt1 viruses and assayed 5 days later for expression of EGF receptors and generation of neurospheres in response to EGF (10 ng/ml). $N=5$ experiments for panel A, $N=5-9$ for panel B. * $p=0.02$, ** $p\leq 0.0005$. (C) Sections of wild-type and smoothened-null E16 cortex were stained with anti-pAkt substrate antibody to assess the status of Akt signaling. No significant difference was observed in smoothened-null cortex with respect to the number of pAkt substrate⁺ precursors (VZ + SVZ) in a 150 μm wide $\times$ 100 μm deep region near the PSB at a mid-anterior/posterior level (46.9 ± 1.6 pAkt substrate⁺ cells in wild-type, compared to 35.9 ± 8.9 in smoothened-null cortex, $n=2$ brains per genotype). LV, lateral ventricle; LGE, lateral ganglionic eminence. Bar = 25 μm .

Fig 7.

Schematic illustrating possible mechanisms of interaction between Akt, EGF receptor, and Hh signaling. Akt signaling could be important for promoting the self-renewal of stem/TA cells at several steps during embryonic development. For example, early (E13–14) cortical precursor cells that express elevated/activated Akt interpret a low concentration of Shh as though it were higher, leading to up-regulation of EGF receptor expression. At a later stage (E15–16) when the EGFR is expressed at a high level, Akt signaling could act downstream of EGFR activation to promote self-renewal. Precursors from EGFR-null, smoothed-null, and EGFR kinase-inhibited precursors all lack sufficient EGFR activity to allow Akt signaling to promote self-renewal.