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Regulation of neural stem cell differentiation in the forebrain

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Summary In the developing forebrain, mounting evidence suggests that neural stem cell proliferation and differentiation is regulated by growth factors. In vitro in the presence of serum, stem cell proliferation is predominantly mediated by fibroblast growth factor-2 (FGF-2) whereas neuronal differentiation can be triggered by FGF-1 in association with a specific heparan sulphate proteoglycan. On the other hand, astrocyte differentiation in vivo and in vitro appears to be dependent on signalling through the leukaemia inhibitory factor receptor (LIFR). The evidence suggests that in the absence of LIFR signalling, the stem cell population is present at approximately the same frequency and can generate neurons but is blocked from producing astrocytes that express glial fibrillary acidic protein (GFAP) or have trophic functions. The block can be overcome by other growth factors such as BMP-2/4 or interferon-γ, providing further evidence that the inhibition to astrocyte development does not result from loss of a precursor population. Signalling through the LIFR, in addition to stimulating astrocyte differentiation, may also inhibit neuronal differentiation, which may explain why this receptor is expressed at the earliest stages of neurogenesis. Another signalling system which also exerts its influence on neurogenesis through active inhibition is Delta-Notch. We show in vitro that at high cell densities which impede neuronal production by FGF-1, lowering the levels of expression of the receptor Notch by antisense oligonucleotide results in a significant increase in neuronal production. Thus, stem cell differentiation appears to be dependent on the outcome of interactions between a number of signalling pathways, some which promote specific lineages and some which inhibit.

Key words: cortical development, glia differentiation, growth factors, leukaemia inhibitory factor, neuron differentiation.

Introduction

Concept of the neural stem cell

The concept of a stem cell whose properties include the ability to give rise to a multitude of cell types and to self-renew has been well established in many systems, particularly the haematopoietic system, and yet it is only recently been deemed applicable to the central nervous system. This was partly because we were unable to grow stem cells in vitro or to monitor their progeny in vivo. Perhaps a more important impediment to the acceptance of the stem cell concept has been the unwillingness to embrace the property of self-renewal, mainly because this implied an ongoing presence of stem cells in the mature nervous system.

The last few years has, however, provided cogent support for this concept through the ability to grow populations of stem cells in vitro and then, more importantly, to clone individual stem cells and formally demonstrate their multipotentiality and self-renewal properties; the use of retroviral markers to demonstrate the multipotential character of stem cells in vivo; and finally, the identification of stem cells in the brains of animals at times beyond the neurogenic period and into adulthood, confirming the considerable extent of self-renewal within the stem cell population.

It is not the intention of the present review to give an overview of stem cell biology because we have done this elsewhere; instead we will focus on results, predominantly from our own laboratories, which address the mechanisms regulating the differentiation of stem cells in the forebrain of embryonic and adult mice.

Fibroblast growth factor and stem cell regulation

Proliferation

The first suggestion that the fibroblast growth factor (FGF) family may influence stem cell growth came from in vitro studies which demonstrated that of a variety of growth factors tried, FGF-2 and FGF-1, in the presence of serum and insulin-like growth factor (IGF)-1, were the most effective agents in stimulating cell division in populations of neuro-epithelial cells obtained from the embryonic day-10 (E10) mouse forebrain. Although it was demonstrated that both neurons and astrocytes could be generated from dividing cells, no conclusion could be drawn about the
nature of the precursor because the experiments were performed at high cell density.

The subsequent demonstration that FGF-2 could be used to stimulate single cells from E10 neuro-epithelium to produce clones consisting of several thousand cells which, in the presence of a glial-derived conditioned medium, produced neurons in addition to astrocytes, confirmed the suspicion that FGF-2 could stimulate the proliferation of stem cell populations.\(^4\) The frequency of precursors with the ability to give rise to clones of a significant size (> 100 cells) was on average ~5% of the population. Close examination of the proliferation curves obtained from bulk cultures stimulated with FGF-2\(^4\) also suggested that the majority of cells generated at the end of a 3-day culture period arose from a small subpopulation of cells no larger than 10%. A similar frequency of cortical clones was observed under culture conditions that did not include serum.\(^10\) Thus, approximately one in 10 cells in the E10 cortical neuro-epithelium has the ability to generate a large number of progeny even though it is clear that > 99% of cells at this stage are dividing.\(^5\) The remaining dividing cells appear to undergo fewer divisions and generate a small number of progeny, as is evident using retroviral markers to identify cortical clones in vivo. Alternatively, they do not respond to FGF-2. Clearly, there is a hierarchy in both the proliferative and lineage potential of precursor cells within the developing forebrain, but a hallmark of the true stem cell is their ability to generate large numbers of progeny. In addition, the FGF-2 responsive clones have the property of self-renewal: > 80% of the clonal progeny cells gave rise to new clones.\(^6\)

### Differentiation

Although our initial studies of high-density cultures indicated that FGF-2 could generate neurons, especially at higher concentrations, subsequent clonal examination revealed that the FGF-2-stimulated clones in the presence of serum rarely produced neurons, although astrocytes did arise.\(^4\) Subsequent studies revealed that neuronal differentiation could be inhibited by FGF-2, which appears to predominantly drive proliferation. Earlier, however, it was observed that immortalized precursors from the E10 forebrain did differentiate in response to FGF-2,\(^4\) and subsequent studies in serum-free medium\(^10\) have shown that cortical clones generated in high doses of FGF-2 contain both neurons and oligodendrocytes. It was also reported that clones generated in low levels of FGF (0.1 ng/mL) contained only neurons, as did the small number of clones arising without FGF-2.\(^10\) This suggests several things: first, that serum inhibits neuronal generation and second, the level of FGF-2 determines the cell-type constituents of the clone. However, closer examination of these results reveals their similarity to the serum-generated clones because the number of neurons generated per clone, regardless of FGF-2 concentration and clone size, is small (average of 15). Thus, like the serum-derived clones, FGF-2 at higher concentrations, which generate substantial-sized clones, appears to inhibit further neuronal production by the stem cell. Whether low doses of FGF-2 actually induce neuronal differentiation seems, as the authors state, \(^11\) unlikely, but it is compatible with a primary role in expanding the precursor population. In addition, like the serum-plus FGF-2 clones, the large serum-free clones contain a majority of glial cells. There is, however, one major difference: the serum-plus clones contain astrocytes with virtually no oligodendrocytes whereas the serum-free clones are almost exclusively comprised of oligodendrocytes. Astrocyte production requires additional factors provided by glial conditioned medium, which as we will discuss, may function by stimulation through the leukaemia inhibitory factor (LIF) receptor complex. It is well established that oligodendrocyte production is enhanced by serum-free conditions.\(^13\)

Thus, it appears that the production of neurons from the majority of precursors within a large clone requires additional factors to FGF-2; Ghosh and Greenberg showed that neurotrophin-3 (NT-3) could stimulate neurogenesis in FGF-2-stimulated cultures,\(^12\) and we have shown that conditioned medium from an astrocyte cell line can result in a significant number of FGF-2 expanded clones producing neurons after FGF-2 withdrawal.\(^4,13\) One strong candidate for providing a neurogenic stimulus was FGF-1 since we had demonstrated that it was expressed at E11 just as neurogenesis begins in the mouse cortex; whereas FGF-2 was present much earlier at E9.5 prior to the commencement of neuronal production. However, initially we were not able to demonstrate a differential action of FGF-1 compared to FGF-2 in our cultures regardless of the presence or absence of heparin. Nevertheless, Guillemot and Cepko had shown that FGF-1 was far more potent than FGF-2 in promoting the differentiation of retinal ganglion cells.\(^14\)

### Role of heparan sulphate proteoglycans in FGF responsiveness

In the process of demonstrating that FGF-1 and FGF-2 were produced by neuro-epithelial cells from mouse forebrain, it was discovered that the majority of the FGF was bound to a single dominant heparan sulphate proteoglycan (HSPG)\(^15,16\) which we have since identified as a variant of Perlecan.\(^17\) The most interesting finding, however, was the binding specificity of this HSPG isolated from the developing forebrain at different times: HSPG from E10 brains (HS-1) preferentially bound FGF-2, whereas HSPG from E12 brains (HS-1) preferentially bound FGF-1.\(^15\) Recently we have shown that this shift is associated with an increase in the number of sulphated domains and increased heparan sulphate glycaminoglycan (HS) side-chain length.\(^18\) It is known that the charge-domains created by sulphation are critical to FGF-1 and FGF-2 binding and also are thought to influence interaction of FGF with its cognate receptor(s). One current hypothesis which we favour is that HS serves to couple FGF to specific HS-binding regions on specific FGF receptors (FGFR) to form an activated signalling complex of FGF/HS/FGFR.

It was found that precursor proliferation in high-density cultures was significantly enhanced when FGF-1 was used with HS-1, or FGF-2 with HS-2.\(^15\) confirming the importance of this type of presentation mechanism. It
suggests there is either a different array of receptors, or present FGF-1 in the appropriate manner. There is endogenous HSPG on the cell membrane which can produce neurons in clonal cultures in response to FGF-2 is FGF-1 can stimulate neuronal production in these clones, contained within the E17 forebrain population.6 The ex-200 cells plated. Again, recent experiments have shown that its embryonic counterpart, forming large undifferentiated population of precursors within the adult forebrain which could be stimulated to produce neurons in response to FGF-7. More recent clonal experiments using precursor from the SVZ of the lateral ventricle of adult mice have shown that the adult stem cell responds in a similar way to its embryonic counterpart, forming large undifferentiated clones in response FGF-2 at the frequency of ~ 1 in every 200 cells plated. Again, recent experiments have shown that FGF-1 can stimulate neuronal production in these clones, but does not require the addition of HS-1 to these cultures (GJ Brooker and PF Bartlett, unpubl. obs. 1998). This suggests there is either a different array of receptors, or there is endogenous HSPG on the cell membrane which can present FGF-1 in the appropriate manner.

The only stem cell population we have found that produces neurons in clonal cultures in response to FGF-2 is contained within the E17 forebrain population.6 The explanation for this is not clear, but it may indicate that this precursor population isolated just after the termination of neurogenesis has received the appropriate signals prior to isolation, whereas the precursor from the adult has resumed a more embryonic state.

Factor regulation of astrocyte differentiation

As discussed in the previous section, there is good evidence for the bi-potential stem cell’s choice of lineage being determined, at least in part, by environmental factors such as growth factors. Previously we had shown, in vitro, that leukaemia inhibitory factor (LIF) could stimulate precursors from the E10 spinal cord to express glial fibrillary acidic protein (GFAP).19 In addition, the present study also showed that antibodies to the LIF receptor (LIFR) significantly reduced the number of astrocytes that developed in the absence of exogenous growth factors, suggesting that endogenous ligands acting through the LIFR influence astrocyte development. Other ligands that signal through the LIFR complex (a heterodimer composed of LIFR and gp130) such as ciliary neurotrophic factor (CNTF), also have been shown to promote GFAP expression in central nervous system (CNS) precursor populations.20 Thus, the in vitro results strongly suggest that ligands that signal through the LIFR complex may have a role in regulating astrocyte differentiation.

The role of LIFR in regulating astrocyte production was supported by the demonstration that E19 embryonic mice with a targeted disruption of the low-affinity LIF receptor gene, which appear to have normal CNS development, have a deficiency of GFAP-positive cells in the developing hindbrain.21 Unfortunately, because these animals die at E19 (which is just 2 days after the first appearance of GFAP22) it was difficult to determine whether this astrocyte deficiency was due to general retardation in development or a failure in astrocyte generation due to lack of signalling through the LIF receptor. To explore these possibilities further, the properties of precursor cells from the forebrain of LIFR-deficient mice were examined in vitro.23 It was shown that precursors from the forebrains of mice homozygous for the LIFR null mutation (LIFR−/−) failed to generate significant numbers of GFAP-positive cells even after 3 weeks in vitro. To determine if the lack of GFAP expression in LIFR−/−precursors fully reflected a failure in astrocyte development, an assay to assess astrocyte function was performed. Previously it has been shown that astrocytes promote neuronal differentiation and/or survival in a number of systems;4,24 thus, the ability of established monolayers derived from LIFR +/+ , +/–, and −/− forebrains to support the neuronal differentiation was tested. No difference was found in the number of neurons produced on the LIFR +/+ or +/– monolayers, but there was ~10-fold fewer neurons found on the LIF−/− monolayers.23 The study showed that signalling through the LIFR is required for the generation of functional astrocytes, not just for the expression of GFAP. This is an important point because it has recently been shown that one of the downstream signalling pathways activated by signalling through LIFR, the JAK-STAT pathway, can directly activate the GFAP gene. It has been shown that STAT 3 can directly bind to a consensus site in the promoter region of the GFAP gene.25 Thus, the regulation of GFAP expression can be directly regulated through the LIFR complex; both LIFR and gp130 appear to be required for this signal.23

It was subsequently shown that the precursor population in the LIFR−/− forebrain was in fact present because stimulation with bone morphogenetic protein (BMP)-2, a member of the transforming growth factor-β (TGF-β) growth factor family previously shown to stimulate GFAP expression in astrocytes, contained a significant percentage of GFAP-positive cells after 10 days in vitro. In addition, long-term passaging in vitro (> 5 weeks) revealed significant numbers of GFAP cells in LIFR−/− cultures which supported neuron generation and/or survival.23 We also found that there was no decrease in the total number of neural clones generated from the LIFR−/− mouse forebrain precursors with FGF-2; also strongly suggesting that LIFR signalling was not essential for the maintenance of precursor cells. As mentioned in the previous section, we had shown that FGF-2-stimulated forebrain precursors...
have the ability to generate two types of clones: clones that contain both neurons and glia, or clones restricted to astrocytes. However, because the frequency of neuron-containing clones generated with FGF-1 and HSPG-1 is also unaltered in the LIFR−/− population, it suggests that there is no change in the relative frequency of either the bipotential or astrocyte-restricted clones in these animals.

The question arises as to whether signalling through the LIF receptor instructs a precursor to become committed to the astrocyte pathway. Several pieces of evidence support such an hypothesis: first, it has been shown that in the presence of LIF > 80% of precursors become GFAP positive in vitro; second, that STAT-3, which is directly activated by LIFR signalling, can bind to the promoter region of the GFAP gene and regulate its expression; and third, that stimulation with LIF can significantly inhibit the neuronal differentiation of clones (GJ Brooker and PF Bartlett, unpubl. obs. 1998). This latter finding is also true in clones derived from adult subventricular zone (SVZ). Although this favours the idea that signalling through the LIFR may actively promote astrocyte differentiation, an alternative interpretation is that LIFR signalling may inhibit neuronal differentiation leading to astrocyte production by default. Thus, it may be that LIFR signalling actively keeps the precursor in an undifferentiated, or stem cell state: as it does for pluripotential embryonic stem cells. Thus, neurogenesis may result from individual stem cells overcoming this inhibitory signal. A candidate for mediating this type of action is the recently discovered suppressors of cytokine signalling (SOCS) family, which have been shown to inhibit signalling through the LIFR.

It is not known which LIFR ligand mediates this effect; we have shown that mice with a targeted deletion in the LIF gene have reduction in the number of astrocytes in the hippocampus but it is in no way complete.23 Because CNTF also has been shown to promote astrocyte formation, it also may play a part. Also, other ligand–receptor pathways may replace LIFR at later stages of development. The finding that long-term cultures from LIFR mice do ultimately start to express GFAP and are functionally active supports this idea, as do recent experiments in which portions of LIFR−/− brains were transplanted to a syngeneic recipient and shown to contain GFAP cells several weeks after transplantation (PF Bartlett and AR Harvey, unpubl. obs. 1998).

**Neuronal differentiation by DisInhibition**

The concept raised in the previous section whereby neurogenesis results from overcoming signals that favour the maintenance of a stem cell state is best exemplified by the action of the neurogenic genes Delta and Notch, which code for a cell surface ligand and receptor, respectively, through a process of lateral inhibition which prevents adjacent precursors from differentiating. This process has been well demonstrated to regulate neurogenesis in *Drosophila* and *Xenopus*, and more recently it has been shown to play a role in mammalian retinal differentiation.22

The key step in this phenomenon is the ability of a single precursor to express more of the ligand Delta than its neighbours, thereby activating the neighbour’s Notch receptor signalling pathway, which inhibits neurogenesis by inhibiting the production of the helix–loop–helix transcriptional regulators Neurogenein and Neuro-D; which in turn regulate Delta levels. To investigate whether the action of the growth factors FGF-2 and FGF-1 could influence this pathway, we have begun to examine neuronal production in high cell density conditions where, as we have previously shown,1 FGF-1 and FGF-2 promote proliferation rather than neuronal differentiation. When Notch-1 expression is reduced by the addition of antisense oligonucleotides to the cultures, it was found that in the presence of FGF-1, but not FGF-2, there was a significant increase in the number of neurons generated (CH Faux, A Turnley and PF Bartlett, unpubl. obs. 1998). Again, this demonstrates the predilection of FGF-1 to promote neuronal differentiation compared to FGF-2 (at similar concentration). It also suggests that neurogenesis in vivo may require both inhibition of Notch signalling and activation of FGF receptor signalling, although there may be a common mechanism whereby Notch expression is further reduced by FGF-1 signalling to levels below the threshold for inhibition. All these possibilities are presently being explored.

**Inhibitory mechanisms in stem cells in the adult SVZ**

We have recently obtained evidence for a similar inhibitory mechanism acting on the precursor population in the adult SVZ.

As reported by Lois and Alvarez-Buylla,28 explants of SVZ grown in vitro do not generate neurons from dividing cells. However, we have recently shown that the dividing cells within the explant have the propensity to give rise to neurons when dissociated and replated at clonal or low cell density. Replating at high cell density leads to inhibition of neurogenesis. The results suggest an inhibitory mechanism similar to lateral inhibition and both Delta and Notch are expressed in the adult SVZ (CH Faux and PF Bartlett, unpubl. obs. 1998). Such inhibitory mechanisms may restrict the ability of precursors within the SVZ to generate neurons apart from those destined for the olfactory bulb. It could be postulated that the olfactory stream provides signals that may reduce these inhibitory effects.

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**References**
