Int. J. Dev. Biol. 46: 661-670 (2002)

Heparan sulfates isolated from adult neural progenitor cells can direct phenotypic maturation

HIRAM CHIPPERFIELD, KULDIP S. BEDI, SIMON M. COOL and VICTOR NURCOMBE*,[1]

School of Biomedical Sciences, Dept. of Anatomy and Developmental Biology and [1]ARC Special Research Center for Functional and Applied Genomics, University of Queensland, St Lucia QLD, Australia

ABSTRACT Multipotent progenitor stem cells that generate both neurons and glia are components of the hippocampus, subventricular zone and olfactory system of adult mammalian nervous system. The lineage choices any stem cell makes are known to be greatly dependent on the constitution of the extracellular matrix to which they are exposed during their development. Here, the adult rat hippocampus was used as a source of cells for clonal culture in order to investigate the effects of the extracellular glycosaminoglycan heparan sulfate (HS). Neurospheres were readily generated from adult tissue and could be used as a source of cells for further experiments. HS species that promote the actions of fibroblast growth factor-2 (FGF2) for embryonic neural progenitors were found to inhibit the actions of this mitogen for adult progenitors. Only HS fractions that promoted the actions of FGF1 had mitogenic effects on these adult cells. The adult cells proved difficult to clone from single cells. However, when endogenous HS was purified from these cells and added back at high concentration to single cells, the clones were capable of generating plentiful neuronal and glial progeny. The adult hippocampal progenitor (AHP) HS is composed of 32 kDa chains bearing 3 sulfated domains. A proportion of primary osteoblast stem cells exposed to the hippocampal HS adopt neuronal phenotypes. Hence, there appears to be a combination of HS-binding extracellular molecules that predispose cells to particular lineages.

KEY WORDS: *Neural stem cells, glycosaminoglycans, transdifferentiation, neuronal development, osteoblasts*

Introduction

A recent spate of *in vitro* studies strongly indicate that multipotent, self-renewing progenitors of neurons and glia can be isolated from several adult brain regions (Reynolds *et al.*, 1992; Richards *et al.*, 1992; Gage *et al.*, 1995; Rietze *et al.*, 2001). The demonstration that the adult brain contains stem cells raises the attractive possibility that endogenous neurogenesis may be manipulated to therapeutic advantage (Magavi *et al.*, 2000). Moreover, the cells seem to be remarkably plastic: adult hippocampal stem cells can give rise to regionally-specific cell types not only in the hippocampus, but also in the olfactory bulb, cerebellum, and retina (Gage *et al.*, 1995; Suhonen *et al.*, 1996; Takahashi *et al.*, 1998). They can even migrate considerable distances, especially after implantation into the neonatal brain (Brustle *et al.*, 1998). Thus, neural stem cells from both embryo and adult seem to be "re-programmable", an idea, drawn from haematopoeisis, that there is a generic, "naïve" brain stem cell capable of being manipulated. What is proving truly remarkable is the range of cell types that can be produced in a culture dish when the environment – growth or trophic factors, in combination with particular substrates - is configured appropriately.

These recent advances in the ability to isolate and culture adult neural progenitor cells are paving the way for the development of new therapies for a variety of neurological disorders and injuries (Brewer, 1999). Several identified growth factors, including LIF, EGF and FGF2, have been found to be necessary to trigger the proliferation of multipotent stem cells (Carpenter *et al.*, 1997; Carpenter *et al.*, 1999; Palmer *et al.*, 1999). The exact combination or cascade of growth factors necessary for this process seems to be largely determined by the stage of differentiation of the stem cells. FGF2 appears to be crucial for the continued proliferation of cells that have reached the stage of being committed to a neural fate (Palmer *et al.*, 1999) and different doses of this factor appear to trigger different phenotypes from the progenitor pool (Qian *et al.*, 1997; Qian *et al.*, 2000).

The FGF family is now known to contain over 20 related proteins that are involved in a diverse range of developmental processes, including germ layer and limb formation as well as in wound healing. FGFs 1 and 2 are the best characterized members of this

Abbreviations used in this paper: AHP, adult hippocampal progenitor; dp, degree of polymerization; FGF, fibroblast growth factor; HS, heparan sulfates.

***Address correspondence to:** Dr. Victor Nurcombe. Dept. of Anatomy and Developmental Biology, University of Queensland, St Lucia QLD, Australia 4072. Fax: +61-7-3365-1299. e-mail: v.nurcombe@mailbox.uq.edu.au

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

family. FGF2 in particular has a powerful mitogenic effect on a multitude of both ectodermal and mesodermal cell types. It plays an important role in neurogenesis in the cerebral cortex (Raballo *et al.*, 2000) but does not appear to be essential for the development of other CNS regions (Ortega *et al.*, 1998). Indeed, it has been speculated that other CNS regions may be influenced by different members of the FGF family (Ford-Perriss *et al.*, 2001).

FGFs signal through the dimerization of cognate FGF receptor (FGFR) tyrosine kinases. There are four FGFRs (FGFR1-4), each of which can be differentially spliced. FGFs show varying degrees of specificity and affinity for these different receptors (Ornitz *et al.*, 1996; Ornitz, 2000), affinities that can be further modulated by different forms of the glycosaminoglycan sugar heparan sulfate (HS). Both FGFs and their high affinity receptors have specific binding sites for HS and require it to form active complexes necessary for intracellular signaling mechanisms. The exact composition of the signaling complex and the role of HS in its creation remains uncertain. A number of *in vitro* studies have shown that separable HS chain isolates can either enhance (Nurcombe *et al.*, 1993; Gallagher, 1997; Guimond *et al.*, 1999; Ornitz, 2000) or inhibit (Guimond *et al.*, 1993; Rahmoune *et al.*, 1998) FGF signaling, depending on their microstructure.

We have previously isolated two HS isoforms, HS1 and HS2, that are expressed in the mouse brain during neurogenesis and neural differentiation respectively (Nurcombe *et al.*, 1993; Brickman *et al.*, 1995; Brickman *et al.*, 1998). The relative selectivities of the HSs matched the onset of synthesis of FGF2 and FGF1 in the embryonic brain tissue. These HS isoforms can modulate proliferation and migration in response to FGF1 and FGF2 in a variety of other cells, including cancer cells (Nurcombe *et al.*, 2000). One current hypothesis is that HSs serve to directly cross-link FGFs to specific HS-binding regions on competent FGF receptors (FGFRs) to form activating ternary complexes (McKeehan *et al.*, 1994; Ornitz, 2000). The details of these experiments are of broad interest because a vast number of developmentally relevant growth factors, adhesion factors and extracellular matrix molecules are also dependent on HS for the expression of their bioactivity (Perrimon *et al.*, 2000).

In this study we first detail the establishment of a self-renewing, multipotential neural progenitor cells from the adult rat hippocampus. We then examined the effects on proliferation of FGF1 and FGF2 on these precursor cells, in combination with a range of different HS chain preparations. It was further found that endogenous HS purified from these adult neural precursors could be used to establish true clonal populations. The remarkable potency of the precursor HS chains was further demonstrated through their effects on cells of mesodermal origin; osteoblast preparations exposed to the neural HS began to adopt neuronal phenotypes. The study thus confirms that particular HS forms can be used to direct the phenotype of uncommitted progenitors and cause committed cells to transdifferentiate across germ layer boundaries.

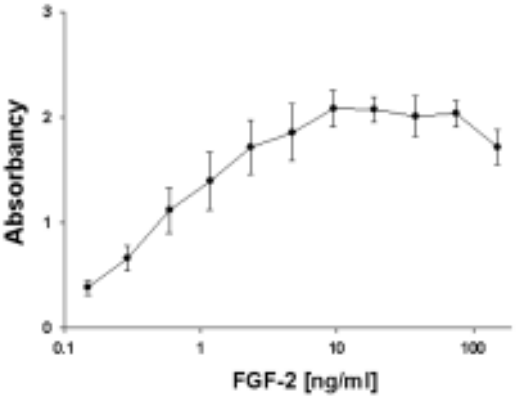

Fig. 2. **Proliferation of neural precursors in response to FGF2**. *Neural precursors were grown in medium containing different amounts of FGF2 for 5 days. The cells were labeled with BrdU overnight and the amount of BrdU incorporated into proliferating cells was measured using colorimetric ELISA. Data points are mean absorbency (n = 3)* ± *SEM.*

Results

Hippocampal Cell Morphology and Growth

The morphology and growth characteristics of the adult hippocampal progenitor (AHP) cells were similar to those previously described (Palmer *et al.*, 1999). They were positive for 200 kDa neurofilament, β-tubulin III and MAP-2 expression, and a proportion also expressed synaptophysin. The cells grew in colonies (Fig. 1) that, on reaching a high density, became detached from the substrate to form "neurospheres" (Svendsen *et al.*, 1998; Svendsen *et al.*, 1999). At high concentrations of heparin or HS1 the cells became non-adherent, even at low density. All subsequent experiments were performed on cells before they reached this non-adherent phase. In any given culture less than 0.5% percent of cells were GFAP-positive. These GFAP-positive cells had processes that were usually surrounded by a mass of proliferating neurofilament-positive cells (Fig. 1). Preliminary experiments showed that the degree of cell proliferation was not influenced by the concentration of FGF1 throughout the concentration ranges examined. However, FGF2 could upregulate cell proliferation over a 5 day period; at concentrations between 10 - 75 ng/ml the rate of BrdU incorporation into the AHP cells was markedly and significantly increased to plateau at approximately 9 ng/ml (Fig. 2). Subse-

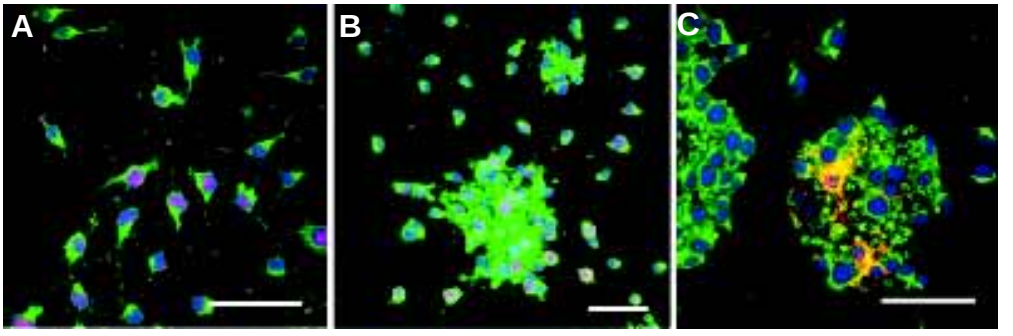

Fig. 1. **Immunostaining of neural precursors**. **(A)** *Precursors stained for the neuronal marker 200-kDa neurofilament in green and* **(B)** *their nuclei in s-phase stained with PCNA shown in pink.* **(C)** *Precursors with 200-kDa neurofilament in green and the glial marker GFAP in orange. The nuclei of all cells were counterstained with propidium iodide shown in blue. The scale bar represents 50 mm.*

quent experiments thus employed a concentration of 10 ng/ml for both FGF1 and FGF2.

The Effects of Characterised HS

Heparin is known to potentiate the binding of all FGFs to all FGFRs and also protect unstable FGFs in solution (Gallagher, 1994; Gallagher, 1997). At low concentration heparin did not significantly alter the proliferative effects of FGF2 for these progenitor cells as monitored by BrdU ELISA (Fig. 3A), indicating both that at maximal proliferation that increasing the binding efficacy of FGF2 has little effect, and that degradation of FGF2 in these cultures was not a significant factor. FGF1 however was potentiated by heparin, producing robust proliferation that peaked at 2 - 10 μg/ml (Fig. 3A). A high concentration of heparin inhibited proliferation for both FGF2 and FGF1. The FGF1 promoting sugar HS1 (Nurcombe *et al.,* 1993; Brickman *et al.,* 1998) had no effect on FGF2 (data not shown); it did however potentiate the proliferative action of FGF1 to a similar extent and at similar molar concentrations to heparin (Fig. 3B). Interestingly it appeared that FGF1 combined with HS1 could stimulate proliferation to a greater extent than FGF2. Surprisingly HS2 inhibited the proliferation of the neural progenitors in response to FGF2 (Fig. 3C). Cells did not display any visible toxic response to HS2, which, combined with the lack of effect of HS1 (which is similar in size and composition; see Nurcombe *et al.,* 1993; Brickman *et al*., 1995; Brickman *et al.,* 1998) indicates that HS2 is most probably inhibiting FGF2 signalling, rather than being toxic. Inhibition of proliferation was competitively rescued by the addition of either heparin or HS1 (Fig. 4). HS2 also did not potentiate FGF1 (data not shown). Thus, both heparin and HS1 required FGF1 to stimulate proliferation; the glycosaminoglycans by themselves had no effect.

FGF and FGF Receptor Expression

The expression of FGFRs was first examined using immunocytochemistry. The potency of the anti-FGFR receptor antibodies we have previously used (Nurcombe *et al.,* 2000) was first tested against cultured primary rat fibroblasts, which readily bound FGFR1, -R2 and -R3 (data not shown). However, the only receptor readily detectable on the neural progenitors was FGFR3 (Fig. 5A); the staining was abolished in the presence of its specific blocking peptide (Fig. 5B). FGFR3 was found throughout the cytoplasm of the progenitors, but was not detectable in neuritic processes. The immunostaining results were supported by RT-PCR. At the conservative number of 30 cycles, only FGFR3-specific primers produced a strong band (Fig. 5C). Increasing the number of cycles resulted in faint bands for the other FGFRs. The results thus indicate that the neural progenitors predominately express FGFR3.

Progenitor cells grew poorly at low densities. RT-PCR was used to test for endogenous FGF expression to determine whether it may play an autocrine/paracrine role. Interestingly, the progenitors express FGF2, but not FGF1 or FGF-8 (Fig. 5D); the latter two FGF species have been reported in the parent hippocampus (see Ford-Perriss *et al.,* 2001 for review).

Adult Progenitor HS Characterization

[^{3}H]-glucosamine-labelled HS was extracted from primary progenitor cell homogenate and the structural features of the HS chains investigated using our established protocols. Samples of purified HS chains derived by Pronase treatment were

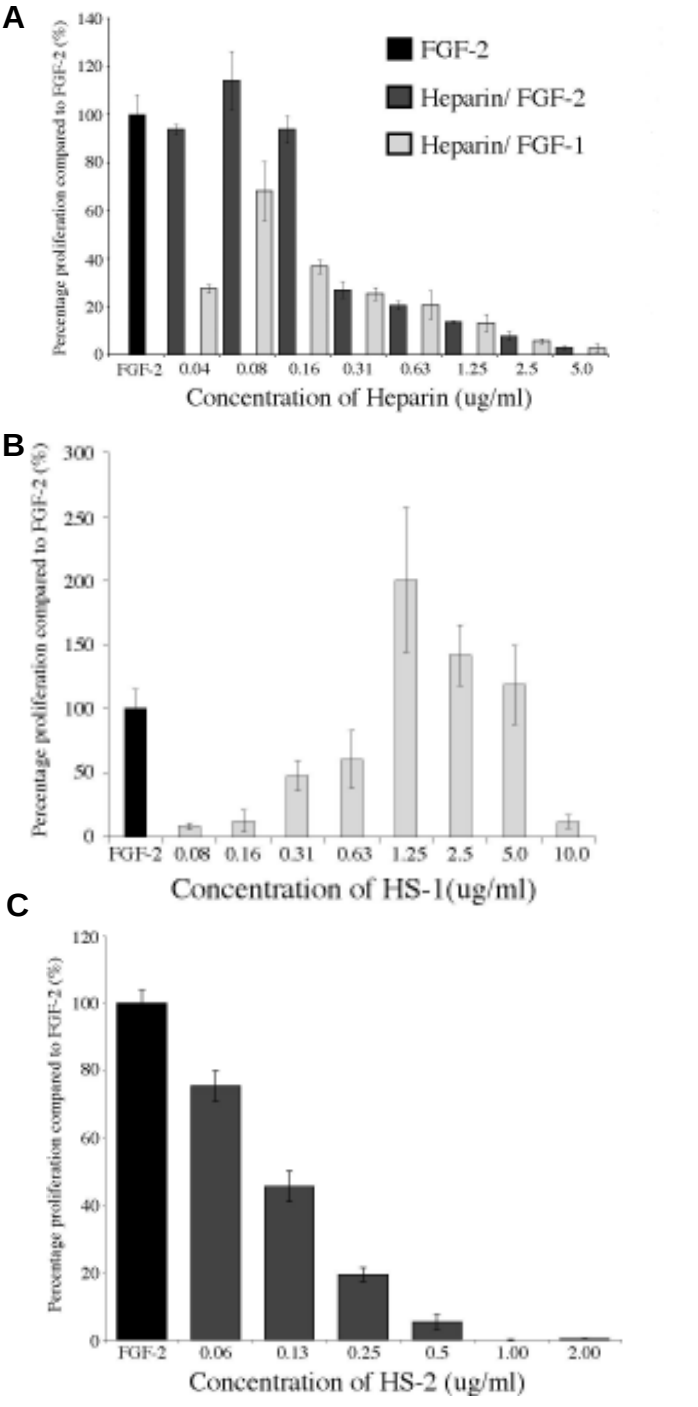

Fig. 3. **The effects of heparin and purified heparan sulfate fractions on the proliferation of neural precursors.** *Cells were grown in medium containing either 10 ng/ml of FGF1 (light gray) or FGF2 (dark gray) with various concentrations of heparin, HS1 or HS2 for 6 days. The number of proliferating cells was assayed using a colorimetric BrdU incorporation ELISA and compared to a positive control group of precursor cells grown in 10 ng/ml of FGF2 (black).* **(A)** *The effects of commercial heparin potentiating the proliferative effect of FGF1 and at higher concentrations the inhibition of FGF2 induced proliferation.* **(B)** *The potentiating of proliferation due to FGF1 due to the addition of HS1.* **(C)** *FGF2-induced proliferation is inhibited by HS2. Data points are mean absorbency (n = 4)* ± *SEM.*

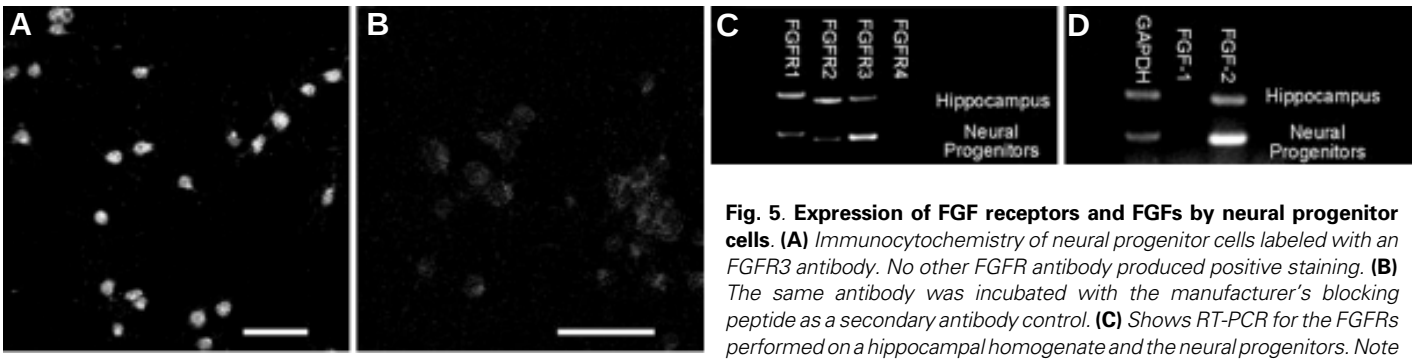

Fig. 5. **Expression of FGF receptors and FGFs by neural progenitor cells**. **(A)** *Immunocytochemistry of neural progenitor cells labeled with an FGFR3 antibody. No other FGFR antibody produced positive staining.* **(B)** *The same antibody was incubated with the manufacturer's blocking peptide as a secondary antibody control.* **(C)** *Shows RT-PCR for the FGFRs performed on a hippocampal homogenate and the neural progenitors. Note the predominance of FGFR3 in the neural progenitors compared to the hippocampal homogenate. No FGFR4 was detected.* **(D)** *RT-PCR for FGFs. The neural progenitors expressed more FGF2 than the hippocampal homogenate. Also shown is the actin control (not shown in C). Scale bars, 50 mm.*

chromatographed through a Sepharose CL-6B column both before and after mild treatment with alkaline borohydride. The HSPG pool showed a partial resistance to proteolysis by Pronase characteristic of proteoglycans, which are densely substituted with polysaccharide chains. This data indicates that there are at least two HS chains per core protein and that their attachment sites are located close together. Before alkali treatment, the HS eluted at a K_{av} of 0.21. After treatment it eluted at a K_{av} of 0.48 (Fig. 6A). These correspond to 72 kDa before treatment and 32 kDa after treatment. Assuming an average molecular weight of 400 Da for a disaccharide, the chain size is approximately 80 disaccharides. Similar chromatographic techniques were employed to determine the size of heparitinase-resistant fragments (Fig. 6B). These fragments indicate the distance between highly sulfated domains in the intact chain.

Chromatography on a Bio-Gel P-10 column of the HS oligosaccharides after low pH HNO_2 treatment gave the elution profiles shown in Fig. 7A. Low pH scission releases the N-sulfate groups from heparan sulfate with subsequent cleavage of the adjacent hexosaminidic bond. These profiles show the typical distribution of *N*-sulfated disaccharides characteristic of heparan sulfate. From these profiles it is possible to calculate the percentage of linkages susceptible to this treatment and thus the percentage of *N*-sulfated glucosamine residues in the HS chains; the progenitor HS was 44% susceptible. Results from the separation of heparinase-treated oligosaccharides on a Bio-Gel P-10 column are depicted in Fig. 7B. The inset (an expanded scale for fractions 60-100) highlights the differences in the composition of the HS reflected by the spacing of heparinase cleavage sites in the sulfated domains. Quantitative analysis revealed that the HS chains are 19.5% susceptible. The major products of this digestion were not resolved on Bio-Gel P-10 columns (V_0 peak) but estimation of their molecular size was possible on a CL-6B column. The major peaks fractioned on the Sepharose CL-6B column have a molecular weight of 10 kDa. The molecular mass of the heparinase-resistant domains in HS corresponds to the average distance between the centres of highly sulfated regions that contain heparinase-susceptible linkages; the heparinase-resistant oligosaccharides are on average 25 disaccharide units in length. Making the assumption that there are broad similarities in structure within the mixture of chains, heparinase therefore trisects the HS. Heparitinase treatment of the samples yielded elution profiles that were also characteristic of HS (Fig. 7C). The majority of the tritium was in the disaccharide peak, unlike the HNO_2 profile where the largest peak corresponds to tetrasaccharides. Quantitative analysis of the profiles demonstrated that 74.9% of the linkages were susceptible to this treatment.

The half-maximal induction of proliferation of the isolated, full-length AHP-HS chains was ~ 4.5 μg/ml when FGF2 was present at 10 ng/ml; the stimulatory activity was markedly greater than that for HS1, HS2 or heparin (Fig. 8A). The adult progenitor HS was then

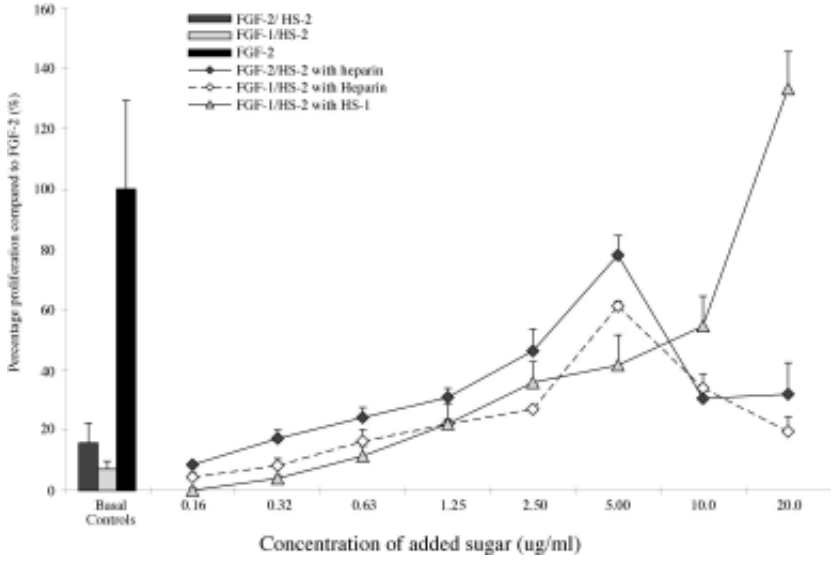

Fig. 4. **The inhibition of proliferation by HS2 is competitive**. *To test if the inhibitory effects of HS2 were both specific and competitive, heparin or HS1 was added to cultures of neural progenitors already supplemented with 1 mg/ml HS2 and either FGF2 or FGF1 (10 ng/ml). After 6 days the number of proliferating cells was assayed using a colorimetric BrdU incorporation ELISA. Proliferation of the cells grown in HS2/FGF2 (dark gray bar) and HS2/FGF1 (light gray bar) was low compared to cells grown in 10 ng/ml of FGF2 alone (black bar). Heparin was able to induce proliferation in both HS2/FGF2 (solid line, dark diamond) and HS2/FGF1 (dashed line, open diamond) with maximal effect at a concentration of 5 mg/ml. HS1 (solid line, light gray triangle) induced proliferation to a greater extent then heparin in HS2/FGF1 but at a higher effective concentration. Data points are mean absorbency (n = 4) ± SEM.*

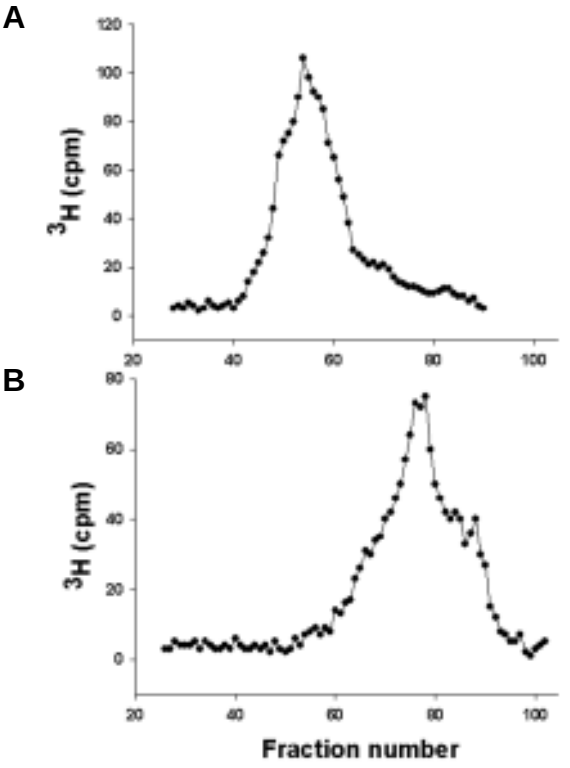

Fig. 6. **Purification of HS chains from AHP cells**. **(A)** *Heparan sulfate from the progenitors was isolated and the size of the full-length chains and* **(B)** *heparinase-resistant fragments determined after Sepharose CL-6B gel filtration.*

tested for its ability to activate FGFR tyrosine phosphorylation in native hippocampal progenitor cells. Only FGFR3 responded to it in the presence of FGF2 (Fig. 8B), consistent with the apparent preponderance of this receptor isotype on these cells. This suggests that the HS fraction leads to R3:R3 homomerisation in these cells.

Cloning of AHP Cells

Previous studies have shown that cortical stem cells grown in serum-free, astrocyte-meningeal cell conditioned medium can generate neurons, astrocytes, and oligodendrocytes (Davis *et al.*, 1994). In contrast, the precursor cells derived here could not be cloned under these conditions. Indeed, no cloning of the precursors was possible until the media was supplemented with relatively high doses of AHP-HS. In cultures where this HS was present at concentrations above 10 μg/ml, cloning became possible. To determine the sequence of cell generation in stem cell clones more precisely, isolated adult progenitor cells were plated at clonal density (1–5 cells per well) in poly-l-lysine-coated Terasaki wells in serum-free, conditioned medium with 10 ng/ml FGF2 added as a mitogen in the presence or absence of 10 μg/ml HS. Heparin and HS2 were used as controls. Neuronal-like cells were the first differentiated cells to appear, based on cell-type-specific antibody markers (Fig. 9). After 3, 6 and 10 days of culture, clones were fixed and stained. Neurons were detected early and continued to expand in number throughout the culture period. Even by 6 days *in vitro*, the majority of stem cell clones contained neurons and undifferentiated cells, with few astrocytes or oligodendrocytes (Table 1). Of the 46 stem cell clones that were assessed at 6 days, only one contained glia (two GFAP-positive astrocytes). After 10 days in culture, the majority of stem cell clones contained both neurons and glia—of 34 stem cell clones examined, >90% contained GFAP-positive astrocytes. Very few O4-positive oligodendrocytes were ever noted. When AHP-HS was omitted and hippocampal stem cells grown in serum-free medium with 10 ng/ml FGF2 and either heparin or HS2, no cells were ever observed. The fact that the typical "neurons then glia" order of cell production (Qian *et al.*, 2000) occurred in the clones suggests that the HS has the capacity to appropriately direct the phenotypic cascade within single cortical stem cells *in vitro*.

Mesodermal Cell Transdifferentiation

Primary newborn mouse calvarial osteoblasts were isolated and grown in standard medium with sodium chlorate to suppress

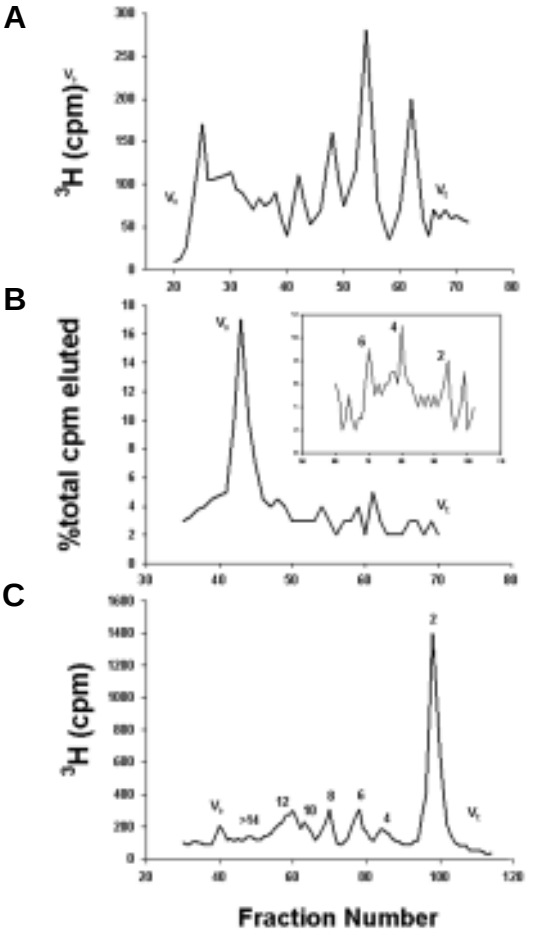

Fig. 7. Gel filtration of oligosaccharides produced by various depolymerising agents. *HS was fractionated after the following treatments.* **(A)** *Low pH HNO_2. This profile was used to identify the purity of the HS sample and to calculate the percentage of susceptible linkages.* **(B)** *Depolymerisation by heparinase. Inset: fractions 64-115 of the heparinase scission profile with an expanded scale in order to reveal the proportions of low-M_r products. The non-resolved V_0 peak was pooled, freeze-dried and resolved on a Sepharose CL-6B.* **(C)** *Depolymerisation by heparitinase. The susceptibility of each species was calculated from this profile. The degree of polymerisation (dp) of each peak is represented by the number above that peak and was subsequently used in the calculations.*

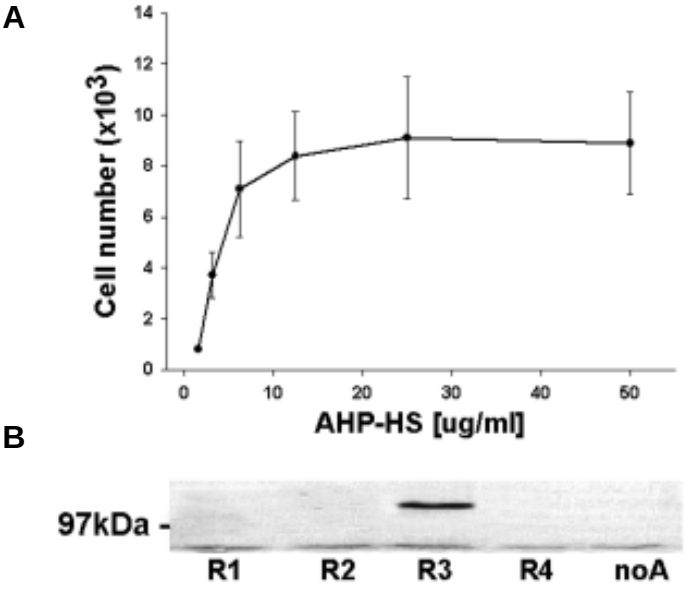

Fig. 8. **Activity of the AHP-HS**. **(A)** *Dose-response curve for the AHP-HS chains revealed that the half-maximal activity for cell proliferation of ~ 4.5 mg/ml when FGF2 was present at 10 ng/ml; this was markedly greater than that for HS1, HS2 or heparin.* **(B)** *The AHP- HS was then tested for its ability to activate FGFR tyrosine phosphorylation in clonal native hippocampal progenitor cells. Cells were exposed to FGF2 (10 ng/ml) and AHP-HS (5 mg/ml). Activity was determined by immunoprecipitation of lysates with anti-FGFR-specific antibodies and revealed on gels with the anti-phosphotyrosine 4G10 monoclonal antibody.*

endogenous HS production, and supplemented with increasing levels of AHP-HS. After 72 h they were then stained for vimentin for mesodermal expression or MAP-2, β-tubulin III, neurofilament and GFAP for neuroectodermal expression and assessed by confocal microscopy (Fig. 10). When the concentration of the HS reached 10 μg/ml, substantial numbers of neuronal-like cells could be identified from amidst the vimentin-stained cells. They contained rounded cell bodies, long, neuritic-like processes and identifiable growth cones. The effects are quantified in Fig. 10E. Approximately 10 $\pm$ 2 % of the osteoblasts could be made to adopt a neuronal-like phenotype when the AHP-HS reached 10 μg/ml; higher concentrations with the fixed FGF2 concentrations proved less effective.

Discussion

The results of this study confirm that clonal progenitor cells derived from adult neural tissue are multipotential. Moreover, they secrete HS chains that dispose osteoblastic progenitor cells towards neural phenotypes. Recently many of the boundaries previously thought to demarcate cell lineages and transdifferentiation have been breached; for example, neuronal cells have been turned into blood, and skin cells have been coerced into adopting a neural phenotype (Vescovi *et al.,* 2001). Bone marrow cells have been differentiated into a wide variety of cell types. Many of these experiments have relied on growth factors, including FGF2, as well as other extracellular supplements. The work here suggests that HS is sufficient in and of itself to induce cells to adopt different phenotypes. HSs could in theory be interesting therapeutic tools as they are much more stable chemically than the growth factors that they modulate.

The question arises as to just how homologous the isolated and expanded AHPs are to embryonic neural stem cells. The AHPs predominately express FGFR3 and we provide preliminary evidence that activation of this receptor is the major source of intracellular signalling. This is somewhat in contrast with embryonic neural stem cells and breast cancer cells, which use FGFR1 for both proliferation and differentiation. The FGFR3 in these cells seems to be involved with more differentiative aspects of these cells' behaviour (Brickman *et al.,* 1995; Nurcombe *et al.,* 2000). It also becomes important to determine how the endogenous FGF2 being synthesised by the AHPs is being directed by the AHP-HS outside the cell. The AHPs grow extremely poorly at low densities, presumably because they require certain threshold levels of receptor saturation in order to grow and develop.

Exactly which HS-driven combination of extracellular molecules directs this particular lineage choice is now accessible to investigation; it is becoming clear that the same ligands can stimulate very different reactions from cells at different stages of their differentiation. For example, FGFR1 and 2 have been shown to be involved in the functional control of osteoblast cells, and are expressed at the onset of osteogenesis by progenitor cells (Molteni *et al.*, 1999). These are thought to maintain the balance between proliferation, through FGFR2, and differentiation, through FGFR1 (Iseki *et al.*, 1999). In contrast, FGFR3 is thought to be a negative regulator of bone growth (Molteni *et al.*, 1999). Interestingly, whether directly or indirectly, the AHP-HS clearly leads to signalling through FGFR3 in the neural progenitors. Although FGF2 has been used to induce the proliferation of adult stem cells by a number of groups, surprisingly little is known about the signalling processes involved. The population of stem cells described here that express glial and neuronal markers after exposure to FGF2, but not LIF or EGF, as other groups have reported. In our culture conditions the AHPs predominately adopted a neural phenotype with only a few glial cells; this however could be a product of the concentra-

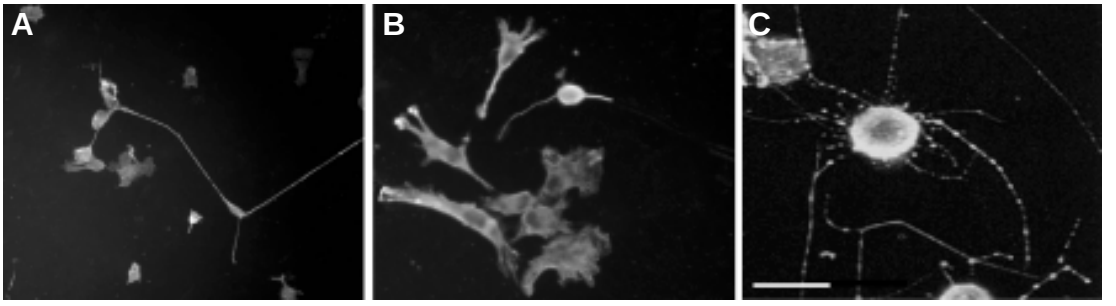

Fig. 9. **AHP cells growing in clonal culture generate neurons**. *Progenitor cell clones derived from adult hippocampus were fixed and stained with cell-type-specific markers after 3 days.* **(A)** *Neurons (indicated by MAP2 staining) appear early and strongly, while* **(B)** *GFAP-staining astrocytes only appear at later stages.* **(C)** *Very few O4+ oligodendrocytes were ever seen. Hence the AHP cells grown at clonal density can generate identifiable neurons. Calibration bar represents 50 mm for A and B, and 20 mm for C.*

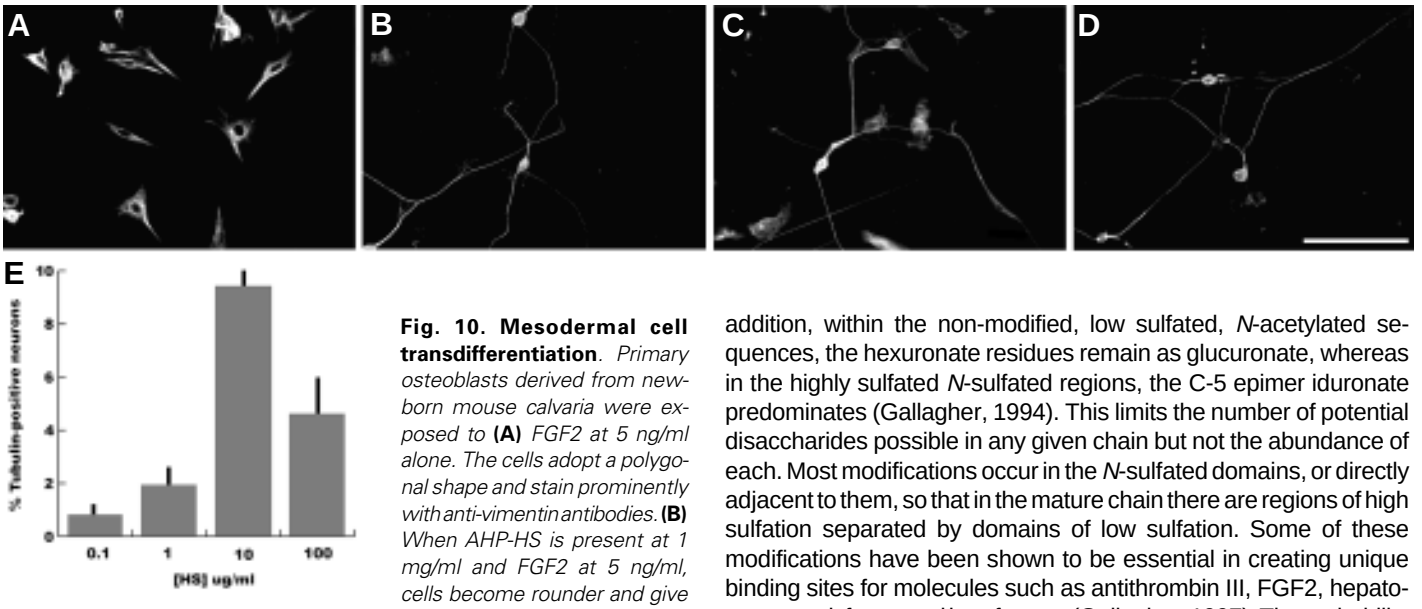

Fig. 10. Mesodermal cell transdifferentiation. *Primary osteoblasts derived from newborn mouse calvaria were exposed to* **(A)** *FGF2 at 5 ng/ml alone. The cells adopt a polygonal shape and stain prominently with anti-vimentin antibodies.* **(B)** *When AHP-HS is present at 1 mg/ml and FGF2 at 5 ng/ml, cells become rounder and give rise long, thin, growth cone-capped processes that stain with tubulin-III.* **(C)** *When AHP-HS is present at 10 mg/ml and FGF2 at 5 ng/ml, the processes also stain with MAP2. Note the non-staining osteoblast-like cells in the background.* **(D)** *AHP-HS at 10 mg/ml and FGF2 at 5 ng/ml and staining for 200-kDa neurofilament.* **(E)** *The percentage of detectable β-tubulin III-positive cells in osteoblast cultures exposed to constant levels of FGF2 (5 ng/ml) but increasing levels of AHP-HS. Values are the average ± SEM for 4 separate cell preparations.*

tion of FGF2 used, as there is some evidence that differing levels of FGF2 favour glial differentiation (Qian *et al.,* 1997).

As specific HS species are essential to FGF activation, knowledge of the structures is clearly crucial to understanding their potentiating mechanism. HS is the most structurally complex of all glycosaminoglycans as it contains highly variable patterns of sulfation, suggesting that its roles are related to specific interactions within its environment and not just to its high negative charge density. HS chains are initially synthesised as polymers of alternating glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) units (Gallagher, 1997). Variation in HS species arises from the synthesis of non-random, highly sulfated clusters of sugar residues along the length of any single HS chain. The initial conversion of *N*-acetylglucosamine to *N*-sulphoglucosamine creates a focus for other modifications, including epimerisation of glucuronic acid to iduronic acid and a complex pattern of *O*-sulfations on glucosamine or iduronic acids (Kjellen *et al*., 1991; Gallagher, 1997; Turnbull *et al*., 2001). In addition, within the non-modified, low sulfated, *N*-acetylated sequences, the hexuronate residues remain as glucuronate, whereas in the highly sulfated *N*-sulfated regions, the C-5 epimer iduronate predominates (Gallagher, 1994). This limits the number of potential disaccharides possible in any given chain but not the abundance of each. Most modifications occur in the *N*-sulfated domains, or directly adjacent to them, so that in the mature chain there are regions of high sulfation separated by domains of low sulfation. Some of these modifications have been shown to be essential in creating unique binding sites for molecules such as antithrombin III, FGF2, hepatocyte growth factor and interferon-χ (Gallagher, 1997). The probability therefore exists that such modifications may create specific binding sites for many other extracellular matrix molecules.

The heparin/HS structures required for growth factor binding are slowly becoming known at the molecular level. IdoA(2-OSO_3) and $GlcNSO_3$(6-OSO_3) residues appear to be required for the FGF2-induced activation of FGFR1, although 2-*O*-sulfate groups alone are sufficient to mediate binding to FGF2 (Guimond *et al*., 1999). However, many previous FGF experiments, including crystallization studies (Pellegrini *et al*., 2000; Schlessinger *et al*., 2000), have not been not been easy to interpret as they have often been presented in association with commercial porcine heparin, which may cross-link a vast series of physiologically irrelevant heparin-binding molecules (Turnbull *et al*., 2001). The evidence presented here also concurs with the model proposed by Guimond *et al.* (Guimond *et al*., 1993) and Wang *et al.* (Wang *et al*., 1995) in which highly sulfated HS subdomains of a single HS chain molecule interact with multiple susceptible partners.

Much work remains to be done to properly identify the specific ligands that the AHP-HS helps couple together. Data are now accumulating that specific domain sub-fragments of HS sugar chains are capable of bringing precise isoform-specific ligand pairs together for downstream signaling and subsequent phenotypic development (Guimond *et al*., 1999; Perrimon *et al*., 2000). Indeed, although the HS appears to play a role in activating FGFR3 receptors on the progenitor cells, it is not clear that this is a direct effect on the receptor itself, or a downstream effect through other HS binding-moieties such as NCAM (Cavallaro *et al*., 2001). The HS chains isolated here contain on average three sulfated domains, and are thus potentially capable of three different mitogenic/adhesion/extracellular matrix interactions. It might be the combination of specific influences that predisposes a naïve mesodermal stem cell to transdifferentiate down neural lineages. Preliminary evidence reveals that only full-length HS chains can bring about this effect (data not shown).

HS, of course, binds multiple species of not only growth factors, but also morphogens such as Wnt and Sonic Hedgehog (Dhoot *et al*., 2001), and most of the large glycoproteins in the extracellular matrix (Perrimon *et al*., 2000). The role of HS may be to allow extremely precise and specific molecular interactions to occur at the right times

TABLE 1

CELLULAR COMPOSITION ARISING FROM SINGLE CELL CLONES

Day	Total Clones	Average Clone Size	Total Number of Clones Containing Neurons	Astrocytes	Oligodendrocytes
3	26	6	22	0	0
6	46	24	29	1	0
10	34	112	33	34	4

Single cells were cultivated in Terasaki wells supplemented with AHP-HS (10 μg/ml). Clones were fixed and stained at day 3, 6 and 10 after plating. Morphologically-distinct, MAP2 and β-tubulin III-staining neurons were detected at the earliest periods, with astrocytes only becoming prominent after 10 days.

TABLE 2

FGFR-SPECIFIC PRIMERS FOR RT-PCR

Target	Forward Primer	Reverse Primer	Product length	Accession Number
FGFR1	5'CCGTCAATGTCTCAGATGCA3'	5'ATAGAGTTACCCGCCAAGCA3'	696	D12498
FGFR2	5'AAGGTACGAAACCAGCACTG3'	5'AGGCGATTAAGAAGACCCC3'	549	Z35138
FGFR3	5'AGAACAAGTTTGGCAGCATC3'	5'AAGCGAGAGACCTTGTGCA3'	540	AF277717
FGFR4	5'CTCTGGCAAGTCAAGTTTGTCC3'	5'TCGGAGGCATTGTCTTTCAG3'	260	M91599
FGF1	5'TGAAGGAGCCCAAAGAACC3'	5'GCTCGCAGACACCAAATGA3'3'	352	NM_012846
FGF2	5'GCAGCATCACTTCGCTTCC3'	5'TGGAAGAAAGAGTATGGCCTTCTG3'	436	NM_019305
GAPDH	5'CTTGCCTCTCAGACAATGCC3'	5'GCAAGTTCAACGGCACAGT3'	403	AF106860

and places during, maturation and regeneration. Certainly the heterogeneity seen in HS chains during development may reflect the multiple interactions being regulated at each developmental stage. One advantage of this tripartite system is that a particular responsiveness (that is, intracellular signaling) is not selected by levels of a growth factor or its receptors but by the targeting of a growth factor to a particular receptor by a specific motif on an HS chain (Chang *et al.*, 2000).

Until quite recently there was a consensus that the fate of adult cells was restricted to their tissues of origin. However, the recent spate of findings that adult cells could be "re-programmed" to express genes typical of differentiated cell types across all three of the mesodermal, endodermal, and ectodermal lineages when fused to cells in heterokaryons has been informative (Brazelton *et al.*, 2000; Mezey *et al.*, 2000; Blau *et al.*, 2001). This degree of plasticity within a cell demonstrated that the differentiated state is readily reversible and requires continuous regulation to maintain the balance of differentiating factors present. Findings involving the cloning of amphibians demonstrated that genes previously silent could be activated in adult nuclei (Gurdon *et al.*, 1999). Although promising, these examples of plasticity all involved extensive experimental manipulations. More recently, findings have been made that show stem cells can assume diverse fates under physiologic conditions. Bone marrow cells can yield not only all cells of the blood but also cells with a liver phenotype (Petersen *et al.*, 1999); perhaps most interestingly, the preferred migration of bone marrow-derived cells to damaged muscle in irradiated dystrophic *mdx* mice has been recently shown (Gussoni *et al.*, 1999). Muscle-derived and CNS-derived stem cell-like populations have also been reported to reconstitute the blood and rescue lethally irradiated mice (Mezey *et al.*, 2000; Vescovi *et al.*, 2001).

In conclusion, our results indicate that an appropriately configured extracellular environment with sufficiently specific HS co-receptors is enough to encourage very precise phenotypic choices amongst susceptible cells, even if they are derived from adults.

Materials and Methods

Materials

Tissue culture media were obtained from LifeTECH (Sydney, Australia) and unless otherwise noted all other reagents were obtained from Sigma-Aldrich (Sydney, Australia). All flasks and plates were coated with poly-D-lysine (50 μg/ml in H_2O) for 2 h at room temperature and then rinsed once with PBS and once with NeurobasalA/B-27 before use. All surgical procedures and dissections were carried out under sterile conditions.

Tissue Culture

Neural progenitors were isolated with essentially standard techniques (Brewer, 1999) with some modification. Hibernate A and Neurobasal A were both supplemented with 2% B-27 supplements, 0.5mM glutamine, 25 units/ml of penicillin and 25 μg/ml of streptomycin before use. Adult female Dark Agouti (DA) rats were given a lethal dose of a mixture of ketamine and xylazine. The hippocampus was quickly removed from one side of the brain, placed into ice-cold Hibernate A/B-27, macerated, transferred to a tube containing Hibernate A/B-27 at room temperature and the volume adjusted to 2 ml. The tissue was dissociated by trituration using a 1 ml pipette and after the debris had settled, the supernatant containing the isolated cells was removed into a fresh test tube. Percoll was added to the isolated cell suspension to a final concentration of 35%. This mixture was spun at 2,000 g for 15 min, the cell pellet removed and re-suspended in 5 ml of fresh HibernateA/B-27. This was re-spun at 800 g for 5min to remove any remaining Percoll. Finally, the cells were re-suspended in NeurobasalA/B-27 containing 20 ng/ml of fresh rhFGF2, plated into 6-well tissue culture plates and placed in an incubator. This was containing a humidified atmosphere of 95% air and 5% carbon dioxide maintained at 37°C.

Within 1 - 2 weeks approximately 5-10 distinct colonies of cells with a neuronal phenotype could be seen in each well. At this stage some cells that displayed glial characteristics were also observed, although but these did not appear to proliferate as rapidly as those with a neuronal phenotype. The glial cells adhered to the tissue culture dish more strongly than the neuronal precursors. The latter cells were removed from the dish by gently washing them out with fresh medium taking care not to disturb the glial cells. These near-pure neuronal precursor cells were re-plated into 25cm^2 tissue culture flasks and allowed to proliferate. The cells were fed once every 2-3 days with Neurobasal-A containing 20 ng/ml of fresh FGF2. Cells from this source were found to maintain their phenotype for up to six months in tissue culture and could also be stored by freezing in Neurobasal A and 5% DMSO.

Immunostaining

The primary antibodies used were mouse anti-neurofilament-200, mouse anti-GFAP, mouse anti-PCNA, mouse anti-MAP2, rabbit anti-neurofilament, rabbit anti- β-tubulin III, O4, vimentin (all from Sigma-Aldrich, Sydney, Australia) and rabbit anti-FGFR1, anti-FGFR-2, anti-FGFR3 and anti-FGFR4 (all from Santa Cruz Biotechnology, CA, USA) at dilutions of 1/100. Labelled cells were visualised using the appropriate secondary antibodies conjugated with Cy5 or FITC and viewed under a BioRad 1024 confocal microscope. A poly-D-lysine-coated 12mm-diameter glass coverslip was placed in each well in a 24-well plate and approximately 1-5 x 10^3 cells plated into each well. The cells were allowed to grow for a period of 1 - 3 days. Following this the cells were rinsed in PBS and fixed for 30 min in either 4% paraformaldehyde (PFA) or in a mixture 90% ethanol and 10% glacial acetic acid. The fixed cells were rinsed in several changes of PBS and treated with a blocking solution consisting of 0.5% Triton X-100 and 10% FCS in PBS for 1 h. The cells were then incubated for 1-2 h in the primary antibodies, washed in several changes of PBS, incubated in the secondary antibodies for between 1-2 h, re-rinsed in PBS before mounting on glass slides. On some occasions cells were counter-stained with propidium iodide (500 nM) for ~ 3 min in order to visualise their nuclei.

Proliferation Assays

The proliferative effect of FGF1, FGF2, HS1 and HS2 on the neural precursor cells was first examined for each individual growth factor and sugar. Subsequently, combinations of each growth factor with the different sugars were studied systematically as nominated. Cell proliferation was assessed using a chromogenic BrdU ELISA kit (Roche). Cells were plated into poly-D-lysine coated 96 well plates at an initial density of 1000 cells/ well in basal medium. The cells were allowed to adhere to the plate and equilibrate in this medium for 1-2 h. The medium was then replaced with fresh medium containing different concentrations of FGF1 (Promega), FGF2 (LifeTech), HS1 (*vide infra*), HS2 (*vide infra*) and heparin. The cells were incubated for 5 d with the medium changed every alternate day. On the fourth day BrdU was added to the medium at a concentration of 10 μm. On the following day the cells were fixed for 30 min in the fixative provided in the kit, incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min. After thorough rinsing a colour reaction was developed in the wells using 3,3', 5,5'-

tetramethylbenzidine (TMB). Absorbances were measured at 490 nm using a BioRad benchmark microplate reader.

RT-PCR

The expression of FGF–1, FGF2 and FGFRs 1-4 in the neural precursors and in the adult female Dark Agouti rat hippocampus was examined using RT-PCR. Details of the primers used are found in Table 1. Total RNA was extracted using an RNeasy kit (QIAGEN, Australia) according to the manufacturer's instructions. RT-PCR was performed using a OneStep RT-PCR kit (QIAGEN, Australia). The PCR mix consisted of 250 ng of RNA, 0.6 μM of each primer, 400 μM of each dNTP, 2.5 mM MgCl and the recommended quantity of DNA polymerase. The RNA was reverse transcribed for 30 min at 50ºC, the HotStarTaq DNA polymerase (QIAGEN, Australia) then activated for 15 min at 95ºC followed by 30 cycles of PCR consisting of denaturation for 45 sec at 95ºC, annealing for 30 sec at 59ºC and elongation for 75 sec at 72ºC. The PCR products were resolved on a 1.0% Agarose gel, stained with ethidium bromide and photographed.

HS Fragment Preparation

HS1 and HS2 were prepared as described previously (Brickman *et al.*, 1995; Brickman *et al.*, 1998). Full-length HS chains were isolated from ~ 100 ml of neural progenitor cell homogenate. Cells, cell matrix and cell conditioned medium were combined and lysed with ice-cold lysis buffer (1% Triton X100, 150 mM NaCl, 10 mM Tris pH 7.4, 2 mM EDTA, 0.5% NP 40, 0.1% SDS) containing protease inhibitors (1 mM sodium orthovanadate, 10 μg/mL leupeptin, 1 μg/mL aprotinin and 1 mM PMSF). This slurry was then separated over a Econo-Pac 5 ml Q-Sepharose column (Bio-Rad, Sydney Australia) according to our established procedures (Brickman *et al.,* 1995). Bound HSPGs were released with a single cut-off step of 1.5 M NaCl. To remove HS chains from core proteins, samples were incubated in 500 mM NaOH/1M $NaBH_4$ for 16 h at 4°C, neutralised to pH 7 with glacial acetic acid and concentrated ammonium bicarbonate added. Gel chromatography of intact HS chains derived from the HSPGs released from the adult progenitor cells or their scission products was performed on Sepharose CL-6B (1 X 120 cm) columns with a running buffer consisting of 0.5 M NH_4HCO_3. Samples were routinely eluted at 4 ml/h with 1 ml fractions collected. Estimates of the size of fragments resolved on Sepharose CL-6B were based on our published calibrations (Brickman *et al.,* 1998). For HS depolymerisation reactions, heparitinase (heparitinase I), heparitinase II and heparitinase IV (Seikagaku Corp., Tokyo, Japan) were used at a concentration of 25 mU/ml in 100 mM-sodium acetate/0.2 mM-calcium acetate, pH 7.0. Heparinase was used at a concentration of 50 mU/ml in the same buffer. Samples were digested in the presence of 100 μg non-labelled carrier HS (porcine mucosal HS). Each sample was separately incubated at 37°C for 16 h and then a second aliquot of enzyme added and incubated for a further 4 h. Saccharides of varying length were generated by partial heparitinase scission terminated by rapid boiling after 2 h.

Receptor Phosphorylation

Binding of FGF/HS combinations was carried out according to our previously described methods (Nurcombe *et al.*, 2000). Briefly, 5 x 10^6 cells were resuspended in basal medium overnight and then exposed to different FGF2 (5 ng/ml) and adult progenitor HS combinations for 5 min. The cells were pelleted (800 rpm x 5 min) and lysed on ice. Lysates were clarified by the addition of Protein A-Sepharose 4B (Pansorbin, Calbiochem, CA, USA) followed by centrifugation and protein determination. Equal amounts of total cell protein were immunoprecipitated with anti-FGFR1, R2, R3 or R4 antibodies (Lin *et al.*, 1997) and then subjected to 5% SDS-PAGE. Gels were electroblotted onto PVDF membranes and the activated receptor complexes revealed with the anti-phosphotyrosine 4G10 monoclonal antibody (Upstate Biotechnology, NY, USA) overnight at 4ºC. Blots were rinsed and incubated with radioiodinated Protein A for 1 h and then placed in an autoradiography cassette with preflashed Kodak X-Omat AR X-ray film.

Clonal Cell Culture

Dissociated progenitor cells were plated at clonal density (1–10 cells/well) into poly-l-lysine-coated microwells in Terasaki plates. Each well contained 12 μl of basal medium with 0.1, 1, or 10 ng/ml FGF2. For some experiments, 50% serum-free preparations of progenitor HS were added to basal medium with 10 mg/ml FGF2. For subcloning experiments, cells were plated at clonal density into basal medium with 10 ng/ml FGF2 on poly-D-lysine-coated 35 mm tissue culture dishes and allowed to generate clonal progeny for 3–10 days. Cells were the dislodged from the coverslips by gentle pippetting, collected by centrifugation, resuspended in 20 ng/ml FGF2 and nominated doses of progenitor HS, and replated at single cell densities onto poly-D-lysine-coated coverslips. Clones were fixed and immunostained for cell type specific markers as described above.

Primary Bone Cell Culture

One-day-old Quackenbush mice were obtained from the University of Queensland Animal Production Department. Animals were euthanased, the calvaria removed and the skin excised to release the skullcap. Brain tissue was removed and the skullcap washed several times in sterile PBS. Parietal bones were harvested, well clear of the developing sagittal suture, and digested in PBS containing 0.1% collagenase and 0.2% dispase at 37ºC for 10 min. The solution was removed to a fresh sterile tube (fraction 1). This procedure was repeated with fresh solution five more times (fractions 2-6). Fractions 2-6 were combined and the cells pelleted by centrifugation at 5000 x g for 5 min. Cells were seeded in culture flasks containing EMEM (LifeTECH, Ontario, Canada) supplemented with 10% FCS, 2 mM/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (LifeTECH, Ontario, Canada) and 20 mM sodium chlorate to suppress endogenous HS expression (herein after referred to as standard medium). After 3-4 days, the cells were detached with 0.25% trypsin 0.5mM EDTA and passaged in standard medium. Cells were seeded at 2 x 10^4 in 500 μl on to poly-L-lysine-coated 12 mm grade 1 sterile glass coverslips in standard medium and allowed to adhere (2 h). The cells were then grown in standard medium supplemented with 10 μg/ml AHP-HS and after 72 h immunostained for vimentin for mesodermal expression or β-tubulin III, GFAP or O4 for neuroectodermal expression as described above.

Acknowledgements

The authors are grateful to the Wesley Research Institute, Brisbane, Australia (Grant No 2000100294) and the Australian Research Council (Grant No 4-21-1135-72-000) for their support of this project. We also thank Mr. Anthony Chan and Ms. Lien Banh for their technical assistance with the experiments.

References

BLAU, H. M., BRAZELTON, T. R., and WEIMANN, J. M. (2001). The evolving concept of a stem cell: entity or function? *Cell* 105, 829-41.

BRAZELTON, T. R., ROSSI, F. M., KESHET, G. I., and BLAU, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290, 1775-9.

BREWER, G. J. (1999). Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture. *Exp Neurol* 159, 237-47.

BRICKMAN, Y. G., FORD, M. D., SMALL, D. H., BARTLETT, P. F., and NURCOMBE, V. (1995). Heparan sulfates mediate the binding of basic fibroblast growth factor to a specific receptor on neural precursor cells. *J Biol Chem* 270, 24941-8.

BRICKMAN, Y. G., NURCOMBE, V., FORD, M. D., GALLAGHER, J. T., BARTLETT, P. F., and TURNBULL, J. E. (1998). Structural comparison of fibroblast growth factor-specific heparan sulfates derived from a growing or differentiating neuroepithelial cell line. *Glycobiology* 8, 463-71.

BRUSTLE, O., CHOUDHARY, K., KARRAM, K., HUTTNER, A., MURRAY, K., DUBOIS-DALCQ, M., and MCKAY, R. D. (1998). Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. *Nat Biotechnol* 16, 1040-4.

CARPENTER, M. K., CUI, X., HU, Z. Y., JACKSON, J., SHERMAN, S., SEIGER, A., and WAHLBERG, L. U. (1999). *In vitro* expansion of a multipotent population of human neural progenitor cells. *Exp Neurol* 158, 265-78.

CARPENTER, M. K., WINKLER, C., FRICKER, R., EMERICH, D. F., WONG, S. C., GRECO, C., CHEN, E. Y., CHU, Y., KORDOWER, J. H., MESSING, A., BJORKLUND, A., and HAMMANG, J. P. (1997). Generation and transplantation of EGF-responsive neural stem cells derived from GFAP-hNGF transgenic mice. *Exp Neurol* 148, 187-204.

CAVALLARO, U., NIEDERMEYER, J., FUXA, M., and CHRISTOFORI, G. (2001). N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. *Nat Cell Biol* 3, 650-7.

CHANG, Z., MEYER, K., RAPRAEGER, A. C., and FRIEDL, A. (2000). Differential ability of heparan sulfate proteoglycans to assemble the fibroblast growth factor receptor complex in situ. *Faseb J* 14, 137-44.

DAVIS, A. A., and TEMPLE, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* 372, 263-6.

DHOOT, G. K., GUSTAFSSON, M. K., AI, X., SUN, W., STANDIFORD, D. M., and EMERSON, C. P., JR. (2001). Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science* 293, 1663-6.

FORD-PERRISS, M., ABUD, H., and MURPHY, M. (2001). Fibroblast growth factors in the developing central nervous system. *Clin Exp Pharmacol Physiol* 28, 493-503.

GAGE, F. H., COATES, P. W., PALMER, T. D., KUHN, H. G., FISHER, L. J., SUHONEN, J. O., PETERSON, D. A., SUHR, S. T., and RAY, J. (1995). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92, 11879-83.

GALLAGHER, J. T. (1994). Heparan sulphates as membrane receptors for the fibroblast growth factors. *Eur J Clin Chem Clin Biochem* 32, 239-47.

GALLAGHER, J. T. (1997). Structure-activity relationship of heparan sulphate. *Biochem Soc Trans* 25, 1206-9.

GUIMOND, S., MACCARANA, M., OLWIN, B. B., LINDAHL, U., and RAPRAEGER, A. C. (1993). Activating and inhibitory heparin sequences for FGF2 (basic FGF). Distinct requirements for FGF1, FGF2, and FGF-4. *J Biol Chem* 268, 23906-14.

GUIMOND, S. E., and TURNBULL, J. E. (1999). Fibroblast growth factor receptor signalling is dictated by specific heparan sulphate saccharides. *Curr Biol* 9, 1343-6.

GURDON, J. B., and COLMAN, A. (1999). The future of cloning. *Nature* 402, 743-6.

GUSSONI, E., SONEOKA, Y., STRICKLAND, C. D., BUZNEY, E. A., KHAN, M. K., FLINT, A. F., KUNKEL, L. M., and MULLIGAN, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401, 390-4.

ISEKI, S., WILKIE, A. O., and MORRISS-KAY, G. M. (1999). Fgfr1 and Fgfr2 have distinct differentiation- and proliferation- related roles in the developing mouse skull vault. *Development* 126, 5611-20.

KJELLEN, L., and LINDAHL, U. (1991). Proteoglycans: structures and interactions. *Annu Rev Biochem* 60, 443-75.

LIN, H. Y., KAPLOW, J., JAYE, M., and HAYMAN, M. J. (1997). Ligand-binding specificity of human fibroblast growth factor receptor-3 IIIc. *FEBS Lett* 411, 389-92.

MAGAVI, S. S., LEAVITT, B. R., and MACKLIS, J. D. (2000). Induction of neurogenesis in the neocortex of adult mice. *Nature* 405, 951-5.

MCKEEHAN, W. L., and KAN, M. (1994). Heparan sulfate fibroblast growth factor receptor complex: structure- function relationships. *Mol Reprod Dev* 39, 69-81; discusison 81-2.

MEZEY, E., CHANDROSS, K. J., HARTA, G., MAKI, R. A., and MCKERCHER, S. R. (2000). Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* 290, 1779-82.

MOLTENI, A., MODROWSKI, D., HOTT, M., and MARIE, P. J. (1999). Differential expression of fibroblast growth factor receptor-1, -2, and -3 and syndecan-1, -2, and -4 in neonatal rat mandibular condyle and calvaria during osteogenic differentiation *in vitro*. *Bone* 24, 337-47.

NURCOMBE, V., FORD, M. D., WILDSCHUT, J. A., and BARTLETT, P. F. (1993). Developmental regulation of neural response to FGF1 and FGF2 by heparan sulfate proteoglycan. *Science* 260, 103-6.

NURCOMBE, V., SMART, C. E., CHIPPERFIELD, H., COOL, S. M., BOILLY, B., and HONDERMARCK, H. (2000). The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates. *J Biol Chem* 275, 30009-18.

ORNITZ, D. M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* 22, 108-12.

ORNITZ, D. M., XU, J., COLVIN, J. S., MCEWEN, D. G., MACARTHUR, C. A., COULIER, F., GAO, G., and GOLDFARB, M. (1996). Receptor specificity of the fibroblast growth factor family. *J Biol Chem* 271, 15292-7.

ORTEGA, S., ITTMANN, M., TSANG, S. H., EHRLICH, M., and BASILICO, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci U S A* 95, 5672-7.

PALMER, T. D., MARKAKIS, E. A., WILLHOITE, A. R., SAFAR, F., and GAGE, F. H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19, 8487-97.

PELLEGRINI, L., BURKE, D. F., VON DELFT, F., MULLOY, B., and BLUNDELL, T. L. (2000). Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* 407, 1029-34.

PERRIMON, N., and BERNFIELD, M. (2000). Specificities of heparan sulphate proteoglycans in developmental processes. *Nature* 404, 725-8.

PETERSEN, B. E., BOWEN, W. C., PATRENE, K. D., MARS, W. M., SULLIVAN, A. K., MURASE, N., BOGGS, S. S., GREENBERGER, J. S., and GOFF, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science* 284, 1168-70.

QIAN, X., DAVIS, A. A., GODERIE, S. K., and TEMPLE, S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. *Neuron* 18, 81-93.

QIAN, X., SHEN, Q., GODERIE, S. K., HE, W., CAPELA, A., DAVIS, A. A., and TEMPLE, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28, 69-80.

RABALLO, R., RHEE, J., LYN-COOK, R., LECKMAN, J. F., SCHWARTZ, M. L., and VACCARINO, F. M. (2000). Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J Neurosci* 20, 5012-23.

RAHMOUNE, H., CHEN, H. L., GALLAGHER, J. T., RUDLAND, P. S., and FERNIG, D. G. (1998). Interaction of heparan sulfate from mammary cells with acidic fibroblast growth factor (FGF) and basic FGF. Regulation of the activity of basic FGF by high and low affinity binding sites in heparan sulfate. *J Biol Chem* 273, 7303-10.

REYNOLDS, B. A., and WEISS, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707-10.

RICHARDS, L. J., KILPATRICK, T. J., and BARTLETT, P. F. (1992). De novo generation of neuronal cells from the adult mouse brain. *Proc Natl Acad Sci U S A* 89, 8591-5.

RIETZE, R. L., VALCANIS, H., BROOKER, G. F., THOMAS, T., VOSS, A. K., and BARTLETT, P. F. (2001). Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* 412, 736-9.

SCHLESSINGER, J., PLOTNIKOV, A. N., IBRAHIMI, O. A., ELISEENKOVA, A. V., YEH, B. K., YAYON, A., LINHARDT, R. J., and MOHAMMADI, M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 6, 743-50.

SUHONEN, J. O., PETERSON, D. A., RAY, J., and GAGE, F. H. (1996). Differentiation of adult hippocampus-derived progenitors into olfactory neurons *in vivo*. *Nature* 383, 624-7.

SVENDSEN, C. N., CALDWELL, M. A., and OSTENFELD, T. (1999). Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol* 9, 499-513.

SVENDSEN, C. N., TER BORG, M. G., ARMSTRONG, R. J., ROSSER, A. E., CHANDRAN, S., OSTENFELD, T., and CALDWELL, M. A. (1998). A new method for the rapid and long term growth of human neural precursor cells. *J Neurosci Methods* 85, 141-52.

TAKAHASHI, M., PALMER, T. D., TAKAHASHI, J., and GAGE, F. H. (1998). Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. *Mol Cell Neurosci* 12, 340-8.

TURNBULL, J., POWELL, A., and GUIMOND, S. (2001). Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends Cell Biol* 11, 75-82.

VESCOVI, A. L., GALLI, R., and GRITTI, A. (2001). The neural stem cells and their transdifferentiation capacity. *Biomed Pharmacother* 55, 201-5.

WANG, F., KAN, M., XU, J., YAN, G., and MCKEEHAN, W. L. (1995). Ligand-specific structural domains in the fibroblast growth factor receptor. *J Biol Chem* 270, 10222-30.