Sprouty genes prevent excessive FGF signalling in multiple cell types throughout development of the cerebellum

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SUMMARY

Fibroblast growth factors (FGFs) and regulators of the FGF signalling pathway are expressed in several cell types within the cerebellum throughout its development. Although much is known about the function of this pathway during the establishment of the cerebellar territory during early embryogenesis, the role of this pathway during later developmental stages is still poorly understood. Here, we investigated the function of sprouty genes (Spry1, Spry2 and Spry4), which encode feedback antagonists of FGF signalling, during cerebellar development in the mouse. Simultaneous deletion of more than one of these genes resulted in a number of defects, including mediolateral expansion of the cerebellar vermis, reduced thickness of the granule cell layer and abnormal foliation. Analysis of cerebellar development revealed that the anterior cerebellar neuroepithelium in the early embryonic cerebellum was expanded and that granule cell proliferation during late embryogenesis and early postnatal development was reduced. We show that the granule cell proliferation deficit correlated with reduced sonic hedgehog (SHH) expression and signalling. A reduction in Fgfr1 dosage during development rescued these defects, confirming that the abnormalities are due to excess FGF signalling. Our data indicate that sprouty acts both cell autonomously in granule cell precursors and non-cell autonomously to regulate granule cell number. Taken together, our data demonstrate that FGF signalling levels have to be tightly controlled throughout cerebellar development in order to maintain the normal development of multiple cell types.

KEY WORDS: Cerebellum, Sprouty, FGF, SHH, Granule cell, Mouse

INTRODUCTION

The cerebellum is an important control centre for fine motor coordination. The mammalian cerebellum consists of a medial vermis flanked by two hemispheres. Several cell types are found within the cerebellum, including granule cells (GCs, the most numerous type of neuron in the brain) (Hatten and Heintz, 1995), Purkinje neurons (Purkinje cells, PCs), interneurons and Bergmann glia (BG). These cells are organised in layers and folded into characteristic folia. Much is known about the mechanisms responsible for establishing the cerebellar territory in the early embryo; however, the nature of the signalling molecules and regulators required for generating the correct numbers of different types of neurons and glia to establish the complex architecture of the cerebellum is still poorly understood (Hatten and Heintz, 1995; Herrup and Kuemerle, 1997).

The cerebellum is derived from the most anterior segment of the hindbrain, rhombomere 1 (r1). Classical embryological studies in the chick embryo have shown that the isthmic organiser (IsO), located between the mesencephalon (mes) and r1, directs formation of the cerebellum (for a review, see Nakamura et al., 2008). Since the demonstration that fibroblast growth factor 8 (FGF8) can mimic the activity of the IsO in the chick embryo (Crossley et al., 1996; Martinez et al., 1999), a large body of evidence implicating FGF signalling in the survival, specification and patterning of r1 has accumulated (for reviews, see Partanen, 2007; Nakamura et al., 2008). In the mouse, the loss of Fgf8 expression from the IsO by embryonic day (E) 7.5 is associated with loss of the entire mes and r1 (Chi et al., 2003). Recently, we have shown that embryonic tissues that are located directly adjacent to the IsO (posterior mes and anterior r1) and that are, therefore, exposed to the highest concentration of FGF ligand, are dependent upon these high levels of FGF signalling for development (Basson et al., 2008). Furthermore, deletion of Fgf8 at later stages of development demonstrated a requirement for prolonged FGF signalling for the normal development of these structures (Sato and Joyner, 2009). The strength or duration of FGF signalling can affect cell fate during mes/r1 development. Ectopic expression of an Fgf8 splice variant, Fgfb, which encodes an FGF8 isoform with high affinity for FGF receptors (Olsen et al., 2006), in the mouse embryo can induce the expression of markers of r1 and cerebellar fate, such as Gbx2 (Liu et al., 1999). By contrast, ectopic expression of Fgfr8a, which encodes an FGF8 isoform with much lower affinity for FGF receptors (Olsen et al., 2006; Zhang et al., 2006), only expands the mes and does not appear to promote cerebellar fate (Lee et al., 1997; Liu et al., 1999).

After establishment of the cerebellar territory from r1, neural progenitors are born in germinal zones and migrate to specific locations within the anlage, where they proliferate and undergo further differentiation and maturation into mature neurons (for reviews, see Hatten and Heintz, 1995; Sotelo, 2004; Zervas et al., 2005; Sillitoe and Joyner, 2007). With the exception of the granule neurons that are derived from the upper rhombic lip (Wingate, 2001; Machold and Fishell, 2005), other classes of neurons and glia are born within the ventricular zone of the cerebellar anlage from ~E11.5 of development. These neuronal precursors migrate radially towards the pial surface on glial fibres. Granule cell precursors (GCps) migrate tangentially over the pial surface of the cerebellum to form the external granular layer (EGL). GCps proliferate...
extensively from ~E16.5 through to postnatal day (P) 15 in response to cell surface ligands and secreted growth factors (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Klein et al., 2001; Solecki et al., 2001; Corrales et al., 2006) to generate a vast number of granule neurons. Upon cell cycle exit, these cells migrate inwardly on Bergmann glial fibres to form the inner granular layer (IGL) (Sotelo and Changeux, 1974; Hatten et al., 1984; Yacubova and Komuro, 2003).

A key signalling molecule that is required for GCp proliferation is sonic hedgehog (SHH), which is produced by PC precursors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Kenney et al., 2003; Lewis et al., 2004). Exogenous FGF2 has been shown to inhibit the responsiveness of GCPs to SHH in vitro and in vivo, resulting in reduced GCp proliferation (Wechsler-Reya and Scott, 1999; Fogarty et al., 2007). Whether endogenous FGF signalling has a direct role in controlling GCp proliferation in vivo remains to be determined. We and others recently reported that many genes encoding FGF ligands and receptors are expressed throughout cerebellar development, including postnatal stages when GCPs are proliferating (e.g. Yaguchi et al., 2009). However, with the exception of FGF9, which has been shown to regulate GC migration to the IGL (Lin et al., 2009), no other FGF ligand has been implicated in later stages (>E12.5) of cerebellar development.

Several regulators of embryonic FGF signalling have been identified over the last decade. These include dual specificity phosphatases, Sef (II17rd – Mouse Genome Informatics), sprouty and spry proteins (Echevarria et al., 2005; Lin et al., 2005; Mason et al., 2006; Bündschu et al., 2007; Li et al., 2007). Sprouty genes encode feedback antagonists of FGF signalling and three Sprouty genes (Spry1, Spry2 and Spry4) are expressed in the IsO, posterior mes and anterior r1 (Minowada et al., 1999). We investigated the function of these genes in development of the mouse cerebellum, and found that these genes are required to prevent excessive FGF signalling at multiple stages of cerebellar development. The effects of deregulated signalling on cerebellar morphology depended on the time and cell type in which Sprouty genes were deleted, revealing several distinct functions of these genes during cerebellar morphogenesis.

MATERIALS AND METHODS

Mouse lines

Conditional floxP-flanked and null alleles of Sprouty1 (Spry1) (Basson et al., 2005), Sprouty2 (Spry2) (Shim et al., 2005), Sprouty4 (Spry4) (Klein et al., 2006), Fgfr1 (Xu et al., 2002) have been described. These lines were intercrossed and crossed with the following Cre lines: En1cre/+, Math1-Cre (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Corrales et al., 2006) as indicated. All lines were maintained on a mixed genetic background. Tail DNA preparations were genotyped by PCR as described in the original publications. All experimental procedures were approved by the UK Home Office.

Histology, in situ hybridisation and immunohistochemistry

For in situ hybridisation and immunohistochemistry on sections, embryos and brains were dissected in ice-cold PBS, fixed overnight in 4% paraformaldehyde (PFA) and embedded in paraffin wax. Serial sections were cut at 10 µm thickness and dried overnight at 42°C. Some sections were stained with Cresyl Violet for histological analyses. In situ hybridisation was carried out using standard methods. Digoxigenin-labelled antisense probe for En5 (Chen et al., 2005), Gli1 (Corrales et al., 2004), Pich1 (Wechsler-Reya and Scott, 1999), Shh (Corrales et al., 2004), Spry1, Spry2 and Spry4 (Minowada et al., 1999) were used. Immunohistochemistry was carried out with the following primary antibodies: anti-BLB (Chemicon, AB9558; 1:500), anti-calbindin (Swant, CB-38a; 1:2000), anti-GFAP (Dako, 0334; 1:500), anti-PCP2 (kind gift from Brad Denker, Harvard University, Boston, MA, USA; 1:200), anti-p27 (Santa Cruz; 1:50), anti-S100 (Dako, Z0311; 1:200), anti-BrDU (BD Biosciences, 347580, 1:100; or Abcam, Ab6362; 1:50) and anti-SHH (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA, 5E1; 1:50). Primary antibodies were detected using Alexafluor-conjugated species-specific secondary antibodies (Invitrogen; 1:200) or biotinylated secondary antibodies detected using the Vectastain ABC Kit (Vector Laboratories) and visualised using 0.03% diaminobenzidine (DAB; Sigma).

TUNEL

Cerebella were collected and processed as for immunohistochemistry. TUNEL on paraffin sections was performed by using the In Situ Cell Death Detection Kit Fluorescein (Roche). Sections were dehydrated in xylene and dehydrated through a descending ethanol series to PBS before treating with proteinase K for 15 minutes at 37°C. After washing in PBS for 10 minutes, antigen retrieval was carried out by microwaving sections in 10 mM sodium citrate buffer (pH 6.0) for 2 minutes at low power. Slides were incubated with reaction mix (10% enzyme solution: 90% label solution) at 37°C for 1 hour. After a few washes in PBS in the dark, samples were counterstained with Hoechst 33258.

Whole-mount in situ hybridisation

E9.5 embryos were dissected in ice-cold PBS, fixed overnight in 4% PFA at 4°C and processed for in situ hybridisation as previously described (Wilkinson et al., 1989b). Digoxigenin-labelled antisense probe for Etv5, Gbx2 (Wassarman et al., 1997), Hoxa2 (Wilkinson et al., 1989a), Otx2 (Simeone et al., 1993), Spry1, Spry2 and Spry4 (Minowada et al., 1999) were used.

GCP cell cycle analysis

To quantify the number of cells in S-phase, pregnant females were injected intraperitoneally with 50 mg BrdU (5-bromo-2′-deoxyuridine, Sigma) per kg body weight. Newborn mice up to P10 were injected with 20 mg BrdU per kg body weight. Mice were sacrificed 1 hour later, brains collected and processed as described above. After immunodetection, BrdU labelled and total number of cells in the EGL were counted in 100×100 µm areas (two to three fields in each lobule). The labelling index (LI) was calculated as the BrdU-labelled/total number of GCPs. To assess the Q fraction, P4 pups were injected with 20 mg IdU (iododeoxyuridine, Sigma) per kg body weight, followed 2 hours later by six BrdU injections at 3-hourly intervals. Mice were sacrificed 30 minutes after the last injection, sections immunostained for BrdU and IdU (Martyngola et al., 2005) and the Q fraction calculated as described (Takahashi et al., 1993; Takahashi et al., 1996).

Western blot

E18.5 mouse cerebella were dissected in ice-cold PBS and frozen on dry ice immediately. Tissues were washed and homogenised in freshly made 20 mM Tris-HCl (pH 7.3) with a cocktail of protease inhibitors (Roche). Proteins were quantified with BSA Protein Assay Reagent (Pierce). Four cerebella of each genotype were analysed. Total protein (10 µg) was resolved on a NuPAGE 3-8% Tris Acetate gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with 10% goat serum in PBS with 0.25% Tween and incubated with the rabbit anti-Gli3 antibody (Santa Cruz, H-280, sc-20688, 1:100) for an hour at room temperature. After incubating with HRP-conjugated anti-rabbit IgG secondary antibody (1:200, DakoCytomation), signal was detected using ECL Plus detection (GE Healthcare) on an X-ray film (Kodak, BioMax, XAR film, Sigma). Band intensity was scanned and measured using ImageJ.

RESULTS

Sprouty genes are expressed throughout cerebellar development

Previous studies have shown that Spry1, Spry2 and Spry4 are expressed in and around the IsO at E9.5 and E10.5 (Minowada et al., 1999; Liu et al., 2003). We recently reported that several genes...
encoding FGF ligands, receptors and downstream target genes are expressed in the developing cerebellum after E10.5 (Yaguchi et al., 2009). To determine whether transcripts of sprouty genes are also present during these later stages of cerebellar development, we analysed gene expression by in situ hybridisation at E12.5, E16.5, P0 and P7. Spry1 and Spry2 expression were maintained at high levels in the IsO, posterior mes and throughout r1 at E12.5 (Fig. 1A,B). Spry4 was expressed at low levels (Fig. 1C). At E16.5, all three genes were expressed in the ventricular zone and in cells throughout the cerebellar anlage. Gene expression in the PC/BG cell layer was particularly prominent, and some cells in the EGL and dura were also positive (Fig. 1D-F). Spry2 transcripts were present at high levels in cells in the posterior EGL (Fig. 1E). By P0, the expression patterns of Spry1 and Spry2 were similar to E16.5, with high expression in the PC/BG layer (Fig. 1G,H). Spry4 expression was low in PC/BG layer and relatively high in GCps around the secondary fissure in the posterior cerebellum (Fig. 1I, sec). At P7, the strongest sprouty gene expression was observed in the PC/BG layer and outer layer of the IGL (Fig. 1J-L). Double immunohistochemistry (IHC) confirmed that most of the sprouty gene expression localised to GFAP+Bergmann glia underneath the calbindin-positive Purkinje cell layer (PCL; Fig. 1M-P). As sprouty genes are expressed throughout development, we hypothesised that these genes might control several aspects of cerebellar development.

**Spry1, Spry2 and Spry4 are required for normal cerebellar development**

Analysis of Spry2−/− (Shim et al., 2005) mutants revealed that cerebellar development was only mildly affected in these mutants, with a small number of neurons in the most anterior, medial portion of the cerebellar vermis being slightly disorganised (see Fig. S1 in the supplementary material). Spry1−/− (Basson et al., 2005) and Spry4−/− (Klein et al., 2006) cerebella appeared normal (see Fig. S1 in the supplementary material). As a high level of functional redundancy between the sprouty genes during cerebellar development might account for these mild defects, we produced mice lacking more than one sprouty gene (e.g. Spry1−/−;Spry2−−). These mice died at birth owing to multi-organ defects (Mahoney Rogers et al., 2011; Taniguchi et al., 2007). To circumvent these lethal defects, we simultaneously deleted two or three conditionally targeted (flanked by loxp sites, ‘lox’; sprouty alleles specifically from the mes/r1 region of the embryo using an En1cre/+ line (Kimmel et al., 2000; Basson et al., 2008) (see Fig. S2 in the supplementary material). These conditional mutants survived to adulthood, which allowed us to determine the functions of sprouty genes during postnatal cerebellar development. Examination of whole brains revealed a change in the overall dimensions of the cerebellar vermis, compared with littermate controls (Fig. 2A). The anteroposterior length of the vermis was reduced in En1cre/+;Spry1flox/flox;Spry2flox/flox;Spry4flox/flox mice, whereas the mediolateral width was increased (Fig. 2B). A mediolateral expansion of the vermis is consistent with our previous findings showing a reduction or loss of vermis tissue when FGF signalling is reduced (Basson et al., 2008). However, the reduction of vermis size in the anteroposterior dimension was unexpected and could not be readily explained by increased FGF signalling from the IsO. Based on previous studies, increased FGF signalling might be predicted to have two possible effects on midbrain development: an enlarged posterior midbrain or fate transformation of midbrain tissue into cerebellum (Crossley et al., 1996; Lee et al., 1997; Liu et al., 1999; Martinez et al., 1999; Nakamura et al., 2008). We found that the posterior midbrain (inferior colliculus, IC) was enlarged in these mutants with no sign of transformation to cerebellum (Fig. 2B). The additional depletion of Spry4 in En1cre/+;Spry1flox/flox;Spry2flox/flox;Spry4flox/flox animals exacerbated both these defects (Fig. 2C).

Sagittal sections through the cerebellar vermis of these mutants confirmed the marked reduction in size in the anteroposterior dimension, which appeared to be due to thinner GC layers with fewer GCs (Fig. 2D-F). Furthermore, the normal organisation of GCs in smooth folia was also disrupted in these mutants. Neuronal hypocellularity and disorganisation was most severe in the anterior zone of the En1cre/+;Spry1flox/flox;Spry2flox/flox cerebella, suggesting
Sprouty genes function as FGF antagonists in the IsO

Our data thus far indicated that sprouty genes were expressed throughout cerebellar development and that several cell types required sprouty gene function for their normal development. The next step was to identify the exact developmental defects caused by sprouty gene loss of function. Before focusing on the development of particular cell types, we first asked whether sprouty genes functioned as FGF antagonists in the context of the embryonic IsO. To determine whether FGF signalling in mes/r1 cells was affected by the loss of sprouty genes, we analysed the expression of genes transcriptionally regulated by FGF signalling. Etv5 (Erm) is expressed in the IsO and in cells in the posterior mes and anterior r1 flanking the IsO (Fig. 3A). Etv5 expression was significantly upregulated and expanded around the IsO of En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl embryos (Fig. 3B). Etv5 expression was further increased and expanded in En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl embryos (Fig. 3C), suggesting that all three sprouty genes functioned redundantly as FGF antagonists in mes/r1. Next, we tested whether increased FGF signalling affected gene expression patterns in the mes/r1 region. Exogenous FGF8b has been shown to promote cerebellar fate by inducing the expression of the r1 marker Gbx2 (Hidalgo-Sanchez et al., 1999; Liu et al., 1999). Gbx2 expression was increased and expanded in sprouty mutant embryos (Fig. 3D-F), consistent with a previous study (Lin et al., 2005). FGF signals can also repress the mesencephalic marker Otx2 and GBX2 and Otx2 mutually repress each other’s expression (Joyner et al., 2000). Compared with control embryos in which Gbx2 and Otx2 gene expression sharply abuts at the IsO (Fig. 3D,G), Gbx2 expression appeared to be expanded anteriorly and Otx2 expression appeared to be repressed in a complementary pattern in sprouty mutant embryos (Fig. 3E,F,H,I). Using Hoxa2 expression as a marker of the posterior border of r1 in combination with Otx2 to indicate the anterior limit of r1 (Fig. 3G), we confirmed that r1 was significantly expanded in sprouty mutant embryos (Fig. 3H,I). Interestingly, this expansion of r1 tissue correlated well with the enlarged Gbx2 expression domain in anterior r1, suggesting that the expansion of r1 was due to an increase in the size of anterior r1, the region immediately adjacent to the source of FGF signalling.

Genetic inducible fate mapping studies have shown that the anterior portion of r1 (marked here by high Gbx2 expression) is fated to become the cerebellar vermis (Sgaier et al., 2005). Thus, the expansion of the Gbx2 positive anterior r1 in sprouty mutant embryos provided an explanation for the enlarged vermis along the mediolateral plane that characterised these mutant cerebella by P21 (Fig. 2G). In sprouty mutants, glial processes appeared thicker and disorganised (Fig. 2H,I). PCs are large neurons organised in a monolayer between the molecular and GC layers with elaborate dendritic trees extending into the molecular layer (Fig. 2J). PC soma appeared smaller and disorganised in sprouty mutants (Fig. 2K,L). We concluded that Spry1, Spry2 and Spry4 were essential for the normal morphogenesis and cytoarchitecture of the cerebellum. All three of these genes appear to function largely overlapping roles, as no unique function could be ascribed to an individual gene and all sprouty-deficient phenotypes were enhanced by a further reduction in sprouty gene dosage.

Sprouty loss of function results in expansion of the vermis and abnormal cytoarchitecture. (A–C) Dorsal views of control Spry1fl/fl;Spry2fl/fl (A), En1cre/+;Spry1fl/fl;Spry2fl/fl (B) and En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl (C) whole-mount P21 mouse cerebella. The yellow line indicates the anteroposterior length of the vermis, and the red line indicates the mediolateral width. (D–F) Cresyl Violet-stained sagittal sections of P21 control (D,D'), En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl (E,E') and En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl (F,F') cerebella; anterior is to the left. D'–F' are higher magnification views of boxed areas in D–F. (G–I) GFAP immunostaining (red) on P21 sagittal sections to detect Bergmann glial fibres, counterstained with Hoechst (blue). High magnification views of the molecular layer are shown. (J–L) PCP2 immunostaining (red) on P21 sagittal sections to detect Purkinje cells. IC, inferior colliculus; SC, superior colliculus.

that the anterior zone was more sensitive to deregulated FGF signalling (Fig. 2E,E'). Interestingly, the milder phenotype in the posterior cerebellum was markedly exacerbated by the additional loss of Spry4 (Fig. 2F). Thus, En1cre/+;Spry1fl/fl;Spry2fl/fl;Spry4fl/fl cerebella exhibited severe hypocellularity and disorganisation throughout, indicating that Spry4, to a large extent, compensated for the loss of Spry1 and Spry2 in the posterior region of En1cre/+;Spry1fl/fl;Spry2fl/fl cerebella. This ability of Spry4 to compensate for the loss of the other sprouty genes in the posterior cerebellum is consistent with the higher expression of Spry4 in this region (Fig. 1F,I,L). Immunostaining for GFAP and calbindin to visualise Bergmann glia and PCs, respectively, revealed abnormalities in these cells. Regularly spaced, thin Bergmann glial processes that are perpendicular to the pial surface normally span the molecular layer (Fig. 2G). In sprouty mutants, glial processes appeared thicker and disorganised (Fig. 2H,I). PCs are large neurons organised in a monolayer between the molecular and GC layers with elaborate dendritic trees extending into the molecular layer (Fig. 2J). PC soma appeared smaller and disorganised in sprouty mutants (Fig. 2K,L). We concluded that Spry1, Spry2 and Spry4 were essential for the normal morphogenesis and cytoarchitecture of the cerebellum. All three of these genes appear to function largely overlapping roles, as no unique function could be ascribed to an individual gene and all sprouty-deficient phenotypes were enhanced by a further reduction in sprouty gene dosage.
was reduced during development. To determine whether the production of granule neurons in Sprouty mutant cerebella are characterised by reduced thickness of the GC layer, suggesting that the production of granule neurons is compromised, we visualised these cells shortly after their generation, comparing the number of GCs from rhombic lip was compromised, we visualised these cells shortly after their production was initiated (Machold and Fishell, 2005). At E13.5, the cerebellar anlage of sprouty mutant embryos appeared slightly larger than control littermates, in agreement with the expansion of r1 observed at E9.5 (Fig. 3H), No difference in the number or distribution of Math1 (Atoh1 – Mouse Genome Informatics) + cells was observed in sprouty mutants at E13.5, excluding a major defect in the specification or production of these cells from the rhombic lip (Fig. 4A,B). Furthermore, Math1 + cells were present along the whole pial surface of the cerebellar anlage, up to its most anterior edge, excluding any abnormalities in tangential migration. By E16.5, when GCs in the EGL had started proliferating, the EGL appeared slightly thinner in the anterior zone of mutant cerebella (Fig. 4C,D). The analysis of developing cerebella at birth (P0) revealed that foliation was initiated as normal with all fissures present. However, some fissures were more shallow and the EGL appeared thinner in the mutants. In addition, the EGL appeared to be completely absent in some areas, especially at the base of some of the forming fissures in the anterior zone (Fig. 4E,F). By P7, the mutant cerebella were smaller and the EGL was thinner than that of control littermates (Fig. 4G,H). Mutant granule cells were disorganised, especially in the anterior region. Furthermore, small regions could be observed where the EGL was almost absent, presumably owing to the loss of GCs (inset in Fig. 4G,H).

These data suggested that the reduction in GC numbers was not due to defects in the formation or migration of GCs from the rhombic lip, but rather due to a failure to maintain the rapidly dividing population of GCs in the EGL from E16.5 onwards. To find evidence for this, we first quantified the number of proliferating GCs at P0, when the first clear evidence of abnormalities in the EGL emerged in the sprouty mutants. The fraction of proliferating progenitors that incorporated BrdU was significantly reduced throughout the mutant cerebella (Fig. 4I-K). To determine whether the failure to maintain sufficient numbers of proliferating progenitors was associated with enhanced GCp differentiation, we stained sections for TAG1 (CNTN2 – Mouse Genome Informatics), an early differentiation marker of cells in the EGL (Bizzoca et al., 2003), and p27Kip1 (p27; CDKN1B – Mouse Genome Informatics), a cyclin-dependent protein kinase inhibitor, which is present at high levels in postmitotic GCs in the inner EGL (Miyaizawa et al., 2000). Consistent with previous reports, TAG1-expressing pre-migratory cells in the inner EGL represented approximately one-third of the total EGL in control cerebella (Fig. 4L). By contrast, the inner EGL corresponded to approximately half of the EGL in sprouty mutant cerebella (Fig. 4M). Furthermore, the number of cells in the inner EGL with high levels of p27 appeared to be increased in mutant cerebella compared with controls, and p27-positive cells were also detected in the outer EGL in sprouty mutant cerebella (Fig. 4N,O). To quantify cell differentiation within a specific time window, we labelled proliferating cells by injecting pups with the thymidine analogue IdU, followed 2 hours later by the cumulative labelling of proliferating cells by five BrdU injections at 3 hourly intervals. The fraction (Q) of IdU-labelled cells that exited the cell cycle and differentiated without incorporating BrdU, relative to IdU-labelled cells that continued cycling and therefore incorporated BrdU during the 17.5-hour period was calculated (Takahashi et al., 1993; Takahashi et al., 1996). In control cerebella, ~5-10% of GCs stopped cycling and differentiated during a 17.5-hour window (Q fraction=0.05-0.1; Fig. 4P-R). By contrast, 11-22% of GCs exited the cell cycle and differentiated in sprouty mutants (Q fraction=0.11-0.22; Fig. 4Q,R). We concluded that the reduced number of GCs and generation of GC precursors from the rhombic lip was compromised, we visualised these cells shortly after their production was initiated (Machold and Fishell, 2005). At E13.5, the cerebellar anlage of sprouty mutant embryos appeared slightly larger than control littermates, in agreement with the expansion of r1 observed at E9.5 (Fig. 3H). No difference in the number or distribution of Math1 (Atoh1 – Mouse Genome Informatics) + cells was observed in sprouty mutants at E13.5, excluding a major defect in the specification or production of these cells from the rhombic lip (Fig. 4A,B). Moreover, Math1 + cells were present along the whole pial surface of the cerebellar anlage, up to its most anterior edge, excluding any abnormalities in tangential migration. By E16.5, when GCs in the EGL had started proliferating, the EGL appeared slightly thinner in the anterior zone of mutant cerebella (Fig. 4C,D). The analysis of developing cerebella at birth (P0) revealed that foliation was initiated as normal with all fissures present. However, some fissures were more shallow and the EGL appeared thinner in the mutants. In addition, the EGL appeared to be completely absent in some areas, especially at the base of some of the forming fissures in the anterior zone (Fig. 4E,F). By P7, the mutant cerebella were smaller and the EGL was thinner than that of control littermates (Fig. 4G,H). Mutant granule cells were disorganised, especially in the anterior region. Furthermore, small regions could be observed where the EGL was almost absent, presumably owing to the loss of GCs (inset in Fig. 4G,H).

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![Image](53x463 to 296x732)

**Fig. 3. Sprouty genes function as negative regulators of FGF signalling at the ISL in the early embryo, whereafter they are required for the normal production of granule neurons.**

(A-I) Whole-mount in situ hybridisation of Evx5 (A–C), Gbx2 (D–F) and Otx2;Hoxa2 (G–I) on E9.5 control (Spry1fl/fl;Spry2fl/fl; A–D, G) and Nestin-Cre;Spry1fl/fl;Spry2fl/fl; Spry4fl/fl (C, F) mouse embryos. Red dashed lines in D–F indicate the anterior limit of Gbx2 expression and white arrows the approximate size of anterior r1 (ar1) as marked by Gbx2. Red dashed lines in G–I mark the anterior and posterior limits of r1 as indicated by Otx2 and Hoxa2, respectively, and black arrows the approximate size of r1. The white dashed lines in H and I indicate the approximate position of the Gbx2 expression limit in the control embryo for comparison. (J) Dorsal view of a whole-mount Nestin-Cre;Spry1fl/fl;Spry2fl/fl cerebellum at P21. (K, K’) Cresyl Violet-stained sagittal sections from the brain shown in J. K’ is a higher magnification view of the boxed area in K. IC, inferior colliculus; SC, superior colliculus.

defects were due to the absence of sprouty gene function after E9.5, we produced conditional mutants in which sprouty genes were deleted in all neural and glial progenitors between E10 and E11 of development using a Nestin-Cre line (see Fig. S2 in the supplementary material). Nestin-Cre;Spry1fl/fl;Spry2fl/fl mutant cerebella exhibited a thinner GC layer, abnormal foliation and disorganised neurons and glia, indicating that these defects were not secondary to abnormalities during r1 specification in the early (E9.5) embryo (Fig. 3J-K’).

As deletion of two or three sprouty genes in mes/r1 resulted in qualitatively similar phenotypes, we continued our analyses of cerebellar development in En1Cre;Spry1fl/fl;Spry2fl/fl; Spry4fl/fl mutants to minimise the numbers of animals used in our experiments.

**Sprouty genes are required to maintain a self-renewing GCp population and prevent their premature differentiation**

Sprouty mutant cerebella are characterised by reduced thickness of the GC layer, suggesting that the production of granule neurons was reduced during development. To determine whether the
thinner GC layers in the mutant cerebella at P21 were due to fewer GCps in the postnatal EGL being maintained in a proliferative, undifferentiated state.

To exclude the possibility that cell death was responsible for reduced granule cell numbers, apoptotic cells were identified by TUNEL at E18.5, P0 and P3. No differences were detected between the mutants and littermate controls (see Fig. S3 in the supplementary material).

Cerebellar defects in sprouty mutant animals are caused by increased FGF and reduced SHH signalling

Having established that sprouty genes are required for normal levels of GCp proliferation in the postnatal cerebellum, we wanted to understand how loss of sprouty function resulted in this defect. As the SHH pathway has been implicated in GCp proliferation and exogenous FGF2 has been shown to inhibit the responsiveness of GCps to SHH (Wechsler-Reya and Scott, 1999; Fogarty et al., 2007), we tested whether these signalling pathways were deregulated in the early postnatal cerebellum. As expected, the expression of genes positively regulated by FGF signalling, Etv4 (Pea3) (not shown) and Etv5 (Erm), were upregulated in the postnatal cerebellum, confirming that sprouty genes also functioned as FGF antagonists at this stage of development (Fig. 5A,B). The expression of Gli1 and Ptc1, transcriptional targets of SHH signalling in the cerebellum (Corrales et al., 2004), was downregulated (Fig. 5C-F), consistent with a model whereby SHH signalling was inhibited by excessive FGF signalling (Wechsler-Reya and Scott, 1999).
As an additional read-out of SHH signalling, we quantified the post-translational processing of GLI3. GLI3 exists in the cell as either a full-length protein (190 kDa), which acts as a transcriptional activator of genes regulated by SHH signalling, or a cleaved 80 kDa form, which acts as a repressor. SHH signalling inhibits this processing of the full-length protein (Wang et al., 2000). We measured the ratio between the full-length GLI3 (GLI3A) and the cleaved isoform (GLI3R) (Fig. 5G). At E18.5, the ratio of GLI3A:GLI3R was 0.87±0.04 in control cerebella (n=4), compared with sprouty mutants, in which this ratio was significantly decreased to 0.71±0.04 (P<0.05). This observation further supports our conclusion that SHH signalling is downregulated in sprouty-deficient cerebella.

To prove that the reduced SHH signalling was caused by excessive FGF signalling and that increased FGF signalling was indeed responsible for the observed phenotypes in sprouty-deficient cerebella, we reduced FGF signalling during development. To achieve this, the dosage of the FGF receptor 1 gene, which encodes a high affinity FGF receptor expressed throughout cerebellar development (Trokovic et al., 2003), was reduced. Fgfr1+/– mice were normal, indicating that halving the Fgfr1 gene dosage is not sufficient to disrupt normal development (data not shown). At P21, En1cre/+;Spry1fl/fl;Spry2fl/fl;Fgfr1+/– cerebella (n=4) exhibited a striking rescue of the sprouty phenotype (Fig. 5H-J). Although the granule cell layers (GCLs) were still slightly thinner compared with normal controls, the cerebellum were of normal size and neuronal disorganisation was restricted to the most anterior portion of the cerebellum (lobule I, Fig. 5J). The phenotypic rescue correlated with a rescue of excessive FGF signalling (Fig. 5K-M) as well as a rescue of the reduced SHH signalling in the early postnatal cerebellum (Fig. 5N-P). The latter observation demonstrated that increased FGF signalling was responsible for the reduced SHH signalling in the developing cerebellum, in agreement with previous studies where exogenous FGF was shown to inhibit SHH signalling and GCP proliferation (Wechsler-Reya and Scott, 1999; Fogarty et al., 2007). To confirm that the normalisation of FGF and SHH signalling levels in En1cre/+;Spry1fl/fl;Spry2fl/fl;Fgfr1+/– cerebella was indeed associated with a rescue of GCP proliferation levels, proliferating cells were labelled by BrdU in newborn (P0) brains. GCP proliferation was increased in these..
Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> mutants (rescue) such that the BrdU labelling indices were statistically different from those in the corresponding regions of mutant cerebella, with the exception of lobules IV/V (Fig. 5Q).

To test whether sprouty genes function cell-autonomously within GCps to maintain them in a proliferative state, we deleted these genes specifically in GCps using a Math1-Cre transgene (Schuller et al., 2007) (see Fig. S2 in the supplementary material). The EGL in Math1-Cre;Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> cerebella appeared only slightly thinner at P3 (Fig. 6A-D) and foliation and neuronal organisation appeared normal (Fig. 6A,B). GCP proliferation was reduced in the postnatal EGL of Math1-Cre;Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> animals (Fig. 6C-E). However, this reduction in GCP proliferation was less pronounced than in the En<sup>F<sub>1</sub>Cre;</sup>Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> mutants and only significant in the anterior (I-II) and posterior (IX) lobules. These data suggest that sprouty genes can regulate GCP proliferation cell-autonomously, albeit to a limited degree. Analysis of Math1-Cre;Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> cerebella at P21 confirmed that the effects of deleting sprouty genes in GCps could not fully recapitulate the phenotype observed in the En<sup>F<sub>1</sub>Cre;</sup>Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> mutants (Fig. 6F-I). The observation that the loss of sprouty genes in GCps could not fully account for the GC phenotype in the En<sup>F<sub>1</sub>Cre;</sup>Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> mutants is consistent with their low expression levels in this cell type (Fig. 1). We therefore sought to analyse the development of other cell types that could contribute to this phenotype.

**Abnormal differentiation of Bergmann glia and PCs in sprouty mutants**

Sprouty genes are expressed at high levels in Bergmann glia and these cells exhibit structural abnormalities by P21 (Fig. 2G-I). To identify the origin of these defects, we followed their development during embryogenesis. The first sign of differentiated Bergmann glia in the cerebellar anlage was the appearance of brain lipid binding protein (BLBP<sup>+</sup>) glial fibres in the dorsal-anterior region of the cerebellar anlage (Fig. 7A,A'). It was difficult to detect the presence of BLBP<sup>+</sup> radial glia in the mutants at this stage, suggesting that the differentiation of these cells is affected by the loss of sprouty genes (Fig. 7B,B'). By E14.5, a small population of BLBP<sup>+</sup> glial cells could be observed in the anterior-dorsal region, extending glial fibres from their cell bodies out to the pial surface where they contacted the basement membrane in so-called 'end feet' structures (Fig. 7C). Glial fibres in the sprouty mutant cerebella appeared twisted and shorter and no clear organisation was evident (Fig. 7D). By P0, most BG cell bodies (positive for S100) were present in the BG/PCL in normal cerebella (area between dotted white lines in Fig. 7E), whereas many S100<sup>+</sup> cells were found in ectopic positions in the molecular layer of the mutants (white arrowheads in Fig. 7E). By P7, disorganised, abnormally thick GFAP<sup>+</sup> glial fibres could be observed in the mutant cerebella (Fig. 7G,H).

A similar analysis of PC differentiation indicated that PC precursors developed normally during embryogenesis and that these cells were normally distributed in newborn cerebella (Fig. 7I-J). However, by P7, the PCs in mutant cerebella failed to organise in monolayers and extend dendritic arborisations as they do in controls (Fig. 7K,L). As this defect in PC differentiation appeared late in development, i.e. after BG and GC defects became evident, and sprouty gene expression in these cells was extremely low (Fig. 1), we concluded that these structural PC defects were most likely to be secondary to defects in BG and GCps.

**Sprouty genes are required to maintain normal levels of SHH expression in the postnatal cerebellum**

As the cell type-specific deletion of sprouty genes from GCps only had a small effect on GCP proliferation, modulation of GCP responsiveness to SHH could not fully explain the reduction in GCP proliferation in En<sup>F<sub>1</sub>Cre;</sup>Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> cerebella. Thus, we considered the possibility that defects in the other cell types affected by the loss of sprouty genes, PCs and Bergmann glia might contribute to this phenotype. As PCs appear to be the major source of SHH in the developing cerebellum (Lewis et al., 2004), we considered the possibility that SHH production was abnormal in sprouty mutants. Indeed, fewer PCs expressed Shh and the overall levels of Shh expression appeared to be reduced in En<sup>F<sub>1</sub>Cre;</sup>Spry1<sup>fl/fl</sup>;Spry2<sup>fl/fl</sup> cerebella by E18.5 (Fig. 8A-B'). Immunohistochemical detection of SHH protein at P4 confirmed the absence of PCs with high SHH protein levels (Fig. 8C,D). As SHH is required for normal PC differentiation, this observation might also explain the postnatal PC defects (Dahmane and Ruiz i Altaba, 1999). To test whether reduced Shh expression was also...
caused by hyperactive FGF signalling, we compared Shh expression between the various mutants and found that reducing Fgfr1 gene dosage also rescued this defect in the sprouty mutants (Fig. 8E-G). These observations suggest that a reduction in the availability of SHH ligand, in combination with the hypo-responsiveness of GCps to SHH, was responsible for the observed defects in GCp proliferation.

DISCUSSION
Since the initial demonstration that FGF8 is sufficient to mimic isthmic organiser activity in the mes/r1 region of the embryonic brain, a large body of work has accumulated to support an essential role for FGF signalling in the establishment, maintenance and patterning of r1 in the early embryo. In this paper, we show that the appropriate level of FGF signalling from the IsO is maintained by sprouty gene expression and that deregulated signalling in the absence of these genes affects normal growth and patterning. We show that all three sprouty genes expressed in the embryo are involved in this activity. In addition, we provide the first evidence for a continued role of these genes during later stages (post-E12.5) of cerebellar morphogenesis. Excessive FGF signalling in the absence of these genes affects the differentiation of multiple cell types and the proliferation of GCps.

Earlier studies on the role of FGF in the IsO have shown that high amounts of FGF8, ectopically applied on beads or via electroporation of Fgf8-expressing constructs, can transform the mes to r1 or induce mes expansion, depending on the strength of the FGF signal provided (Lee et al., 1997; Liu et al., 1999; Sato and Nakamura, 2004; Olsen et al., 2006). Overexpression of Spry2 or dominant-negative Spry2 in the chick mes/r1 inhibits or enhances FGF signalling resulting in mes/r1 fate changes (Suzuki-Hirano et al., 2005). By deleting endogenous antagonists in the mouse embryo we studied the consequences of increased FGF signalling in the context of normal expression (i.e. not ectopic) and found evidence for both a fate transformation early in development as well as an expansion of the mes later in development. Our observation of an expansion of the Gbx2-expressing anterior r1, apparently at the expense of Otx2-positive mes, is consistent with cell fate at the IsO being sensitive to the level of FGF signalling. However, we cannot rule out the possibility that a small increase in cell proliferation in anterior r1 might be the cause of this apparent change in gene expression. Furthermore, our observation that the posterior midbrain is expanded in sprouty mutant mice confirms previous observations showing that increased FGF signalling can also increase the size of the midbrain. The fact that the genetic manipulations we report here increase FGF signalling throughout cerebellar development might account for the fact that both an anterior expansion of r1 fate early in development (E9.5) and larger midbrain (by P0) are observed.

A number of previous studies have shown that the differentiation of cell types in the developing cerebellum are intimately linked. For example, Purkinje cell differentiation has been shown to require GCps (Berry and Bradley, 1976; Caddy and Herrup, 1990; Baptista et al., 1994). The regulation of GCp proliferation by PCs is well established (Lewis et al., 2004) and PC-derived signals control aspects of glial differentiation (Fukaya et al., 1999; Tam et al., 2010). In addition, the genetic ablation of glial cells has been shown to affect both PC differentiation and GCp proliferation (Delaney et al., 1996). Taking together our observations that BG express high amounts of sprouty transcripts (compared with the low or absent expression in other cell types) and that BG differentiation defects are present prior to GCp
proliferation defects becoming apparent, raises the possibility that these early glial defects might affect the development of several other cell types, including GCps. Interestingly, GCps have been shown to respond to at least one factor secreted by cerebellar astroglia, FGF2 (Hatten et al., 1988). As the deletion of sprouty genes in other developmental contexts are sometimes associated with increased FGF gene expression (Klein et al., 2008), we looked for changes in FGF gene expression in En1cre/+;Spry1fl/fl;Spry2fl/fl mouse cerebella. However, no changes in Fgf2, Fgf3, Fgf9 or Fgf15 expression could be observed (data not shown). A recent study demonstrated that the deletion of FGF receptors during later stages of cerebellar development using Nestin-Cre, resulted in major cerebellar defects including GCp proliferation and migration defects (Lin et al., 2009). As the deletion of FGF receptor genes in GCps has little effect on development (Rob Wechsler-Reya, personal communication), the most likely explanation for the GCp defects in these conditional mutants is that they are secondary to early abnormalities in glial differentiation. It will be informative to delete FGF receptor and sprouty genes specifically in BG without affecting other cell types; unfortunately the availability of an appropriate Cre driver for simultaneous deletion of multiple conditional alleles remains a practical limitation.

Our study indicates that the maintenance of normal SHH signalling levels during cerebellar development requires sprouty gene function. We found that excess FGF signalling affected SHH signalling at more than one level: first, the expression of Shh is reduced in PCs and second, the level of SHH signalling is reduced in SHH-responsive cell types, including GCps. The latter observation is consistent with previous in vitro findings (Wechsler-Reya and Scott, 1999) but the effects on Shh expression has not previously been reported to our knowledge. The exact molecular mechanisms that underlie these effects remain to be determined and might have implications for other contexts in which SHH and FGF signalling are closely apposed (Kataoka and Shimogori, 2008).

In conclusion, we have found that several cell types in the developing cerebellum are sensitive to deregulated FGF signalling. Future experiments will be required to identify the individual roles of different FGF ligands and the exact interplay between different cerebellar cell types during development.

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Competing interests statement
The authors declare no competing financial interests.

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