Bmp2 antagonizes sonic hedgehog-mediated proliferation of cerebellar granule neurones through Smad5 signalling

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Summary

During development of the cerebellum, sonic hedgehog (Shh) is directly responsible for the proliferation of granule cell precursors in the external germinal layer. We have looked for signals able to regulate a switch from the Shh-mediated proliferative response to one that directs differentiation of granule neurones. Bone morphogenetic proteins (BMPs) are expressed in distinct neuronal populations within the developing cerebellar cortex. Bmp2 and Bmp4 are expressed in the proliferating precursors and subsequently in differentiated granule neurones of the internal granular layer, whereas Bmp7 is expressed by Purkinje neurones. In primary cultures, Bmp2 and Bmp4, but not Bmp7, are able to prevent Shh-induced proliferation, thereby allowing granule neuron differentiation. Furthermore, Bmp2 treatment downregulates components of the Shh pathway in proliferating granule cell precursors. Smad proteins, the only known BMP receptor substrates capable of transducing the signal, are also differentially expressed in the developing cerebellum: Smad1 in the external germinal layer and Smad5 in newly differentiated granule neurones. Among them, only Smad5 is phosphorylated in vivo and in primary cultures treated with Bmp2, and overexpression of Smad5 is sufficient to induce granule cell differentiation in the presence of Shh. We propose a model in which Bmp2-mediated Smad5 signalling suppresses the proliferative response to Shh by downregulation of the pathway, and allows granule cell precursor to enter their differentiation programme.

Key words: Cerebellar development, Granule neuron, Sonic hedgehog, Bone morphogenetic proteins, Smad proteins, Mouse, Chick

Introduction

The cerebellum, comprising the deep cerebellar nuclei, the white matter and the cerebellar cortex, is well described as playing several important roles in the control of motor coordination. The mature cerebellar cortex arises late in development and is organized in three layers: an outer molecular layer (ML) containing granule cell axons and Purkinje cell dendrites; a layer of Purkinje cell bodies (PcL); and an inner layer containing granule neurones, the internal granular layer (IGL). Neurogenesis of the Purkinje cells occurs within the ventricular zone during the early phases of cerebellar development, Purkinje cell precursors migrate to form the first cell layer and thereby set up the framework for the cerebellar cortex. Concomitantly, granule cell precursors appear in an area of the neuroepithelium, which is known as the rhombic lip, just dorsal to the zone where Purkinje cells are generated. Precursor cells within the rhombic lip segregate from the adjacent neuroepithelium, and migrate up onto the surface of the cerebellar anlage. The layer of proliferating cells, which spreads across the roof of the anlage is called the external germinal layer (EGL) (reviewed by Hatten et al., 1997). The EGL exists transiently on the surface of the cerebellar anlage and contains many mitotically active cells (granule cell precursors) (Altman and Bayer, 1997; Ramón y Cajal, 1911). After clonal expansion in the superficial EGL, granule cells migrate through the field of differentiating Purkinje neurones to settle in the IGL.

Recent studies have provided insights into the molecular nature of the signals directing the subsequent steps of cerebellar cortex development. Dorsal midline-derived bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGFβ) superfamily, appear to act on a regionalized cerebellar anlage to induce the generation of granule neuron progenitors that migrate from the rhombic lip to populate the EGL (Alder et al., 1999). Furthermore, sonic hedgehog (Shh) within the EGL and secreted from adjacent Purkinje neurones acts as a potent mitogenic signal to expand the granule cell progenitor population (Dahmane and Ruiz i Altaba, 1999; Kenney and Rowitch, 1999; Pons et al., 2001; Wallace, 1999; Wechsler-Reya and Scott, 1999). Subsequent steps of granule cell differentiation require exit from the cell cycle, initiation of differentiation and migration through the Purkinje cell layer, events that occur within a Shh-rich environment. Therefore, termination of granule cell proliferation is probably not due to reduced exposure to Shh, rather it is likely to result from signals that can suppress the proliferative response to Shh. Extracellular matrix glycoproteins (Pons et al., 2001) and fibroblast growth factors

(FGFs) (Wechsler-Reya and Scott, 1999; Pons et al., 2001) are able to differentially modulate but not to totally suppress Shh-mediated proliferation of granule cell precursors, making them unlikely candidates to account completely for the suppression of the proliferative response. However, hedgehog proteins and BMPs are co-expressed at many sites of cell-cell interaction during development (Bitgood and McMahon, 1995) and are known to have opposing activities in many developmental paradigms (Lee and Jessell, 1999; Mekki-Dauriac et al., 2002; Patten and Placzek, 2002; Zhu et al., 1999). We have therefore undertaken an analysis of BMP protein expression and function in the developing cerebellum, as putative antagonists of Shh-mediated proliferation.

Shh is a secreted protein that signals through its receptor patched (Ptch1), an eleven-pass transmembrane receptor. In the absence of Shh, Ptch1 associates with and sequesters the activity of smoothened (Smo) (for reviews, see Ingham and McMahon, 2001; Ho and Scott, 2002; Nybakken and Perrimon, 2002; Martí and Bovolenta, 2002). In response to the binding of Shh, Ptch1 releases Smo inhibition, which then activates a Gαi subunit to inhibit cAMP production within the cell. The Gli family of zinc-finger transcription factors act at the last known step in the Shh signal-transduction pathway (reviewed by Ruiz i Altaba et al., 2002a; Ruiz i Altaba et al., 2002b). Within the Shh-receiving cell, Gli proteins are regulated in the cytoplasm via multiple distinct molecular mechanisms. The cyclic AMP-dependent protein kinase (PKA) acts as a common negative regulator such that Gli repressor forms are generated by PKA-mediated phosphorylation and that inhibition of PKA activity releases Gli activated forms. Gli proteins then move to the nucleus where, by acting together with co-activators (Goodman and Smolik, 2000) or with co-repressors (Dai et al., 2002), they regulate transcription of target genes.

BMPs are also secreted proteins that use a relatively simple mechanism to signal to the nucleus (for reviews, see Massagué, 2000; Massagué et al., 2000; Shi and Massagué, 2003). BMP ligands bring together members from two families of receptor serine/threonine kinases, known as the type I and type II receptors. Type II receptors activate type I receptors that then propagate the signal by phosphorylating Smads, which are the only known BMP receptor substrates capable of signal transduction. Phosphorylation causes Smads to move to the nucleus where they assemble complexes that directly control gene expression. Each different ligand may have a choice of several type I and type II receptors, and a given cell may express different receptor forms; however, in the case of BMPs, the various type I receptors funnel their activities through one of three different Smads (Smad1 or the closely related Smad5 and Smad8). Phosphorylation of Smad1/5/8 increases their affinity for a particular member of the family, Smad4, that functions as a shared partner (co-Smad), and is required for the assembly of active transcriptional complexes. Activated Smad1/5/8 moves to the nucleus where, acting together with co-activators (Goodman and Smolik, 2000) or with co-repressors (Wang et al., 2000), it activates/represses target genes transcription.

We describe the expression of several BMPs in the developing cerebellar cortex. In search of a putative functional antagonism of Shh activity by BMPs, we show that Bmp2 and Bmp4, but not Bmp7, are able to totally overcome Shh-induced proliferation of granule cell precursors. Furthermore, we show that Bmp2-mediated differentiation of cerebellar granule neurones is mediated by Smad5 signalling and that Smad5 expression is sufficient to trigger granule cell precursor differentiation, thus providing a strong basis for understanding the molecular control of granule neuron proliferation/differentiation.

Materials and methods

Chick and mouse embryos

Eggs from White-Leghorn chickens were incubated at 38.5°C in an atmosphere of 70% humidity. Embryos were staged as described previously (Hamburger and Hamilton, 1951). BALB/c mice from an inbred colony were kept under standard housing, feeding and lighting conditions (22°C, 12 hours light/12 hour dark). The day of birth was taken as day 0 of postnatal life (P0). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication Number 85-23).

Antibodies and chemicals

The monoclonal antibody anti-bromodeoxyuridine (BrdU) was obtained from the Developmental Studies Hybridoma Bank (Iowa). The monoclonal antibody against a unique βIII-Tubulin (Tuj-1) was obtained from MEDPASS (Grand Duché de Luxembourg) and used to identify postmitotic neurons. The rabbit polyclonal anti-GFAP (AB1980) was purchased from CHEMICON. The rabbit polyclonal anti-calbindin-D28K was purchased from Swant. The rabbit polyclonal anti-phospho-histone 3 (P-H3) was purchased from Upstate Biochemicals.

Anti-Smad1, anti-Smad5 and anti-Smad8 were purchased from Santa Cruz. A phospho-specific antibody that recognizes the activated forms of Smad1/5/8 was purchased from Cell Signaling Technologies.

The *E. coli*-produced 19 kDa N-terminal fragment of recombinant sonic hedgehog (based on the human sequence) used in this study was a gift from Biogen (Cambridge, MA).

Bmp2 was obtained from Genetic Institute (Cambridge, MA), Bmp4 was purchased from R&D Systems (Minneapolis, MN) and Bmp7 from Creative BioMolecules (Boston, MA).

In situ hybridisation, immunohistochemistry and RT-PCR

In situ hybridisation was performed on 50 μm vibratome sections following standard procedures. Hybridisation was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim).

The following probes have been previously described: chick *sonic hedgehog* (Riddle et al., 1993); mouse *Shh* (Echelard el al., 1993); chick *bmp2* and *bmp4* (Francis-West et al., 1994); chick *bmp7* (Houston et al., 1994); mouse *Bmp2, Bmp4* and *Bmp7* (Piedra and Ros, 2002). Chick *Smad1* and *Smad5* probes were obtained from Dr Juan Hurlé (University of Cantabria, Spain), and *Smad8* probe was obtained from the chicken EST project (UK-HGMP RC). Mouse *Smad1* and *Smad5* probes were obtained from Dr E. Robertson (Harvard University, MA).

Immunohistochemistry was performed on free-floating vibratome sections (50 μm) based on standard procedures. After single or double staining, sections were mounted, analysed and photographed using a Leica Confocal microscope.

RNA extractions were done following the user manual of the NucleoSpin RNA purification kit from BD Biosciences. RT-PCRs were performed following the user manual of the Titanium One-Step RT-PCR kit from as BD Biosciences. Primer sequences are available on request.

Primary culture of granule cells

Cerebellar cultures were performed using a modification of the procedure described by Meyer-Franke et al. (Meyer-Franke et al., 1995). Chemicals and incubation times were optimised for the

simultaneously processing of four P6 mouse cerebellae. Cerebellae were aseptically removed, washed once in Earl's Balanced Salt Solution (EBSS) (Invitrogen), cut into small pieces (1 mm) and transferred to a 50 ml screw cap tube. Tissue fragments were allowed to settle, the excess EBSS aspirated and 4 ml of EBSS containing 100 U/ml of DNAse (Worthington, Lake Wood, NJ), 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was added and gently mixed with tissue fragments. Finally, 100 U of papain (Worthington) that was pre-activated for 30 minutes at 37°C in 1 ml of activation buffer (EBSS containing 5 mM L-Cys, 2 mM EDTA and 0.067 mM β-mercaptoethanol) was added, air was displaced with 95%O_2/5%CO_2 and the sample was incubated for 90 minutes on a shaking platform at 37°C. At the end of this period, the tube was vortexed at low speed for 1 minute and undigested fragments were allowed to settle, the supernatant was then transferred to a fresh 15 ml screw cup tube and centrifuged at 600 ***g*** for 5 minutes. The supernatant was aspirated and the pellet resuspended in 3 ml of EBSS containing 3 mg of ovomucoid protease inhibitor (Worthington), layered onto an albumin cushion consisting of 5 ml of EBSS containing ovomucoid protease inhibitor and ovoalbumin (Worthington) each at 10 mg/ml and centrifuged at 600 ***g*** for 5 minutes. The resulting pellet was resuspended in neurobasal medium (Invitrogen), and the cell number and viability was assessed using a haemocytometer. The typical yield from this protocol is 12-15 million cells per cerebellum and cell viability is higher than 90%. Cells were plated onto laminin (GIBCO, BRL)-coated tissue culture dishes or glass coverslips at 100,000 cells/cm^2 in neurobasal medium supplemented with B27 (Invitrogen) containing 20 mM KCl and were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. This culture medium has been optimized to support neuronal survival and minimize glial proliferation; more than 95% of the cells displaying neuronal markers after 48 hours in culture. Twelve hours before harvesting cultures were pulsed-labelled with [^{3}H]thymidine (1 μCi/ml) for incorporation of radioactivity. Cultures plated onto glass coverslips were fixed in 4% paraformaldehyde and processed for immunostaining. TUNEL staining was performed using the In Situ Cell Death detection Kit (Roche).

Organotypic slice culture experiments

Cerebellar slice cultures were prepared either from mouse P4-P6 or from chick stage 38-40 [embryonic day (E) 12-13] cerebellae. Brains were aseptically removed and the cerebellae were excised. Tissue pieces were cut into sagittal slices (50 μm) using a tissue chopper (McIlwain Tissue Chopper, Vibrotome 800) and maintained in ice cold Earl's Balanced Salt Solution (EBSS) (Invitrogen). Cerebellar slices were placed onto 1 μm polycarbonate filters (Costar) and the filters were supported by stainless steel grids on the surface of the culture medium (Dulbecco's Modified Eagles Medium/F12 supplemented with 2 mM glutamine, penicillin/streptomycin and B27 (Invitrogen). The medium was changed every 24 hours.

Heparin acrylic beads (Sigma, 80 μm) were used for exogenous application of proteins. Beads were rinsed with PBS and incubated with 5 μl of protein solution at room temperature for 1 hour before use. Beads were soaked either in PBS (control beads) or in recombinant purified human Shh (1 μg/ml), recombinant human Bmp2 (0.1 μg/ml), Bmp4 (0.1 μg/ml) or Bmp7 (0.1 μg/ml). Four hours prior to fixation, cultures were treated with 50 ng/ml BrdU, fixed in 4% paraformaldehyde and processed for immunohistochemistry.

Western blotting

Primary cultures were lysed in 1×SDS loading buffer [10% glycerol, 2% SDS, 100 mM DTT and 60 mM Tris-HCl (pH 6.8)] and the DNA disrupted by sonication. Samples were then separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes; blocked with 8% non-fat dry milk in TTBS (150 mM NaCl; 0.05 Tween-20 and 20 mM Tris-HCl pH 7.4) and probed with the different antibodies used. The blots were developed using anti-rabbit coupled peroxidase plus the ECL system (Amersham). Quantifications were performed using a Molecular Dynamics Densitometer.

Transfection of granule cell precursors

After 24 hours in culture, granular cell preparations were transfected using FuGENE6 reagent (Roche). Briefly, P6 granular cells platted onto PLL/LN coated cover-slips were grown in neurobasal-B27 containing 3 μg/ml of Shh for 24 hours. For the transfection, 50 μl of neurobasal media containing 2 μl of FuGENE plus 0.5 μg of DNA vectors were added to each well of a 12-well culture dish. Full coding regions of Smad1, Smad5 and Smad8 were subcloned in the bi-cistronic vector pCIG (Megason and McMahon, 2001) that contains nuclear EGFP. The cultures were allowed to grow for 48 hours in the same media, fixed in 4% paraformaldehyde and processed for immunocytochemistry.

Results

BMPs are expressed in the developing cerebellar cortex

We first analysed the expression of *Bmp2*, *Bmp4* and *Bmp7* in the developing cerebellum as a prerequisite for a functional interaction with Shh. *Bmp2* and *Bmp4* are expressed in the EGL, at HH38 in the chick and P0 in the mouse, with Bmp2 being expressed more weakly (Fig. 1A,E). As development proceeds, *Bmp2* expression in the EGL is reduced concomitantly with an increased expression in the IGL (Fig. 1B,C). Double labelling with the Purkinje cell marker calbindin revealed that *Bmp2* expression in the IGL is strong in cells just beneath the Purkinje cell layer (Fig. 1D). *Bmp4*, however, is expressed in the EGL at all stages analysed and only weak expression is seen in the IGL (Fig. 1E-G). *Bmp7* in both chick (Fig. 1H-J) and mouse (not shown) cerebellae is expressed in migrating and settled Purkinje neurones in a similar pattern to that previously shown for Shh (Fig. 1K-M). These studies demonstrate that BMPs are expressed in all three layers of the developing cerebellar cortex: in the superficial EGL where proliferation of granule cell precursors occurs, in the Pukinje cell layer and the IGL where granule neuron differentiation is terminated, thus raising the possibility of an interaction between Shh and BMPs during cerebellar development.

BMPs differentially regulate Shh-induced proliferation of granule cell precursors in primary cultures

To assess the putative activity of BMPs in the developing cerebellum, granule cell precursors were cultured on laminin for 48 hours in the presence or the absence of purified Shh (3 μg/ml), Bmp2 (100 ng/ml), Bmp4 (100 ng/ml) or Bmp7 (100 ng/ml). Proliferative activity was analysed by pulsing cultures with either [^{3}H]thymidine or BrdU (50 ng/ml) for a further 12 hours before analysis of incorporated radioactivity, or immunocytochemistry. Under these culture conditions, purified Shh induces a potent and long-lasting proliferative response in granule cell precursors as reported previously (Pons et al., 2001). However, Bmp2, Bmp4 and Bmp7 do not exert any effect on [^{3}H]thymidine incorporation above control levels (Fig. 2A). To assess the phenotype of BMP-treated cultures, at the end of the culture period cells were fixed and immunostained for the pan-neuronal marker βIII-Tubulin and for the glial marker glial fibrillary acidic protein (Gfap). Our culture conditions have been optimized to support neuronal

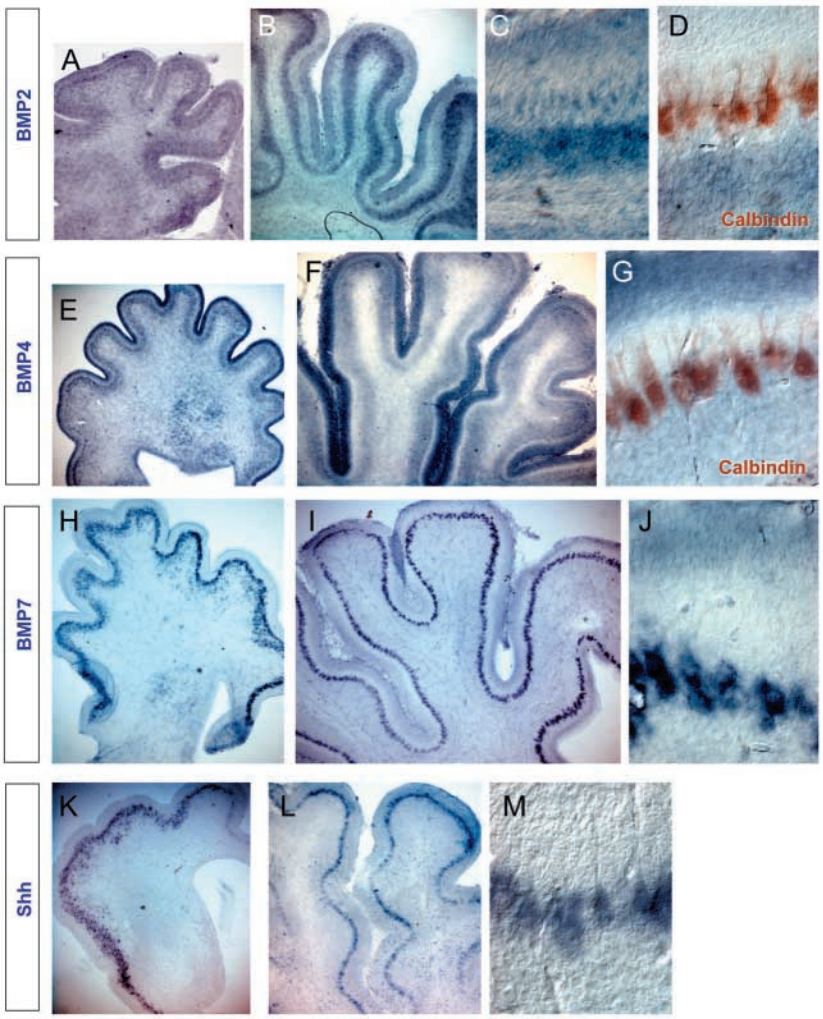

Fig. 1. Expression analysis of BMPs in the developing cerebellum. In situ hybridisation was performed on vibratome sections of mouse and chick cerebellae with probes against *Bmp2*, *Bmp4*, *Bmp7* and sonic hedgehog (Shh). (A) Hybridisation of a postnatal day 0 (P0) mouse with a Bmp2 probe shows expression in the EGL but weak expression in the IGL. (B,C) In a chick HH42 cerebellum, Bmp2 expression is weak in the EGL and strong in the IGL. (D) Double labelling with the Purkinje cell marker calbindin revealed that Bmp2 expression is located just beneath the Purkinje cell layer. (E) Hybridisation with a Bmp4 probe at HH38, shows expression in the EGL and weak expression in the IGL. (F,G) A similar pattern of Bmp4 expression is maintained throughout cerebellar development. (H) Hybridisation with a Bmp7 probe at HH38 reveals expression in migrating Purkinje cells. (I,J) Bmp7 expression is maintained in settled Purkinje neurones at HH42. (K) Hybridisation with a Shh probe at HH38 shows expression in migrating Purkinje cells. (L,M) Shh expression is maintained in settled Purkinje neurones at HH42.

survival and minimize glial proliferation (Pons et al., 2001); therefore, after 48 hours in control cultures, more than 95% of the cells exhibit a neural phenotype and express of βIII-tubulin whereas fewer than 5% of total cells show an astroglial phenotype expressing Gfap (Fig. 2B). In cultures grown in the presence of Shh, the percentage of undifferentiated cells is significantly higher, as indicated by groups of nuclei that are not labelled with either neural or glial markers (Fig. 2C). These cells are actively proliferating as revealed by immunostaining with anti-BrdU (data not shown) (Pons et al., 2001). However, cultures grown in the presence of Bmp2 (Fig. 2D), Bmp4 (not shown) or Bmp7 (Fig. 2E) showed no differences in either the percentages or the phenotypes of neural and glial cells, with respect to the control cultures.

To test whether BMPs exert any effect on the Shh-induced proliferation of granule precursors, cells were grown in the presence of Shh together with Bmp2 (100 ng/ml), Bmp4 (100 ng/ml) or Bmp7 (100 ng/ml). Even in the presence of a highly proliferative concentration of Shh (3 μg/ml) and in cultures plated on laminin (Pons et al., 2001), Bmp2 and Bmp4 totally abolished Shh-induced [^{3}H]thymidine incorporation which returned to control levels. Furthermore, Bmp2/4 suppression of Shh-

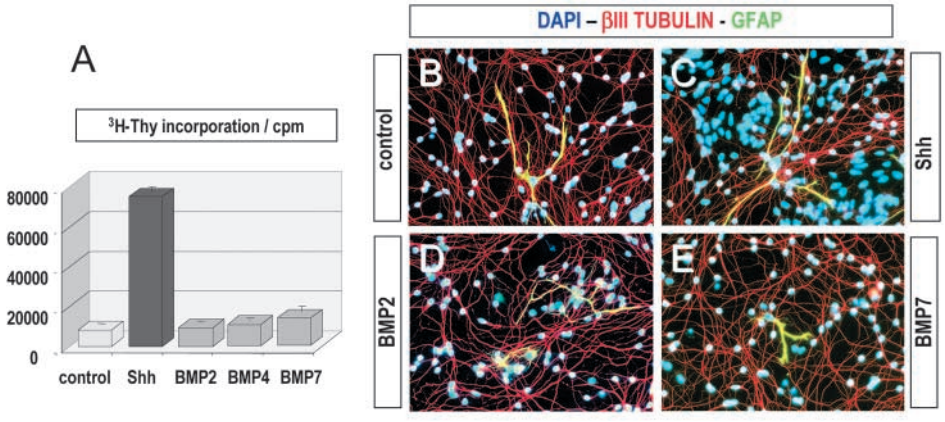

Fig. 2. Effects of treatment with Shh and BMPs on the proliferation of granule cell precursors. Cerebellar granule cells were cultured in medium without additional growth factors (control), or with Shh (3 μg/ml), Bmp2 (100 ng/ml), Bmp4 (100 ng/ml) or Bmp7 (100 ng/ml). (A) After 60 hours in culture, cells were pulsed-labelled with [^{3}H]thymidine for 12 hours, harvested and analysed for incorporation of radioactivity. BMPs had no effect on proliferation, whereas Shh induces a high rate of [^{3}H]thymidine incorporation. (B-E) After 72 hour in culture, cells were immunostained for the neural-specific marker βIII Tubulin (red) and the astroglial marker Gfap (green) in control cultures (B) and cultures treated with Shh (C), with Bmp2 (D) or with Bmp7 (E). Cultures treated with BMPs were not phenotypically different from control cultures. Shh-treated cultures showed the presence of big clumps of precursor cells that showed no immunoreactivity to either neural or glial marker. All cultures are counterstained with the nuclear marker DAPI (blue).

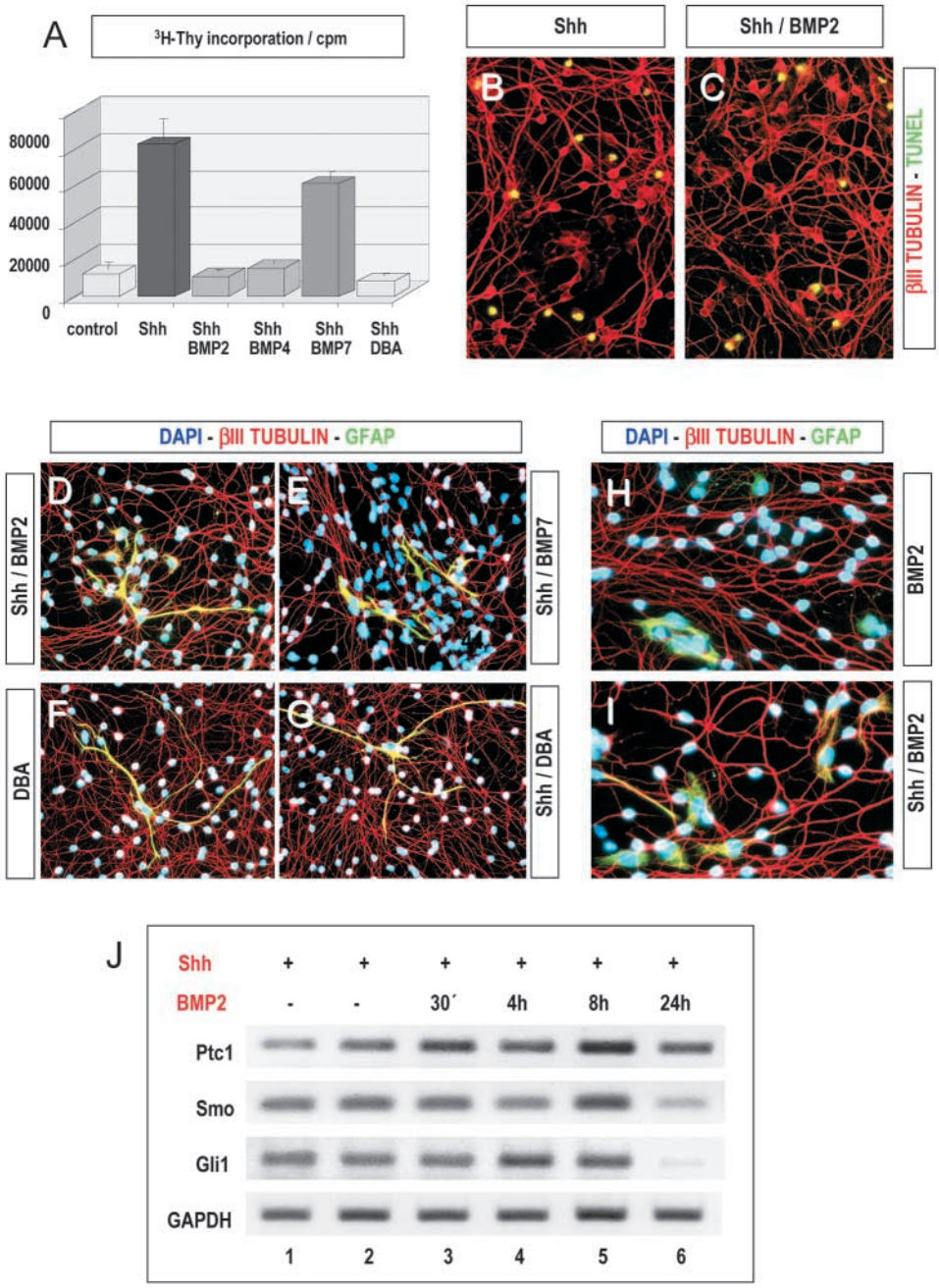

Fig. 3. Modulation of Shh induced proliferation by BMPs. Cerebellar granule cells were cultured in the absence or presence of purified Shh alone (3 μg/ml), or the same concentration of Shh together with Bmp2 (100 ng/ml), Bmp4 (100 ng/ml), Bmp7 (100 ng/ml), or with the PKA activator dibutyril cAMP (DBA). (A) After 60 hours in culture, cells were pulsed labelled with [^{3}H]thymidine for a further 12 hours, harvested and analysed for incorporation of radioactivity. Bmp2 and Bmp4 cause a strong reduction in [^{3}H]thymidine incorporation, equal to the reduction caused by DBA. Bmp7 had no effect on the Shh-induced proliferation. (B,C) After 72 hour in culture, apoptosis was assessed in cultures using the TUNEL reaction (green) and immunostained for the neural specific marker βIII Tubulin (red). There was no significant difference between the numbers of apoptotic cells in cultures grown with Shh alone (B) or with Shh and Bmp2 (C). (D-G) Cells were immunostained for the neural-specific marker βIII Tubulin (red) and the astroglial marker Gfap (green), and counterstained with the nuclear marker DAPI (blue). (D) Cultures treated with Shh and Bmp2 show a phenotype similar to that of control cultures (not shown). (E) Cultures grown in the presence of Shh and Bmp7 show a similar phenotype to those grown with Shh alone (not shown). (F,G) Cultures grown in the presence of DBA, either alone (F) or together with Shh (G) show long astrocytic processes. (H,I) Cultures grown in the presence of Bmp2, either alone (H) or together with Shh (I) show early differentiated astrocytes that express GFAP, even though they have a precursor cell morphology. (J) RT-PCR analysis of RNA isolated from cultures treated with Shh for 48 hours (lane 1), with Shh for 72 hours (lane 2), with Shh for 48 hours and with Bmp2 for the indicated periods of time (lanes 3-6).

mediated proliferation is as efficient as treatment with dibutiryl-cyclic-AMP (DBA) that causes a direct inhibition of the Shh pathway through the intracellular increase of cAMP levels and PKA activation (Fig. 3A). However, Bmp7, which is co-expressed with Shh in Purkinje neurones, has no significant effect on the Shh-mediated proliferation of granule cells (Fig. 3A).

As the lack of [^{3}H]thymidine incorporation could simply reflect an increase in cell death, we compared the rate of apoptosis in the different culture conditions and found no significant changes in the number of TUNEL positive cells in cultures treated either with or without Shh alone or with a combination of Shh and Bmp2 (Fig. 3B,C). This is in contrast to the effect of Bmp2 in medulloblastoma cells in which it is reported to mediate retinoid-induced apoptosis (Hallahan et al., 2003).

Furthermore, cultures treated with Shh together with Bmp2 or Bmp4 showed the same phenotype as control cultures in which more than 95% of the cells are βIII-Tubulin positive and less than 5% cells are GFAP positive (Fig. 3D). However, cells grown in the presence of Shh and Bmp7, in which proliferation is still high, show the presence of groups of undifferentiated cells that are not labelled with either neural or glial markers (Fig. 3E), similar to the phenotype of cultures treated with Shh alone (Fig. 2C). A moderate phenotypic change occurs in glial cells grown in the presence of DBA, either alone or together with Shh, in which astrocyte processes are longer and thinner than controls (data not quantified, Fig. 3F,G). The role of Bmp2

and Bmp4 in astrocytic differentiation has been well characterised both in vitro (Angley et al., 2003; Nakashima et al., 2001; Zhu et al., 1999) and in vivo (Gomes et al., 2003) and, consistent with this role, in our culture conditions we observe the presence of progenitor cells expressing GFAP in cultures grown in the presence of Bmp2 or Bmp4 alone (Fig. 3H), which are more abundant in cultures treated with Shh and Bmp2/4 (Fig. 3I).

We next tested the effect of Bmp2 treatment on components of the Shh pathway. Cultures were grown for 48 hours in the presence of Shh, Bmp2 (200 ng/ml) was then added for different time points (Fig. 3J). RT-PCR analysis of RNA isolated from cultures treated solely with Shh show high expression of key components of the pathway Ptch1, Smo and Gli1, either after 48 hours (lane 1) or after 72 hours (lane 2) in culture. Smo and Gli1 expression are downregulated after 24 hours of Bmp2 treatment (lane 6), whereas Ptch1 expression is not regulated by Bmp2. This is in concordance to the in vivo situation where Smo and Gli1 expression are downregulated along cerebellum development, whereas Ptch1 and Shh expression are maintained to adulthood (Traiffort et al., 1999). Experiment shown reflects observations made in three separate experiments.

Bmp2 and Bmp4 inhibit proliferation of granule cell precursors in the EGL

Our data supports the suggestion that Bmp2 and Bmp4 exert a potent antagonistic activity on the Shh-induced proliferation of granule cell precursors in vitro. However, cell proliferation/differentiation of cerebellar granule neurones is also finely regulated by extracellular matrix glycoproteins (Graus-Porta et al., 2001; Pons et al., 2001) and by proteoglycans (Rubin et al., 2002). Thus, in order to test the in vivo relevance of BMP activity seen in primary cultures, we adapted an organotypic slice culture of cerebellum that maintains cell-cell and cell-matrix interactions occurring during normal granule neuron development. Beads soaked either in PBS (control beads), Shh (1 μg/ml), Bmp2 (0.1 μg/ml), Bmp4 (0.1 μg/ml) or Bmp7 (0.1 μg/ml), were implanted close to the EGL (Fig. 4A), and organotypic slices were maintained for 3 days in culture. Four hours prior to fixation cultures were pulsed-labelled with BrdU (50 ng/ml) to label proliferating cells, fixed and co-immunostained with anti-BrdU antibody and with an anti-calbindin antibody to label Purkinje neurones. Only those beads that at the end of the culture period were properly placed either above or within the Purkinje cell layer (Fig. 4B) were further analysed. At least eight to ten beads loaded with each purified protein were analysed in three independent experiments. The total numbers of BrdU-immunostained nuclei, in a fixed area surrounding the beads, were counted on a Leica confocal microscope and quantitative data were expressed as mean±s.e.m.

Under these culture conditions, Shh beads induce a significant increase in BrdU-positive cells (Shh; 236±37, $P<0.001$ Turkey's test) above control beads (PBS; 212±31), whereas Bmp2 and Bmp4 beads induce a significant reduction in BrdU-positive cells (Bmp2; 131±30, $P<0.001$ and Bmp4; 150±24, $P<0.01$) below control. Bmp7 beads induced a slight but non-significant increase in the number of BrdU-positive cells (Bmp7; 220±27, $P<0.05$) above control beads, a proliferative effect that we do not observe in primary cultures treated with Bmp7 alone (Fig. 2A). This might reflect the combined activity of an exogenous Bmp7 protein source with the endogenous Shh protein secreted from the Purkinje neurones, similar to the cooperation of Bmp7 and Shh observed

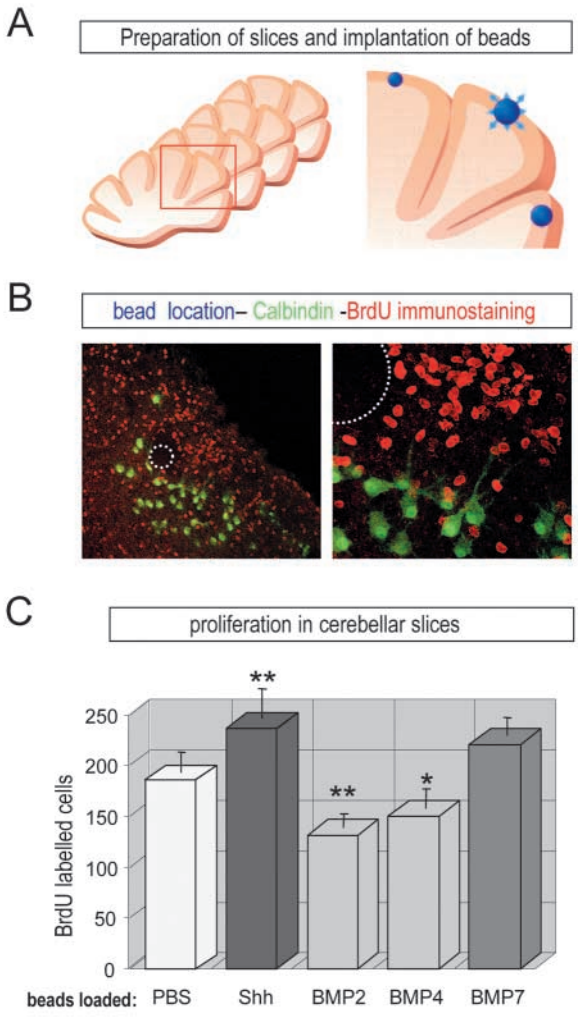

Fig. 4. Modulation of granule cell precursor proliferation in the EGL by Shh and BMPs. Organotypic slice cultures of cerebellum were maintained for 72 hours and analysed for granule cell precursor proliferation by BrdU incorporation. (A) Cerebellar slices were obtained (300 μm), cultured on polycarbonate filters. Beads soaked in either PBS, Shh, Bmp2, Bmp4 or Bmp7 were implanted close to the EGL. (B) Confocal images of cultured slices that were in toto immunostained with anti-calbindin antibody (green cells) and anti-BrdU (red nuclei). White dots indicate the location of the bead. Analysis of each culture was performed by counting total numbers of BrdU-positive cells in a fixed area surrounding the beads. (C) Quantification of proliferation in the EGL of organotypic slice cultures with different beads implanted. Values are expressed as mean±s.e.m. A significant increase in the total number of BrdU-positive cells is observed in slices with Shh beads (**$P<0.001$ Turkey's Test) when compared with PBS-soaked beads. A significant decrease on the total number of BrdU-positive cells is observed in slices treated with Bmp2 (**$P<0.001$) and Bmp4 (*$P<0.01$) when compared with PBS beads. Although Bmp4 seams less active in these experiments, differences between Bmp2 and Bmp4 activities have no statistical significance (Bmp2 versus Bmp4 $P<0.05$). Bmp7-loaded beads induced a slight but not statistically significant increase on the total number of BrdU-positive cells ($P<0.05$) when compared with PBS beads.

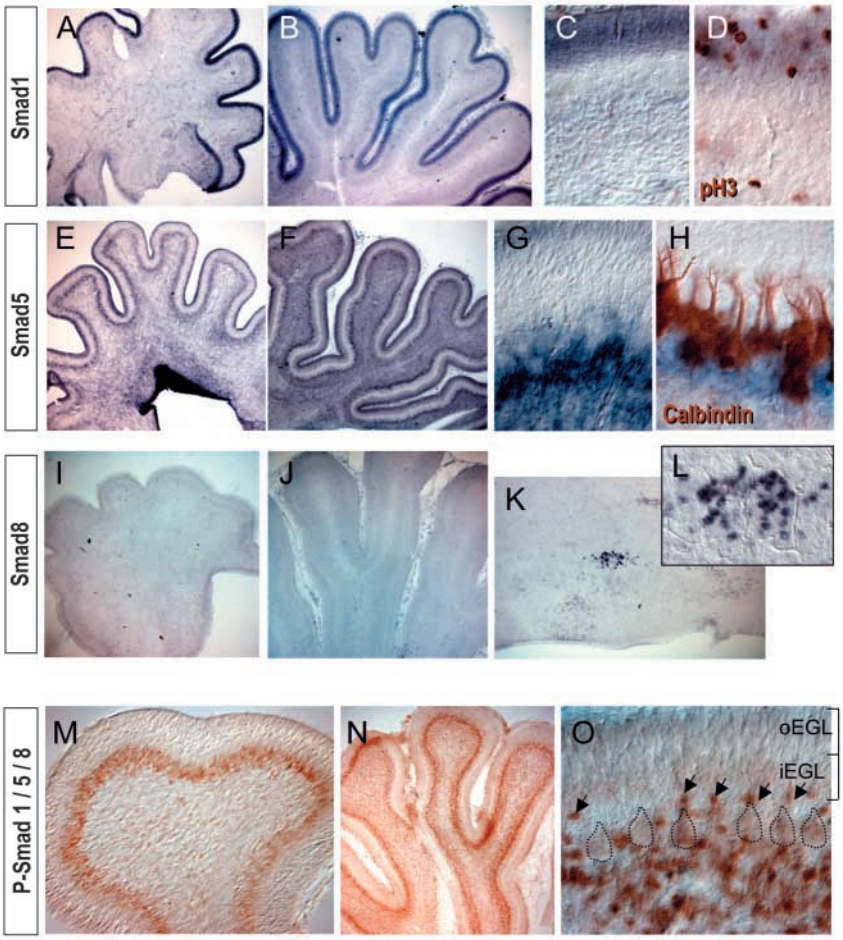

Fig. 5. Expression analysis of Smads in the developing cerebellum. In situ hybridisation was performed on vibratome sections of chick cerebellae with probes to Smad1, Smad5 and Smad8. (A) Hybridisation with a Smad1 probe at HH38 reveals strong expression in the EGL. (B,C) At HH42 Smad1 expression remains restricted to the EGL. (D) Double labelling with Smad1 and the mitosis marker Phospho-Histone3 (brown nuclei) reveals Smad1 expression (blue staining) localised in the highly proliferative external EGL. (E) Hybridisation with a Smad5 probe at HH38 shows expression in the EGL and the IGL. (F,G) At HH42, Smad5 expression is weak in the EGL but strong in the IGL, particularly in cells just below the Purkinje cell layer. (H) Double labelling with Smad5 and the Purkinje cell marker calbindin (brown neurones) reveals Smad5 labelling (blue staining) in granule neurons just below the Pc layer. (I,J) Hybridisation with a Smad8 probe at HH38 (I) and HH42 (J) shows no expression in any layer of the developing cerebellum. (K,L) Expression of Smad8 in cells of a motor nucleus at the ventral medulla. (M-O) Vibratome sections of mouse P4 (M) and chick HH42 (N,O) cerebellae immunostained with the phospho-specific Smad 1/5/8 antibody show the presence of phosphorylated forms of Smad proteins mainly at the IGL, together with few nuclei leaving the iEGL (arrows), which should correspond with Smad5 mRNA expression. The location of Purkinje cell bodies is indicated by broken lines.

during the induction of forebrain ventral midline cells (Dale et al., 1997). These results support the suggestion that the in vivo antagonists of Shh-induced proliferation of granule cell precursors are Bmp2 and/or Bmp4, and raise the question of how these different BMP signals are read and interpreted within the developing cerebellum.

BMP-dependent granule neuron differentiation is mediated by Smad5

BMP proteins signal through membrane localised serine/threonine kinase receptors, which propagate the signal by phosphorylating Smads. Smad1 or the closely related Smad5 and Smad8 proteins are the only known BMP receptor substrates capable of signal transduction. In order to define which Smad protein might be transducing the BMP-mediated antagonism of Shh in granule neuron development, vibratome sections of developing cerebella were hybridized with probes to *Smad1*, *Smad5* and *Smad8*. *Smad1* expression is restricted to the external germinal layer (EGL), from the earliest stage analysed (chick HH38, Fig. 5A) until late in cerebellar development (Fig. 5B-D). *Smad5* however, is expressed in both the EGL and the IGL from HH38 (Fig. 5E). Interestingly, *smad5* expression in the IGL is particularly high in a layer just below the Purkinje cells (Fig. 5G,H), where early differentiated granule neurones reside, an area corresponding to that of *bmp2* expression (Fig. 1D). *Smad8* is not expressed in the developing cerebellar cortex at any stage analysed (Fig. 5I,J). Strong *smad8* expression observed in a motor nucleus (Fig. 5K,L) indicates that the probe and hybridisation procedure are effective and therefore confirms the absence of expression in the cerebellar cortex. As phosphorylation of Smad proteins is a pre-requisite for signal transduction, we immunostained vibratome sections of mouse and chick cerebellae with an antibody against phosphorylated-Smad1/5/8 (P-Smad1/5/8). Immunostained sections revealed the presence of phosphorylated forms of Smad1/5/8 in nuclei that are starting to migrate away from the EGL, in a pattern corresponding to that of *bmp2* and *smad5* mRNAs expression (Fig. 5M-O).

We next evaluated the phosphorylation status of Smad proteins in primary cultures of cerebellar granule precursors grown for 24 hours in the presence of Shh (3 μg/ml) and pulsed with Bmp2 (200 ng/ml) at different time points. Total cell lysates from granular cell cultures, or immunoprecipitates with the anti-Smad5, were separated by SDS-PAGE and the resulting nitrocellulose membranes were blotted with the antibody against phosphorylated-Smad1/5/8 (P-Smad1/5/8). Treatment with Bmp2 increases the level of P-Smad1/5/8 within 5 minutes, an increase that is greater after 15 minutes and decreases within 1 hour of Bmp2 treatment (Fig. 6A). This transient phosphorylation of cultures might correspond to the high level of anti-Smad1/5/8 immunostaining seen in newly differentiated granule neurones, just beneath or within the Purkinje cell layer.

Therefore, we next tested whether elevated levels of Smad5 were sufficient to induce granule neuron differentiation in

vitro. To address this question, we assessed the effects of overexpressing Smad5 on Shh induced proliferation (Fig. 6). Granular cell cultures plated onto LN and treated with Shh were transfected with DNA vectors (pCIG) containing either EGFP (control) (Fig. 6) or EGFP-Smad5 (Fig. 6). Cells were then grown in the same conditions for 2 more days, pulsed-labelled with BrdU (4 hours prior fixation) and immunostained using anti-BrdU antibodies. The percentage of proliferating cells among those expressing the control marker EGFP was evaluated in each transfection group. Interestingly, overexpression of Smad5 decreased the percentage of proliferating cells more than three-fold when compared with the control cultures transfected solely with EGFP (from 54.8±8.5% to 21.5±5.3%). These results suggest that, in the presence of Shh, Smad5 signalling is sufficient to decide between the proliferation or differentiation of granular cells.

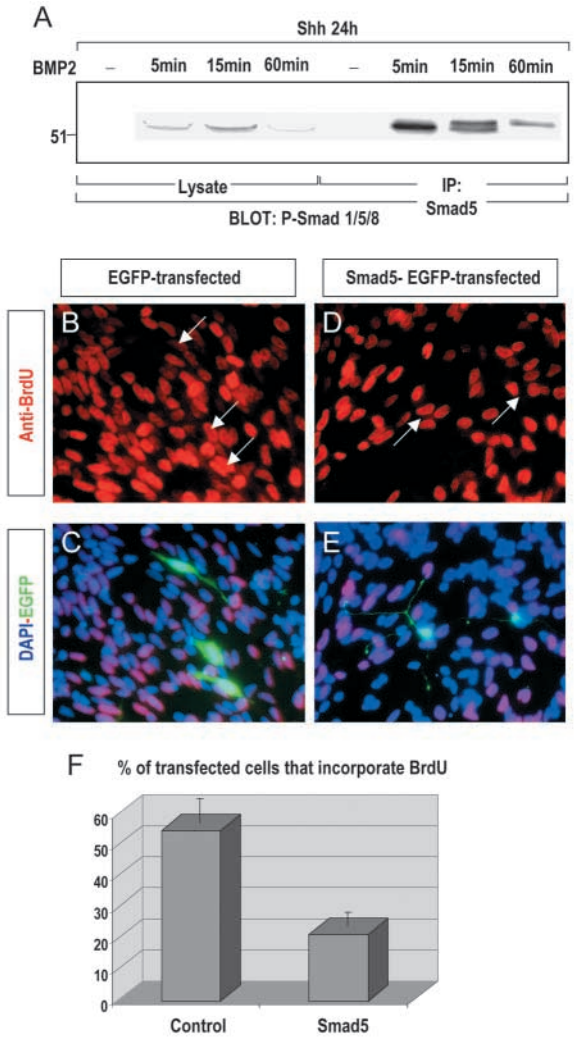

Fig. 6. Smad5 activity in cultures of granule cell precursors. The phosphorylation status of Smad1/5/8 was evaluated by western blotting of cells cultured for 24 hours in the presence of Shh and treated with Bmp2 for different time periods (0, 5, 15 and 60 minutes). Total cell lysates from granular cell cultures were separated by SDS-PAGE and the resulting nitrocellulose membranes blotted with an antibody against phosphorylated-Smad1/5/8 (P-Smad1/5/8) (lysate). Alternatively, cultures were immunoprecipitated with the anti-Smad5 antibody, separated by SDS-PAGE and the resulting nitrocellulose membranes blotted with an antibody against phosphorylated-Smad1/5/8 (P-Smad1/5/8) (IP, Smad5). (B-E) One-day old granular cell cultures plated on laminin and treated with Shh were transfected with DNA vectors containing EGFP (B,C) or EGFP-Smad5 (D,E). Cells were immunostained for proliferation with anti-BrdU (red), with the nuclear marker DAPI (blue) and with GFP (green). White arrows in B point indicate transfected cells (green stained in C) that are BrdU labelled. White arrows in D indicate the position of transfected cells (green stained in F) that are BrdU negative. (F) The percentage of GFP-expressing cells that were differentiated cells (cells that do not incorporate BrdU) was evaluated in each transfection group. Overexpression of Smad5 decreased the percentage of proliferating cells more than threefold when compared with the control group transfected with EGFP (from 54.8±8.5% to 21.5±5.3%).

Discussion

The establishment of the cerebellar primordium depends on early inductive signals involving Fgf and Wnt proteins produced by cells at the mesencephalic/metencephalic boundary (reviewed by Joyner, 1996). Within the cerebellar anlage, a second phase of development commences with the generation and differentiation of specific neural cell types. Precursors of deep cerebellar nuclei and Purkinje cell precursors exit the cell cycle in the ventricular zone and migrate to the cerebellar primordium. Granule neuron precursors, however, undergo a highly unusual developmental programme. Proliferating at the edge of the neuroepithelium (the rhombic lip), this pool of dividing precursor cells streams rostrally up the lip and onto the surface of the anlage, where they establish a displaced germinal zone, the EGL (Ramón y Cajal, 1911). Later in development, a rapid proliferation of the granule cell precursors expands the EGL from a single cell layer to a layer eight cells thick. Precursor proliferation continues within the EGL until late in development, when the zone disappears due to the inward migration of postmitotic cells to form the internal granular layer (IGL). Abnormal developmental processes have been observed in which granule cell precursors become transformed and fail to undergo normal differentiation. This aberrant growth control generates medulloblastoma tumours that are frequently found near the surface of the cerebellum (reviewed by Wechsler-Reya and Scott, 2001). As medulloblastoma is the most common malignant brain tumour in children, it is important to understand the molecular mechanisms controlling the growth and differentiation of granule neurones, both in normal development and in tumourigenesis.

Sonic hedgehog-induced proliferation of cerebellar granule precursors is inhibited by bone morphogenetic proteins

Shh, although initially recognised for its role in embryonic patterning (reviewed by Ingham and McMahon, 2001), has more recently been described as having a role in the proliferation of neural precursors (reviewed by Martí and Bovolenta, 2002; Ruiz i Altaba, 2002a). Shh signalling is required for expansion of neural precursor cells in the EGL of the developing cerebellum and is the most potent known mitogen for cerebellar granule precursors in vitro (Dahmane and Ruiz i Altaba, 1999; Kenney and Rowitch, 2000; Pons et al., 2001; Wallace, 1999; Wechsler-Reya and Scott, 1999). We now know that Nmyc1 is a direct target of the Shh pathway that functions to regulate cell cycle progression in granule cell precursors by increasing G_1 cyclin

expression (Kenney and Rowitch, 2000; Kenney et al., 2003). Furthermore, mutations in several components of the Shh pathway seem to account for most cases of desmoplastic medulloblastomas (reviewed by Rubin and Rowitch, 2002; Wechsler-Reya and Scott, 2001). Thus, the control of Sonic signalling is likely to be a key point in the normal developmental programme of cerebellar granule neurones.

We, and others, have previously reported that extracellular matrix glycoproteins (Pons et al., 2001) and FGFs (Wechsler-Reya and Scott, 1999; Pons et al., 2001) are able to differentially modulate but not to totally suppress Shh-mediated proliferation of granule cell precursors. TGFβ signalling generally has a negative effect on cell growth such that inactivation of this pathway contributes to tumourigenesis (reviewed by Shi and Massagué, 2003). Among the TGFβ superfamily, BMPs have opposing activities to hedgehogs in many developmental paradigms (Lee and Jessell, 1999; Mekki-Dauriac et al., 2002; Patten and Placzek, 2002; Zhu et al., 1999). Thus the expression of *Bmp2* and *Bmp4* in proliferating and early differentiated granule neurones of the cerebellum shown here, indicates the possibility of a functional interaction with Shh in the regulation of granule cell development. In primary cultures of granule cell precursors, Bmp2 and Bmp4 are able to totally overcome Shh-induced proliferation. Furthermore, in a pseudo-in vivo situation, such as organotypic slice cultures, Bmp2 and Bmp4 significantly reduce granule cell precursor proliferation at the EGL, proliferation induced by endogenous Shh. These results strongly suggest that Bmp2 and/or Bmp4 are potent inhibitors of the Shh pathway during normal development of the cerebellum and raise the interesting point of whether these molecules could also control hedgehog pathway activity in medulloblastoma growth. In accordance with this, a recent publication has suggested that Bmp2 may mediate retinoid-induced apoptosis in medulloblastoma cells (Hallahan et al., 2003).

Bmp7, a slightly more divergent member of the BMP family, is expressed in a different cell type population and exhibits an apparently different role. Similar to previous reports for Shh (Dahmane and Ruiz i Altaba, 1999; Traiffort et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999), Bmp7 is expressed in migrating and settled Purkinje neurones. Bmp7 had no significant effect on the Shh-mediated proliferation of granule cell precursors, either in primary cultures or in organotypic slice cultures, thus leaving the role that Bmp7 might be playing in cerebellar development unresolved.

Bmp2 activity in the developing cerebellum is mediated by Smad5

Although the diverse TGFβ ligands elicit quite different cellular responses, they all share a highly conserved signalling pathway. Ligand binding to type I and type II receptor serine/threonine kinases at the cell surface initiates signalling through phosphorylation of the Smad proteins. There are eight distinct Smad proteins among which Smad1, Smad5 and Smad8 are directly phosphorylated and activated by Bmp signalling. Phosphorylated Smad1/5/8 undergoes homotrimerization and formation of heteromeric complexes with Smad4. The activated Smad complexes are then translocated into the nucleus and, in conjunction with other nuclear co-factors, regulate the transcription of target genes (Massagué, 2000; Massagué et al., 2000). We asked which Smad protein might be transducing the Bmp-mediated antagonism of Shh activity in the developing cerebellum. We show that *Smad1* and *Smad5* are both expressed in the developing cerebellar cortex, although in different cell populations. Whereas *Smad1* expression is restricted to the EGL, where granular cell precursors proliferate, Smad5 is expressed in early differentiated granule neurones. We used a phospho-specific antibody to Smad1/5/8 (anti-P-Smad1/5/8) to evaluate the activation of Smad1 and Smad5, and found that at these developmental stages anti-P-Smad1/5/8 only labels nuclei in a pattern overlapping to that of *Smad5* mRNA expression. This expression analysis strongly suggests that the signalling activity of Bmp2 is mediated by Smad5. Bmp4, however, which is expressed in an overlapping pattern to that of *Smad1* at the EGL, seems not to be signalling at this developmental stages as Smad1 is not being phosphorylated. We favour the hypothesis that expression of *Bmp4* at the EGL may be inherited from earlier developmental stages, at which Bmp4 mediates determination of granule cell precursor (Alder et al., 1999), and that later Bmp4 is apparently not active during clonal expansion and/or final differentiation of granule neurones. However, the fact that Bmp receptor (Bmpr) 1a and Bmpr1b are highly expressed in the EGL (Ming et al., 2002) suggests that Bmp4 might be alternatively using a non-canonical Smad1/5/8 signalling that needs to be investigated.

In primary cultures of granule cell precursors grown in the presence of Shh, Bmp2 treatment induces strong and transient Smad5 phosphorylation. Furthermore, we show that Smad5 overexpression is sufficient to suppress the proliferative response to Shh and allow granule cell precursor to enter the differentiation programme. Whether this is achieved by a direct interaction Smad/Gli (Liu et al., 1998), by the competition for common transcriptional co-activators (Goodman and Smolik, 2000) or co-repressors (Dai et al., 2002; Wang et al., 2000) of the Smad and the Gli pathways, or by different mechanisms, remains to be elucidated.

Shh-mediated proliferation of granule cell precursors is as well regulated by components of the extracellular matrix (Graus-Porta et al., 2001; Pons et al., 2001; Rubin et al., 2002). We have previously described that the extracellular matrix glycoprotein vitronectin stimulates CREB phosphorylation using a pathway not involving MAPK, and that CREB signalling was sufficient to induce differentiation of granule cells. These results revealed CREB as an essential signal for granule neuron differentiation (Pons et al., 2001). We described the role of Bmp2 as a potent inhibitor of Shh-induced proliferation, and show that the BMP pathway in the developing cerebellum activates Smad5 phosphorylation. Whether CREB- and Smad5-mediated transcription of target genes are two parallel pathways leading to granule neuron differentiation, or whether there are points of crosstalk between these two pathways remain to be determined, although a cooperation between Smads and CREB to activate transcription in response to TGFβ signalling has already been reported in a different cell context (Zhang and Derynck., 2000).

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