FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development

Hyun-Jung Kim*†, David P. C. Rice*, Päivi J. Kettunen and Irma Thesleff‡

Institute of Biotechnology and Institute of Dentistry, PO Box 56, University of Helsinki, FIN 00014, Finland

*Both authors have contributed equally to this work
†Present address: School of Dentistry, Chung-Gu Dong-in Dong 2Ga, Daegu, Republic of Korea
‡Author for correspondence (e-mail: thesleff@operoni.helsinki.fi)

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SUMMARY

The development of calvarial bones is tightly co-ordinated with the growth of the brain and needs harmonious interactions between different tissues within the calvarial sutures. Premature fusion of cranial sutures, known as craniosynostosis, presumably involves disturbance of these interactions. Mutations in the homeobox gene Msx2 as well as the FGF receptors cause human craniosynostosis syndromes. Our histological analysis of mouse calvarial development demonstrated morphological differences in the sagittal suture between embryonic and postnatal stages. In vitro culture of mouse calvaria showed that embryonic, but not postnatal, dura mater regulated suture patency. We next analysed by in situ hybridisation the expression of several genes, which are known to act in conserved signalling pathways, in the sagittal suture during embryonic (E15-E18) and postnatal stages (P1-P6). Msx1 and Msx2 were expressed in the sutural mesenchyme and the dura mater. FGFR2(BEK), as well as Bmp2 and Bmp4, were intensely expressed in the osteogenic fronts and Bmp4 also in the mesenchyme of the sagittal suture and in the dura mater. Fgf9 was expressed throughout the calvarial mesenchyme, the dura mater, the developing bones and the overlying skin, but Fgf4 was not detected in these tissues. Interestingly, Shh and Ptc started to be expressed in patched pattern along the osteogenic fronts at the end of embryonic development and, at this time, the expression of Bmp4 and sequentially those of Msx2 and Bmp2 were reduced, and they also acquired patched expression patterns. The expression of Msx2 in the dura mater disappeared after birth.

FGF and BMP signalling pathways were further examined in vitro, in E15 mouse calvarial explants. Interestingly, beads soaked in FGF4 accelerated sutural closure when placed on the osteogenic fronts, but had no such effect when placed on the mid-sutural mesenchyme. BMP4 beads caused an increase in tissue volume both when placed on the osteogenic fronts and on the mid-sutural area, but did not effect suture closure. BMP4 induced the expression of both Msx1 and Msx2 genes in sutural tissue, while FGF4 induced only Msx1. We suggest that the local application of FGF on the osteogenic fronts accelerating suture closure in vitro, mimics the pathogenesis of human craniosynostosis syndromes in which mutations in the FGF receptor genes apparently cause constitutive activation of the receptors. Taken together, our data suggest that conserved signalling pathways regulate tissue interactions during suture morphogenesis and intramembranous bone formation of the calvaria and that morphogenesis of mouse sagittal suture is controlled by different molecular mechanisms during the embryonic and postnatal stages. Signals from the dura mater may regulate the maintenance of sutural patency prenatally, whereas signals in the osteogenic fronts dominate after birth.

Key words: Tissue interaction, Sagittal suture, Msx1, Msx2, FGF4, FGF9, FGFR2(BEK), BMP2, BMP4, Shh, Ptc, Intramembranous bone formation, Mouse

INTRODUCTION

Co-ordinate growth of the brain and skull is achieved through a series of tissue interactions between the developing brain, the growing bones of the skull and the sutures that unite the bones. These interactions couple the expansion of the brain to the growth of the bony plates at the sutures (Liu et al., 1995). Cranial sutures develop initially by a wedge-shaped proliferation of cells at the periphery of the extending bone field, called the osteogenic front (OF), whose cells undergo differentiation into osteoprogenitor cells that ultimately lay down the bone matrix (Johansen and Hall, 1982; Decker and Hall, 1985).

Craniosynostosis, the premature fusion of cranial sutures, is a birth defect that results in a profoundly abnormal skull shape (Virchow, 1851). Recently, mutations in the genes encoding for Msx2, TWIST and fibroblast growth factor receptors (FGFRs) have been identified as causes of craniosynostoses (Jabs et al., 1993, 1994; Muenke et al., 1994; Wilkie et al., 1995; El Ghouzzi et al., 1997; Howard et al., 1997) suggesting...
involvement of these genes in the signalling pathways regulating cranial suture maintenance. The functions of these genes in cranial suture development are still unclear and, in general, the molecular basis of signalling during suture morphogenesis remains to be analysed.

There is increasing evidence that the shapes of individual bones and the relative proportions of the skeleton are regulated by complex cascades involving multiple signals, their receptors and transcription factors (Kingsley, 1994; Erlebacher et al., 1995). FGFs and their receptors have been frequently implicated in the coordination of proportional bone growth and development (Givol and Yayon, 1992; Muenke and Schell, 1995). Their significance has been recently supported by the demonstrations of several FGFR mutations in dominantly inherited human skeletal disorders (Shiang et al., 1994; Tavormina et al., 1995; Cohen, 1995; Bellus et al., 1996). The four FGFRs, which are tyrosine kinases in type, have several splice forms and interact with varying specificity and affinity to at least ten FGF ligands, FGF1 to 10 (Yamaguchi and Rossant, 1995; Green et al., 1996; Yamasaki et al., 1996). FGFs regulate various cellular processes, most notably the proliferation and differentiation of cells of mesenchymal and neuroectodermal origin (Ullrich and Schlessinger, 1990; Cobb et al., 1991).

Bone morphogenetic proteins (BMPs) were originally identified by their ability to induce ectopic bone formation (Wozney et al., 1988). Since then, they have been shown to be important signalling molecules in a range of developmental processes, including mesoderm induction, odontogenesis, limb development and skeletal patterning (Kingsley et al., 1992; Vainio et al., 1993; Francis et al., 1994; Northrop et al., 1995). Recently, both BMP2 and BMP4 were shown to be involved in determining the size and shape of skeletal elements in chick limbs (Duprez et al., 1996).

The vertebrate hedgehog gene family consists of three members, Sonic (Shh), Desert and Indian hedgehog (Ihh) (Riddle et al., 1993; Krauss et al., 1993). Shh is expressed in several organising centres, such as notochord, the zone of polarising activity and the enamel knot, regulating the dorsoventral patterning of neural tube, limb polarity and crown pattern of tooth (Yamada et al., 1993; Tabin, 1995; Green et al., 1996; Yamasaki et al., 1996). Msx1 and Msx2 have also been associated with the differentiation of neural crest-derived intramembranous bones in the skull (Takahashi and Le Douarin, 1990; Takahashi et al., 1991), as well as with the formation of the dorsal part of the vertebral cartilage forming the spinous process (Watanabe and Le Douarin, 1996). Furthermore, the Boston-type craniosynostosis results from a mutation in the Msx2 gene (Jabs et al., 1993).

The available data therefore suggest that the FGF, BMP and Hh pathways form complex signalling networks regulating tissue interactions in developing vertebrate embryos. In this study, we have tested the hypothesis that they also regulate the tissue interactions that are responsible for the formation and subsequent maintenance of the sagittal suture during calvarial development. We demonstrate developmentally regulated expression patterns of Msx1, Msx2, Bmp2, Bmp4, FGFR2, Fgf9, Shh and Ptc. In addition, we show that the local exposure of sutural tissue to FGF4 and BMP4 affects suture morphology. Notably, FGF4 accelerates the approximation of the parietal bone margins. Furthermore, FGF4 stimulates the Msx1 gene expression and cell proliferation, whereas BMP4 induces both Msx1 and Msx2 genes. These data suggest specific roles of the FGF, BMP and Shh signalling pathways in membranous bone growth and cranial suture development.

MATERIALS AND METHODS

Preparation of tissues

Calvaria of mice (CBA + NMRI) aged between E15 and P6 were dissected in Dulbecco’s phosphate-buffered saline (pH 7.3) under a stereomicroscope, and they were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. The age of the embryos was determined by the day of the appearance of the vaginal plug (day 0) and confirmed by morphological criteria. Whole-mount material was embedded in methyl methacrylate and dehydrated in a methanol series and stored at −20°C until use. Material for tissue sections, which consisted of parietal bones and the interposed sagittal suture, was dehydrated and embedded in paraffin. Sections of 5 μm were mounted on silanized slides, dried overnight at 37°C and stored at 4°C. Postnatal material for tissue sections was decalcified in 12.5% ethylenediaminetetraacetic acid/2.5% PFA in PBS, changed every 4 day, for about 7–10 days, at 4°C.

Culture of calvaria

Calvaria were dissected from mice aged both E16.5 and P1 mice for the dura mater experiment, and from mice aged E15.5 for the bead experiment. In the dura mater experiment, the dura mater was removed from the sagittal suture. All explants were cultured in a Trowell-type organ culture system. Explants were placed on 0.1 μm pore size Nuclepore filters (General Electron), supported by metal grids and cultured for 24–96 hours in Dulbecco’s minimal essential medium supplemented with 10% foetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. 100 μg/ml of ascorbic acid was supplemented daily and culture medium was changed every other day. Explants were cultured for 24–48 hours (20 explants with FGF4 beads; 20 with BMP4 beads).
and 40 with BSA beads), and in the dura mater experiments explants were cultured for 96 hours (15 with dura and 25 without).

**Treatment of beads**

Beads incubated in recombinant human BMP4 protein (100 ng/μl), FGF4 protein (25 ng/μl) or bovine serum albumin (BSA) (same concentrations as for BMP4 and FGF4) were prepared as previously described by Vainio et al. (1993) and Vahtokari et al. (1996b). Before use, beads were rapidly washed in culture media, then 2 or 3 beads were placed with a mouth-controlled capillary pipette onto the OFs or the mesenchyme of sagittal suture of freshly dissected calvaria explants.

**Preparation of probes and in situ hybridisation**

The preparation of the following RNA probes has previously been described: Mxs1 and Mxs2 (Jowett et al., 1993); Bmp2, Bmp4 and Shh (Vahtokari et al., 1996a), Fgf4 and Fgf9 (Kettunen and Thesleff, 1998). The FGF-F2(BEK) probe was prepared as follows. The corresponding cDNA was cloned by RT-PCR from E15 mouse molar tooth mRNA. The PCR product was subcloned into the pAMP1 vector (Cloneamp System, Gibco BRL); this was sequenced and confirmed to be the desired cDNA by comparison to a previously published sequence (Ort-Urrtregger et al., 1993). The plasmid DNA was digested with EcoRI or BamHI producing a 139 bp fragment, then transcribed with SP6 or T7 RNA polymerase yielding antisense and sense riboprobes, respectively. The PtC probe was prepared from a 841 bp fragment of murine PtC cDNA in pBluescript II KS+; this was digested with BamHI or HindIII and transcribed with T3 or T7 RNA polymerase for antisense and sense riboprobes, respectively.

In situ hybridisation on tissue sections was performed using 35S-UTP-labelled riboprobes as described by Vainio et al. (1991). Following in situ hybridisation, the sections were stained with Delafield’s haematoxylin and mounted with DePeX (BDG). In situ hybridisation on whole mounts was performed using digoxigenin-UTP labelled riboprobes according to the protocol described by Wilkinson and Green (1990) with modifications. Briefly, explants were pretreated with proteinase K (Sigma), refixed in fresh 4% PFA/0.2% glutaraldehyde in PBST and then prehybridised for 5 hours at 55°C in a hybridisation buffer including 50% formamide. After hybridisation, tissues were washed in high-stringency conditions and preblocked in antibody blocking solution, then incubated with preabsorbed antibody. Colour development was performed in NBT/BCIP in NMT solution. Following visualisation of the stain, the tissues were postfixed and cleared in 50% formamide.

**Cell proliferation assay**

Whole-mount tissues were labelled with bromodeoxyuridine (BrdU, Boehringer-Mannheim, 1:1000) for 1 hour, fixed in MeOH overnight at −20°C, and detected by immunoperoxidase staining using a Vector ABC Elite kit.

**Histological staining**

Serial coronal sections of calvaria showing sagittal sutures from mice, aged between E15 and P3, were stained with Harris’ haematoxylin and eosin. Transverse sections of sagittal suture explants from E16.5 or NB mice, cultured for 72 or 96 hours, respectively, were stained with van Gieson stain.

**RESULTS**

**Changes in sagittal suture morphology during development**

The histological appearance of sagittal suture morphogenesis was analysed from frontal sections of calvaria from E15 to P3 (Fig. 1). At the initiation stage, the OFs of parietal bones in the presumptive sagittal suture are widely separated by intervening mesenchyme which is abundant (Fig. 1A). The OFs of parietal bones are pointing toward the underlying dura mater and the suture appeared tear-drop in shape. This morphological picture continues through embryonic development (Fig. 1A-C). After birth, the morphology of sagittal suture is altered, notably, the ends of opposite OFs confront each other in an end-to-end fashion and the amount of mesenchyme between OFs is reduced (Fig. 1D,E).

**The effect of dura mater on cranial suture development**

To evaluate the role of dura mater on mouse sagittal suture development, we dissected calvaria from E16.5 and P1 mice and cultured them in vitro both with and without the underlying dura mater (Fig. 2). After 24 hours in culture, the OFs of parietal bones had approached each other slightly, but no differences could be detected in the morphology between the explants with and without dura mater (data not shown). After 48 hours, the E16.5 calvarial explants showed clear difference: when cultured in the absence of dura mater, the OFs of the parietal bones had approached each other more rapidly than those cultured with dura mater, and they appeared to form contacts (Fig. 2C,D). After 72 hours, the explants cultured without dura mater appeared partially fused (Fig. 2F), whereas with intact dura mater the sagittal suture was still open (Fig. 2E). Histological analysis of the explants after 72 hours of culture supported these findings (Fig. 2G,H). However, in...
Expression of FGFR2 (BEK), Fgf4, Fgf9, Msx1 and Msx2 during sagittal suture development

To address the roles of FGFR2 (BEK), FGF4, FGF-9 (known ligands of the BEK receptor) and the homeobox-containing Msx genes in calvarial development, we examined their expression patterns in serial sections of sagittal sutures by in situ hybridisation. BEK, a splicing alternative of FGFR2, was intensely expressed in the OFs of parietal bones of E15 and E17 mouse embryos, and transcripts were also detected in the superficial dermis of skin (Fig. 3A,C). Postnatally BEK was expressed at the same location but its intensity was diminished (Fig. 3E,G). Interestingly at P6 these areas of expression appeared to join above and below the mid-sutural mesenchyme, possibly indicating the forming periosteum sheathing this area. At E15 Fgf9 was expressed with high intensity in the dural layers, the calvarial mesenchyme and the overlying epidermis (Fig. 3B). By E17 transcripts were most notably located in the dura mater and endocranial portion of the mesenchyme and dermis (Fig. 3D). Postnatally, expression was still noted in the calvarial mesenchyme at a diminished level (Fig. 3F). Fgf4 was not present in these tissues between E15 and P6 (Fig. 3H). Msx1 was expressed in the mesenchyme of sagittal suture and the dura mater during embryonic (Fig. 4A,C,) and postnatal stages (Fig. 4E,G). Msx2 was intensely expressed in the sutural mesenchyme and the dura mater during embryonic stages (Fig 4B,D). Interestingly, after birth, the expression of Msx2 was dramatically diminished in the mesenchyme and it completely disappeared from the dura mater (Fig. 4F,H). Furthermore, analysis of serial sections revealed that the expression of Msx2 was no longer continuous after birth around the OFs, indicating a patched pattern of expression. Msx1 and Msx2 transcripts were intensely expressed in hair follicles (Fig. 4D-H). It should be noted, however, that the hair pigment also contributed an additional component to the authentic expression. Hybridisation with sense probes gave negative results in all cases (data not shown).

Expression of Bmp2 and Bmp4 during sagittal suture development

During embryonic stages, Bmp2 mRNA was detected the OFs and weakly in parietal bones (Fig. 5A,C). After birth, the expression of Bmp2 was greatly reduced (Fig. 5E). Bmp4 mRNA, on the contrary, was localised in the OFs at high levels and at a lower level in mesenchyme of sagittal suture until E17 (Fig. 5B). At E15 Bmp4 was also expressed in the developing falx cerebri and in the dura (Fig. 5B). From the end of embryonic stage (E18), the expression of Bmp4 was decreased and the analysis of serial sections showed that expression was not continuous in the OFs, indicating a patched pattern (Fig. 5D,F). Both Bmp2 and 4 transcripts were expressed in epidermis of skin and from E16 in hair follicles, however, as with Msx probes the hair pigment also contributed somewhat to the authentic expression (Fig. 5A-F). Hybridisation with sense probes gave negative results in all cases (data not shown).

Expression of Shh and Ptc during cranial suture development

The spatiotemporal expression patterns of Shh and Ptc were analysed by whole-mount in situ hybridisation. The first weak signs of Shh gene expression were found at E17 in postero medial OFs of the parietal bones (data not shown). From E18 onwards, Shh transcripts were expressed in a patched pattern in the OFs in sagittal suture as well as in metopic suture (Fig. 6A,C). Expression of Ptc, recently identified as Shh receptor (Stone et al., 1996), was not detected by whole-mount in situ hybridisation until E18 (Fig. 6B). From E18 onwards, Ptc transcripts were localised in the OFs in sagittal and metopic sutures (Fig. 6B,D). The patterns of Ptc and Shh expression were remarkably similar. Control tissues...
hybridised with sense probes gave negative results (Fig. 6E,F). Interestingly, neither Shh nor Ptc was expressed in the area of the coronal suture during these stages (Fig. 6A-D).

**Stimulation of suture closure by local application of FGF4**

To analyse the role of FGFs in cranial suture development, we added FGF4 protein locally, using heparin acrylic beads, on sutural tissue of E15.5 mouse in vitro (Figs 7, 8). When the FGF4 beads were placed directly on OFs, acceleration of sutural closure was evident after 24 hours (Fig. 7E), compared to explants without beads (Fig. 7B), or with control beads soaked in BSA (Fig. 7H). After 48 hours in culture, a partial fusion of the suture was observed in the explants cultured with FGF4 beads placed on the OFs (Fig. 7F). However, when FGF4 beads were placed on the mid-sutural mesenchyme, no acceleration of sutural closure was observed, only a local increase in tissue thickness (Fig. 7J-L). Histological analysis of tissue sections showed increased bone growth of the OFs and partial fusion in explants where FGF4 beads were placed on the OFs (Fig. 8A-C). Cell proliferation was analysed by BrdU incorporation of explants as whole mounts. After 24 hours of culture, FGF4 beads located on both the OFs (Fig. 8D) and the mid-sutural mesenchyme (data not shown) increased cell proliferation around beads. BSA beads showed no signs of activity (Fig. 8E).

**Effect of local application of BMP4 on suture morphology**

Similar experiments to those with FGF4 beads were performed with beads soaked in BMP4 recombinant protein. There was no difference in the width of sagittal suture between BMP and BSA bead explants after 24 hours of culture (Fig. 9B,E,H,K). A small increase in tissue volume around the BMP4 beads was observed after 24 hours of culture (Fig. 9B,H) as compared to BSA control beads (Fig. 9E,K). This increase continued, so that after 48 hours the tissue volume had increased considerably around beads located on both the OFs and in the mid-sutural mesenchyme (Fig. 9C,I). In some instances, this greater tissue volume masked the appearance of the OFs making interpretation more taxing. Control beads showed no such effects (Fig. 9D,F,J-L). The effect of BMP4 beads on tissue volume is illustrated in Fig. 10E,F as a dramatic increase in tissue thickness.

**Induction of Msx1 expression by FGF4 and of Msx1 and Msx2 expression by BMP4**

In situ hybridisation analysis of explants after 48 hours culture showed that, FGF4 beads induced the expression of Msx1 (Fig. 10A), but not Msx2 (Fig. 10B) in the mesenchymal cells around the beads, both when placed on the OFs and the mid-sutural area (data not shown). BMP4 beads placed both on the OFs and the mid-sutural mesenchyme (data not shown) induced expression of both Msx1 and Msx2 around the beads (Fig. 10E,F). No signals were seen around the control BSA beads (Fig. 10C,D,G,H). Hybridisation with sense probes gave negative results in all cases (data not shown).

**DISCUSSION**

In this study, we have analysed the expression of several genes, involved in conserved signalling pathways, during mouse cranial suture development (Fig. 11). In addition, we have used an in vitro model system to examine the effects of the dura mater, as well as the local application of FGF and BMP proteins on sutural closure, parietal bone growth and gene expression. Our results support the hypothesis that sutural morphogenesis is regulated by interactions between at...
least three tissues: the sutural mesenchyme, the OFs of calvarial bones and the dura mater. The data also indicate that the same signalling pathways that are used widely in developmental regulation and which have been conserved in evolution may be involved in the regulation of suture development.

Involvement of BMP signalling in cranial suture development and intramembranous bone growth

There is increasing biochemical and genetic evidence indicating that BMPs are key signalling molecules in the initiation of bone and cartilage formation, and that they regulate the early commitment of mesenchymal cells to the chondrogenic and osteogenic lineages (Wozney, 1992). The composite expression patterns of different BMPs as well as experimental studies affecting their expression suggest that they are likely to control the basic form and pattern of the vertebrate skeleton, and that induction of Msx genes may be involved in the mediation of these effects (Kingsley, 1994; Katagiri et al., 1994; Davidson, 1995). In line with these findings, our in situ hybridisation analysis demonstrated that Bmp2 and Bmp4 was expressed in the OFs. In addition, Bmp4 was expressed in the sutural mesenchyme. Msx1 and Msx2 were expressed in the intervening mesenchyme of sagittal suture. We showed that BMP4 protein, when applied locally by beads on the OFs and sutural mesenchyme, induced the expression of Msx1 and Msx2 and contributed to an increase in tissue mass around beads. This induction maybe a direct effect or a result of an increased number of mesenchymal cells/osteoprogenitors that express Msx1 and Msx2. Msx2 and Msx1 are known to be present in vitro in osteoblastic cells (Hodgkinson et al., 1993; Towler et al., 1994a). There is evidence that Msx2 is involved in the regulation of collagen I and osteocalcin, genes expressed by terminally differentiated osteoblasts (Towler et al., 1994b; Dodig et al., 1996; Hoffmann et al., 1996; Newberry et al., 1997). Interestingly, the MSX2 craniosynostosis phenotype appears to be caused by a gain-of-function gene mutation (Jabs et al., 1993; Liu et al., 1995; Semenza et al., 1995; Ma et al., 1996).

We suggest that the BMPs may be involved in regulating the balance between the undifferentiated and differentiated states of osteogenic cells, that Msx genes are involved in this signalling pathway and, furthermore, that FGFs may act later on committed osteoblasts.

The function of FGF signalling pathway in the calvarial suture morphogenesis

The identification of FGF receptor mutations as causes of several dominantly inherited skeletal disorders, in particular
Signalling in cranial suture development

craniosynostosis, reflects functions of FGF signalling in bone development (Shiang et al., 1994; Cohen, 1995). The FGFR2 mutation causing Crouzon syndrome produces FGF independent, constitutive activation of the receptor due to aberrant intermolecular disulphide-bonding (Neilson and Friesel, 1995; Galvin et al., 1996). Interestingly signalling through FGFRs can upregulate an element in the osteocalcin promoter (Newberry et al., 1996). Hence, in our in vitro experiments, the local application of FGF4, one of the FGF ligands binding to FGFR2 (Green et al., 1996), apparently mimicked the effects of the FGFR mutation in the Crouzon syndrome. Interestingly, we found that beads releasing FGF4 protein accelerated the approximation of the OFs resulting in the closure of sutural space only when they were placed on the OFs. Application on the mid-sutural mesenchyme did not affect the speed of sutural closure. Cell proliferation was, however, stimulated around the FGF4-releasing beads in both locations. One possible explanation for these different responses is that, in the mid-sutural mesenchyme, FGF4 may stimulate the proliferation of undifferentiated mesenchymal cells, whereas, in the OFs, the stimulation of the proliferation of differentiating osteoblasts may increase the number of osteoblasts and the production of bone matrix. BMP4 did not accelerate sutural closure. The effect of FGF4 on the OFs would appear to be related to the high expression of the BEK in the OFs as shown in our in situ hybridisation analysis. BEK is known to bind to several ligands including FGF4 and FGF9 (Ornitz et al., 1996). We suggest that it is valid to use FGF4 in in vitro experiments, as it can activate the FGF receptor and thereby mimic the action of an in vivo ligand. Our evidence is suggestive that one natural ligand of the BEK receptor in the OF is FGF9, but this maybe one of many. Indeed, it is known that the FGFRs bind to a wider range of ligands, including heparan sulphate proteoglycans and neural cell adhesion molecules (Green et al., 1996). Iseki and

Fig. 6. Shh and Ptc expression in mouse calvaria from E18 to P3. In situ hybridisation analysis of whole mounts. (A,C) Shh mRNA is detected in patched pattern lining the osteogenic fronts of the sagittal and metopic sutures from E18 onwards (arrows). (B,D) From E18, Ptc mRNA is expressed in a similar patched pattern. (E,F) Hybridisation with sense probes showing no signs of activity. c, coronal suture; f, frontal bone; m, metopic suture; p, parietal bone; s, sagittal suture. Scale bar 1 mm.

Fig. 7. The effect, in vitro, of the local application of FGF4 on the development of mouse calvarial explants. (A-C) Control explants of E15.5 calvaria cultured for 48 hours. (D-F) FGF4 beads placed on the osteogenic fronts (OFs) of the sagittal suture accelerate the approximation of the parietal bones resulting in the partial obliteration of the mid-sutural space (arrow). (G-I) BSA beads placed on the OFs have no detectable effects. (J-L) FGF4 beads placed on centre of the sutural mesenchyme and cultured for 48 hours cause a local increase mesenchymal thickness (arrowhead), of, osteogenic front; p, parietal bone; s, sagittal suture. Scale bar 1 mm.
coworkers (1997) have suggested that FGF2 is another such ligand.

**Different signalling pathways may regulate suture maintenance prenatally and postnatally**

Macroscopic and microscopic analysis of calvarial explants revealed that the embryonic, but not postnatal, dura mater stimulated the maintenance of the overlying cranial suture. The dura mater is a thin sheet of cells separating the forming calvarial bones from the brain tissue. Based on studies on cultured rat coronal sutures, a role for the dura mater, mediated by soluble factors, in the maintenance of calvarial sutures has earlier been suggested (Opperman et al., 1995). We performed similar experiments with the mouse sagittal suture using a different type of organ culture system.

Our in situ hybridisation analysis showed that the expression of $\text{Msx2}$ and $\text{Bmp4}$ is correlated with the prenatal activity of dura mater. Postnatally, neither of these genes were detected in this location.

Although the expressions of $\text{Msx1}$ and $\text{Msx2}$ were overlapping, they showed clear differences and, in vitro, BMP4 induced the expression of both $\text{Msx1}$ and $\text{Msx2}$, whereas FGF4 induced the expression of $\text{Msx1}$ only. Our recent experiments indicate that, in dental mesenchyme also, FGFs preferentially regulate $\text{Msx1}$, but not $\text{Msx2}$ (Kettunen and Thesleff 1998). These findings are in line with a recent report by Catron et al. (1996) showing that the functions of $\text{Msx1}$ and $\text{Msx2}$ genes are modulated differentially by their non-conserved N-terminal regions. However, it was shown that $\text{Msx1}$ and $\text{Msx2}$ have similar DNA binding and transcriptional properties suggesting redundant functions of the two genes.

Our whole-mount in situ hybridisation analysis showed that Shh as well as its receptor, Ptc, started to be expressed at the end of embryonic development. Their expression appeared as patches on the OFs of the midline sutures. This indicates that, firstly, the target tissue for Shh signalling is in the OF and, secondly, there are site-specific differences in Shh signalling in the calvaria, which may reflect the difference in sutural architecture. The coronal suture whose OFs are overlapping apparently lacked $\text{Shh}$ expression, whereas as $\text{Shh}$ was seen in end-to-end type midline sutures. We suggest that the Shh signalling may be involved in regulating cranial suture development and intramembranous bone formation. It is possible that Shh has an analogous effect on intramembranous bone development as has been shown for Ihh, another hedgehog family member, in endochondral bone formation, where Ihh controls the differentiation of hypertrophic chondrocytes (Vortkamp et al., 1996). The patched expression pattern of these genes may be related to the subsequent development of the serrated morphology of bone surface at the sutures.

There are several examples of interactions between Shh and
Analysis of serial tissue sections indicated that postnatally the expression of Bmp2, Bmp4 and Msx2 was discontinuous along the OFs apparently reflecting a patched pattern thus resembling the expression of Shh and Ptc. We speculate that Shh may interact with Bmps in the OF through a Ptc-dependent pathway which maybe involved in the prevention of precocious sutural closure. It should be highlighted that our evidence for this model is correlative, and that additional functional data will be required to elucidate these speculations.

We propose that the maintenance of suture patency during postnatal calvarial development may be controlled by signalling events at the OFs, with Shh playing an important role. Thus signalling pathways regulating cranial suture development during the embryonic and postnatal stages would differ, with the relative importance of each varying with time.

**Fig. 10.** FGF4 beads induce Msx1 expression, while BMP4 beads induce both Msx1 and Msx2 in calvarial explants. Mouse calvarial explants (E15.5) were cultured for 48 hours with beads incubated with either FGF4, BMP4 or BSA, and analysed by in situ hybridisation. (A,B) FGF4 beads induce Msx1 but not Msx2 expression. (E,F) BMP4 beads induce the expression of both Msx1 and Msx2, and cause an increase in tissue thickness around beads. (C,D,G,H) BSA control beads. b, bone; m, mesenchyme. Scale bar 200 μm.

**Fig. 11.** Schematic diagram to illustrate the genes active prenatally and postnatally in the developing mouse sagittal suture. At E15, Bmp4 is also expressed in the central portion of the mid-sutural mesenchyme and in the dura. Postnatally, Msx2 expression is reduced and seen in a patched pattern. Also conversely, Shh and its receptor Ptc are present at P1 but absent at E15. For clarity, Fgf9 is not shown in this illustration, it is present in the calvarial mesenchyme postnatally and with great intensity prenatally, this prenatal expression is most noteworthy in the dural layers. of, osteogenic front; s, skin; p, parietal bone periosteum; d, dura mater; m, mesenchyme.


The enamel knot as a signaling center in the developing mouse tooth. Mech. Dev. 54, 39-43.


