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Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis

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SUMMARY The development and growth of the skull is a co-ordinated process involving many different tissues that interact with each other to form a complex end result. When normal development is disrupted, debilitating pathological conditions, such as craniosynostosis (premature calvarial suture fusion) and cleidocranial dysplasia (delayed suture closure), can result. It is known that mutations in the *fibroblast growth factor receptors 1*, *2*, and *3* (*FGFR1*, *2*, and *3*), as well as the transcription factors *MSX2* and *TWIST* cause craniosynostosis, and that mutations in the transcription factor *RUNX2* (*CBFA1*) cause cleidocranial dysplasia. However, relatively little is known about the development of the calvaria: where and when these genes are active during normal calvarial development, how these genes may interact in the developing calvaria, and the disturbances that may occur to cause these disorders. In this work an attempt has been made to address some of these questions from a basic biological perspective. The expression patterns of the above-mentioned genes in the developing mouse skull are detailed. The microdissection and *in vitro* culture techniques have begun the task of identifying Fgfrs, Msx2, and Twist interacting in intricate signalling pathways that if disrupted could lead to craniosynostosis.

Introduction

The bones of the skull vault or calvaria are connected by joints called sutures and fontanelles. These joints normally close post-natally in a synchronized manner, allowing the underlying brain and the rest of the skull to reach its full size and shape. Craniosynostosis is a condition where the bones of the calvaria fuse prematurely. This fusion restricts growth between the bones, and as the brain and head continue to develop growth is redirected in an abnormal direction, and deformity results.

Mutations in genes encoding for the homeobox containing transcription factor *MSX2*, the helix-loop-helix transcription factor *TWIST* and *fibroblast growth factor receptors 1*, *-2*, and *-3* (*FGFR1*, *-2*, *-3*) have been identified as causes of craniosynostosis (reviewed by Wilkie, 1997; Hehr and Muenke, 1999). Mutations in *MSX2* and *FGFR1*, *-2*, and *-3* are known to lead to a gain of gene function by both ligand-dependent as well as ligand-independent mechanisms. In addition, mutations found in the *TWIST* gene are thought to cause craniosynostosis through a loss of TWIST function (reviewed by Wilkie *et al*., 2001). However, the exact role of these genes in the developing calvarial bones and sutures is still to be determined.

Msx2, *Twist* and the *Fgfr*s are expressed at numerous locations during early mouse development, including the craniofacial area (Orr-Urtreger *et al*., 1991; MacKenzie *et al*., 1992; Füchtbauer, 1995). However, little is known about their expression during calvarial bone and suture development. In an attempt to address this, *in situ* hybridization analysis was performed to detect the tissues and cells where these genes are active, and an *in vitro* culture system was developed in which the interactions of the various tissues and signalling molecules may be analysed. Using a system of beads soaked in a variety of different growth factors it was analysed at a tissue level how these molecules of interest, primarily those involved in osteoblast differentiation, may interrelate.

In this article, using osteoblastic cell lineage markers it is demonstrated how mouse calvarial bones and sutures develop. Methods of microdissection, tissue culture, and bead induction assays have been developed and are detailed. It was found that genes known to cause craniosynostosis are expressed in a unique spatiotemporal manner. Furthermore, bone morphogenetic protein 2 (BMP2) was found to induce the expression of *Msx2* mRNA in calvarial mesenchymal tissue. With this in mind previously reported gene expression and gene interaction studies are summarized (Kim *et al.*, 1998; Rice *et al*., 2000). Finally it is concluded that FGFs and BMPs, together with TWIST and MSX2, interact in intricate signalling pathways during osteoblast differentiation, that if disrupted could lead to craniosynostosis.

Materials and methods

Preparation of the tissues

Whole heads of mice (NMRI) aged between embryonic days 10 and 15 (E10–E15), and calvaria from mice aged

between E15 and post-natal day 4 (P4) were dissected in Dulbecco's phosphate-buffered saline (PBS) (pH 7.3) under a stereomicroscope. The age of the embryos was determined by the day of the appearance of the vaginal plug (day 0) and by morphological criteria. The approximate line of dissection is illustrated in Figure 1a. Material for tissue sections was cut at 90 degrees to the suture under investigation. Following overnight fixation in 4 per cent paraformaldehyde (PFA) in PBS at 4°C, the tissues were dehydrated in an ethanol series, stained with eosin to aid tissue orientation in paraffin, treated in xylene and embedded in paraffin. Sections of 7 μm were cut and mounted on triethoxysilane-treated slides, dried overnight at 37°C and stored at 4°C. Post-natal material for tissue sections was decalcified in 12.5 per cent ethylenediaminetetra-acetic acid (EDTA)/2.5 per cent PFA in PBS, changed every 4th day, for about 7–14 days, at 4°C. Some histological sections of both *in vivo* and cultured tissues were stained with haematoxylin and eosin.

Where whole pieces of tissue (whole mounts) were to be processed for analysis by *in situ* hybridization, the tissue was denuded of the overlying skin and underlying brain. This was to improve fixation and probe penetration. The tissues were fixed overnight in 4 per cent PFA at 4°C, then dehydrated in a methanol series and stored in absolute methanol at –20°C.

In situ hybridization

In situ hybridization, to detect mRNA of a specific gene, was carried out on both tissue sections and whole mounts using techniques based on protocols described by Wilkinson and Green (1990) and Vainio *et al.* (1991) with modifications (Kim *et al.*, 1998). cDNA containing plasmids were linearized and single-stranded antisense and sense RNA probes generated by *in vitro* transcription (Kim *et al.*, 1998; Rice *et al.*, 1999, 2000). The *Runx2* (*Cbfa1*) probe was prepared from a 270 bp fragment of murine *Runx2* cDNA in pBluescript KS- vector (Stratagene, La Jolla, CA, USA), a gift from G. Karsenty, which was digested with *Xba*I and *Eco*RI for sense and antisense probes, respectively.

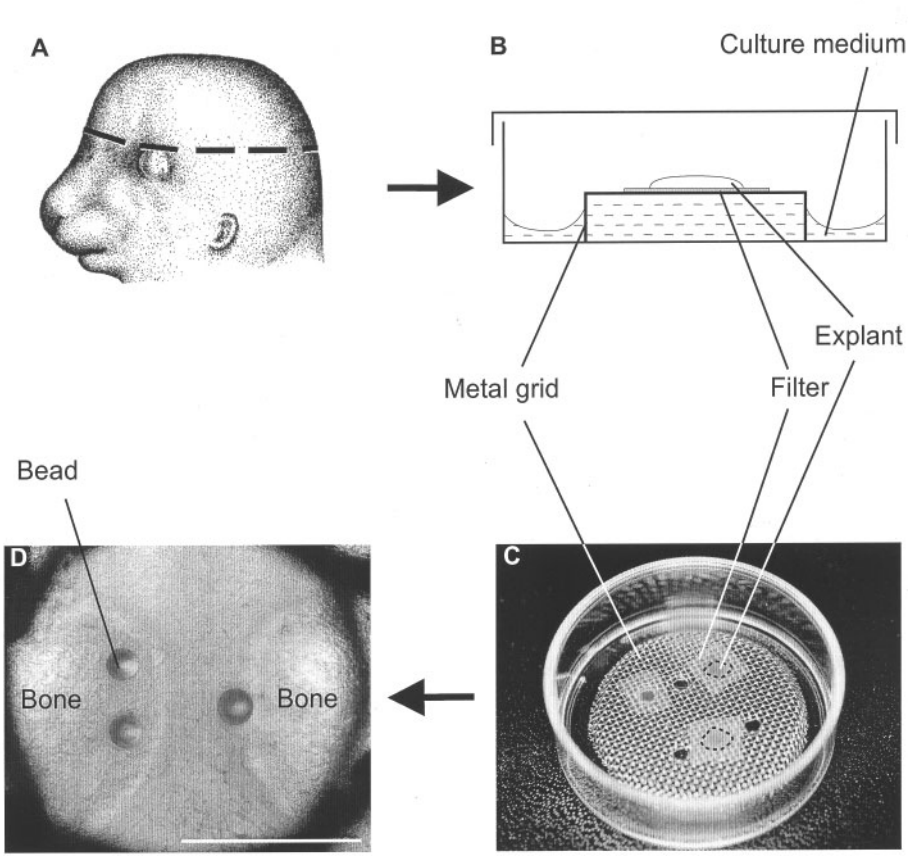

Figure 1 Microdissection and *in vitro* culture of mouse calvaria. (a) Schematic illustration showing the approximate line of dissection for mouse whole mount calvaria. (b and c) Trowell-type *in vitro* culture. The calvarial explant is placed on a filter, which rests on a metal grid. Unlike conventional tissue culture systems where the explant is immersed in liquid, in the Trowell technique the explant lies in the gas/culture medium interface permitting the tissue to draw nutrients from the medium below and allowing gas exchange with the air above. As the explant is situated in the liquid meniscus, the tissue remains wet. (d) Calvarial explant as viewed through a stereomicroscope, showing the two parietal bones, the developing sagittal suture between them, and a bead that can be impregnated with the growth factor under investigation. Scale bar in (d) = 1 mm.

Microdissection and in vitro culture of embryonic calvarial bones and sutures

The aim was to develop an *in vitro* organ culture system, which facilitates the study of molecular signalling in the developing calvarial bones and sutures. The tissue of choice proved to be the mouse calvaria aged E15. At this stage osteogenesis of the calvaria has commenced; however, the suture between the bones is still sufficiently wide to allow manipulation with beads soaked in growth factors or with microinjections of growth factors or growth factor inhibitors into specific tissues. Furthermore, the tissue is thin enough to permit development to continue.

Under a stereomicroscope, whole calvaria, in Dulbecco's PBS, were dissected with Vannasophthalmic scissors (Heiss, Tuttlingen, Germany) (Figure 1). The overlying skin and underlying brain were then removed and the explant placed on a Nuclepore polycarbonate filter (0.1 μm thickness; Pleasanton, CA, USA), which is on a metal grid in a Trowell-type organ culture. In the Trowell technique, unlike conventional tissue culture systems, the explant lies in the gas/culture medium interface, permitting the tissue to draw nutrients from the medium below and allowing gas exchange with the air above. As the explant is in the liquid meniscus the tissue remains wet. Explants were cultured for between 1 and 4 days, after which they were examined as both whole mounts and as sectioned material. Whole mounts were analysed unstained, after eosin staining, and by *in situ* hybridization.

Preparation and application of beads

The culture technique was adapted so that growth factors of particular interest could be introduced into the system and their effects later analysed, for example by *in situ* hybridization. The protein under investigation could be added either directly to the medium or via miniature beads, which have been incubated in the protein. Affi-Gel agarose beads (Biorad, Hemel Hempstead, Herts, UK) were soaked in either 100 ng/μl recombinant human BMP2 or –4 (Genetics Institute, Boston, MA, USA) for 40 minutes at 37°C then stored at 4°C until use. Heparin coated acrylic beads (Sigma, Poole, Dorset, UK) were used to deliver 25 ng/μl recombinant human FGF2 or FGF4 (R&D Systems Ltd, Abingdon, Oxford, UK). The beads were transferred directly onto the site of interest with a suction-controlled drawn Pasteur pipette under a stereomicroscope (Figure 1d).

Results

Localization of osteoblastic markers during calvarial bone and suture development

The aim of the study was to describe calvarial bone and suture development in the developing mouse. The calvarial bones (frontal, parietal, and interparietal) form by intramembraneous ossification, a process in which osteoblasts differentiate directly from mesenchymal cells.

mRNA of two osteoblast markers, namely *Runx2* (*Core binding factor alpha 1*, *Cbfa1*) and *Bone sialoprotein* (*Bsp*) were localized (Figure 2). Runx2 is a transcription factor that regulates osteoblastic cell differentiation. It binds to the promoters of several genes that encode osteoblast proteins, including osteocalcin. *Runx2* is expressed by osteoblasts and is thought be an early marker of osteoblast differentiation. *Runx2* mRNA was first detected at E11 in the head mesenchyme inferior-lateral to the developing brain, just lateral to the temporal cartilages. From these ossification centres of the frontal and parietal bones, the expression spread rapidly towards the apex of the cranium, so that by E15 osteogenic fronts from opposite sides of the head approximated to form a suture consisting of two osteogenic fronts and the intervening mesenchyme. *Runx2* was expressed by both osteoblastic progenitor cells as well as mature osteoblasts, and was detected in a wider area of expression than that of *Bsp*, which was only found in mature osteoblasts. This was seen in E15 calvaria, where *Runx2* expression extended beyond the osteogenic fronts where preosteoblasts are located, into the mid-sutural mesenchyme where the osteoprogenitors are located (compare Figure 2h,i).

Bsp is an extracellular matrix protein produced by osteoblasts. It is thought to have an intimate role in the process of bone mineralization. Transcripts of *Bsp* were first detected at E12 in a similar location to those of *Runx2*, just lateral to the temporal cartilages in a strip medial and superior to the eye, extending occipitally (Figure 2) (Rice *et al*., 2000). Until E17 *Bsp* was expressed throughout the calvarial bones, most notably on their outer surfaces. In contrast, osteoclasts were found mainly on the endocranial surfaces (Rice *et al*., 1997). Thus, as the calvaria expands there is an intimate balance between bone apposition and resorption, which maintains bone thickness and shape. After E17, both *Runx2* and *Bsp* expression became more restricted to areas of high osteogenic activity, notably the bone ends in the sutures.

Localization of Fgfrs, Fgf2, Twist, and Msx2 mRNA in the developing murine calvaria

To elucidate the roles of Fgf signalling family members, Twist, and Msx2 in calvarial bone and suture development, a detailed survey of the gene expression in mice (E10–P4) of *Fgfr1*, *-2*, and *-3* splicing alternatives (c or b), the potential ligand *Fgf2*, as well as the transcription factors *Msx2* and *Twist*, was performed (Kim *et al*., 1998; Rice *et al*., 2000). Distinct patterns of overlapping expression were found, which were compared with the expression of *Runx2* and *Bsp* osteoblastic markers (Figure 3).

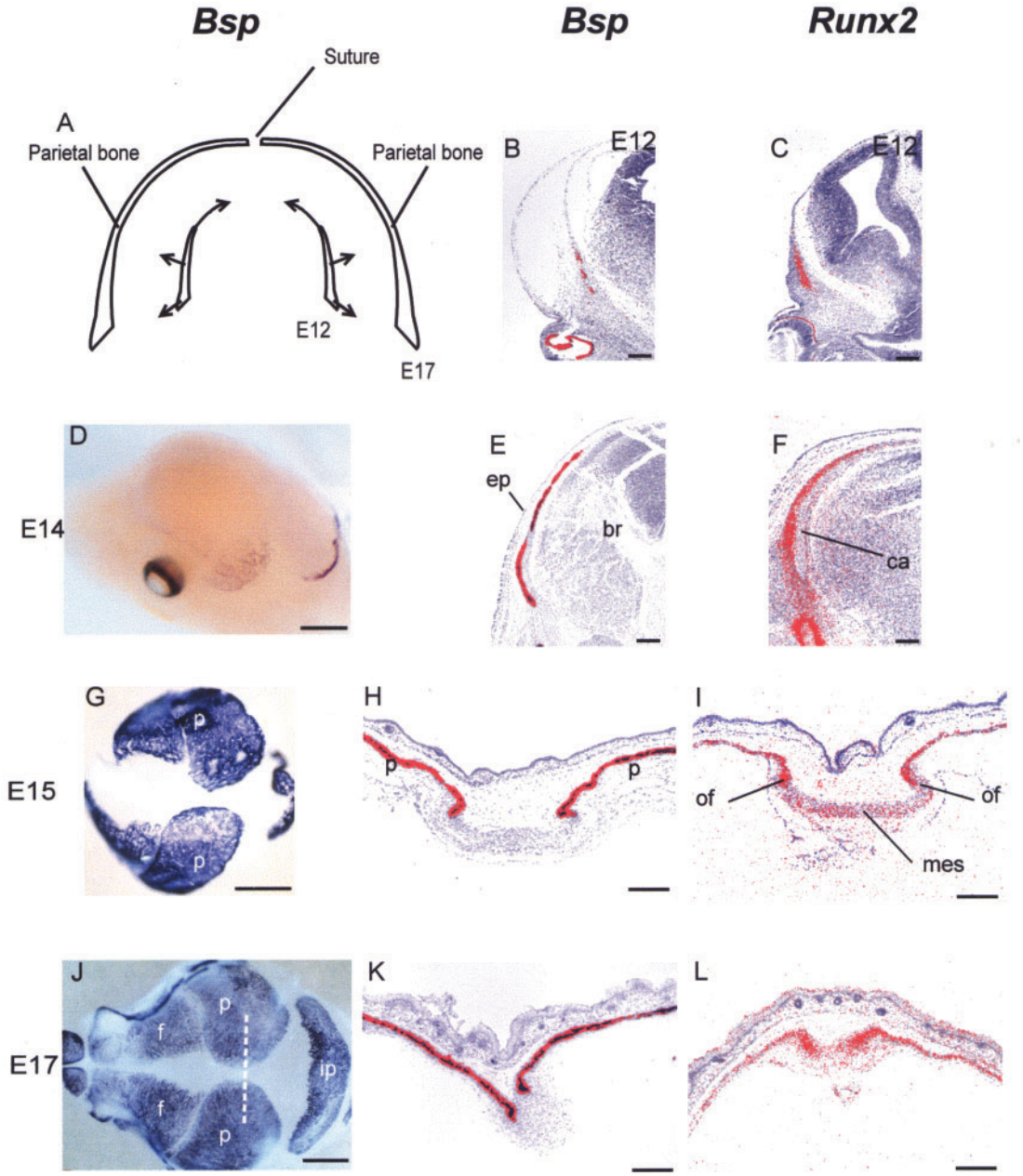

Figure 2 Mouse calvarial bone and suture development. Localization by *in situ* hybridization of *Runx2* and *Bsp* mRNA, early and late markers of osteoblastic differentiation, respectively. (a) Schematic diagram to illustrate calvarial bone growth and suture formation. Formation of the frontal and parietal bones starts on the lateral side of the head at E12. Ossification spreads upward towards the apex of the cranium to meet in the midline where a suture is formed. Growth of the calvarial bones is co-ordinated between the expanding underlying brain and overlying skin. (d, g, and j) Whole mounts showing the distribution of *Bsp* mRNA from E14 to E17. (b, e, h, and k) Frontal sections showing the localization of *Bsp* transcripts from E12 to E17. *Bsp* is expressed by mature osteoblasts, being seen only in cells close to the bone surface. (c, f, i, and l) Frontal sections showing the distribution of *Runx2* transcripts in the developing calvaria. Osteoprogenitor cells, preosteoblasts, and mature osteoblasts are all seen to express *Runx2*. This is illustrated by its larger area of expression as compared with *Bsp*, for example in (i) *Runx2* transcripts can clearly be seen stretching beyond the osteogenic fronts, into the intersutural mesenchyme (compare with h). White dotted line in (j) illustrates the plane of section (frontal) in the histological sections shown. br, brain; ca, cartilage; ep, epithelium; f, frontal bone; ip, interparietal bone; of, osteogenic front; mes, sutural mesenchyme; p, parietal bone. Scale bars: whole mounts, 1 mm; sections, 200 μm.

Except for the *Fgfr1b* and *Fgfr3b* isoforms, all *Fgfr*s studied were expressed in the sutural osteogenic fronts of the calvarial bones. Most notably, *Fgfr2c* transcripts were detected with high intensity (Figure 3a). *Fgfr3b* and *-c* were chiefly found in cartilage, while *Fgfr2b* transcripts were detected in the periostea and perichondria. In contrast to the receptor expression, *Fgf2* mRNA was found in the sutural mesenchyme between the calvarial

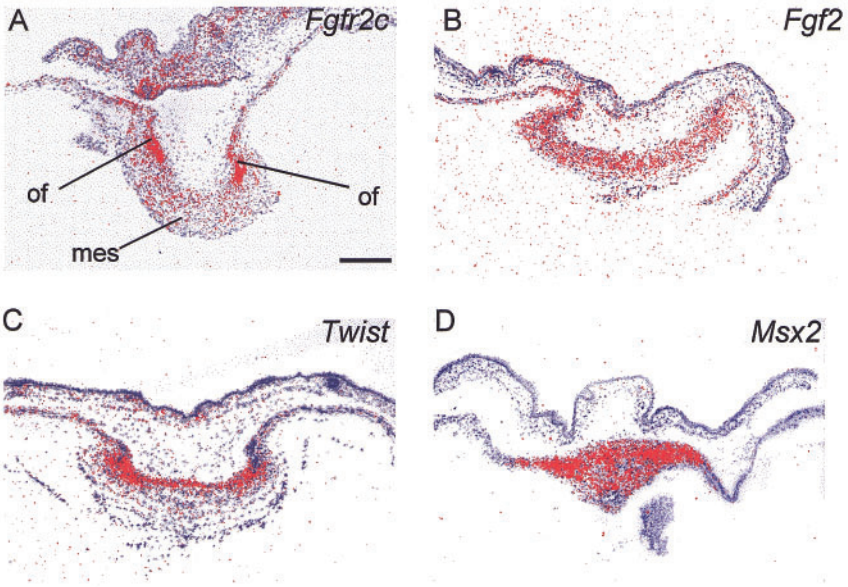

Figure 3 Genes known to cause craniosynostosis were localized in the developing calvaria by *in situ* hybridization. These show unique but overlapping expression patterns. (a) *Fgfrs1*, *-2*, and *-3* are all expressed at the osteogenic fronts of the developing calvarial bones, *Fgfr2c* illustrated here also extends into the sutural mesenchyme, all be it with decreased intensity. (b) *Fgf2* is mainly expressed in the mesenchyme but also in the calvarial bones. (c) *Msx2* is also expressed in the sutural mesenchyme but also by cells at the osteogenic front. (d) *Twist* is expressed in the mesenchyme and as osteoblastic cells increase in maturity *Twist* expression diminishes. Thus it would appear that *Twist* is expressed early in osteoblast differentiation then *Msx2* is switched on, and then the *Fgfr*s. Scale bar, 200 μm.

bones, as well as weakly in the developing calvarial bones and underlying meninges (Rice *et al*., 2000). These results correlate well with recently published gene expression patterns of *FGFR* in human calvaria, and the localization of *Fgf2* mRNA correlates with immunodistribution studies in rats (Gonzalez *et al*., 1990; Delezoide *et al*., 1998). *Msx2* was expressed in the sutural mesenchyme and the dura mater.

Early in head development (E10–11) *Twist* was intensely and extensively expressed in mesenchyme of the first and second branchial arches, and in the cranial mesenchyme just beneath the epithelium. Expression then became more restricted so that by E15 transcripts were seen in the mid-sutural mesenchyme between the osteogenic fronts. *Twist* was not expressed by preosteoblasts at the osteogenic front but by neighbouring osteoprogenitor cells. Thus, as osteoblasts differentiated from mesenchymal cells to osteoprogenitor cells, to preosteoblasts, and to mature osteoblasts, their gene expression profile changed. Firstly expressing *Twist* and *Msx2*, then later expressing *Fgfr2c* and *Msx2*.

A schematic summary of some of the genes that have been localized in the developing mouse suture is shown in Figure 4.

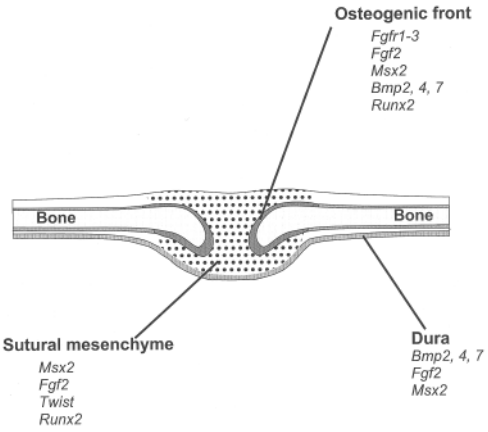

Figure 4 Schematic diagram illustrating some of the genes that are active in a developing mouse suture.

In vitro culture of embryonic calvarial bones and sutures

Explants developed in a manner consistent with suture development *in vivo* (Figure 5). After 4 days in culture, opposing parietal bone ends (osteogenic fronts) approximated and formed sutures. This is illustrated by whole mount *in situ* hybridization detecting *Bsp* mRNA. The osteogenic fronts drew closer together but did not fuse, indeed they often turned back on themselves with the tips of the bones growing slightly away from the mid-sutural mesenchyme. This morphology is reminiscent of the E15 sagittal suture where the osteogenic fronts are turned endocranially. Later, two parietal bones form a characteristic butt joint

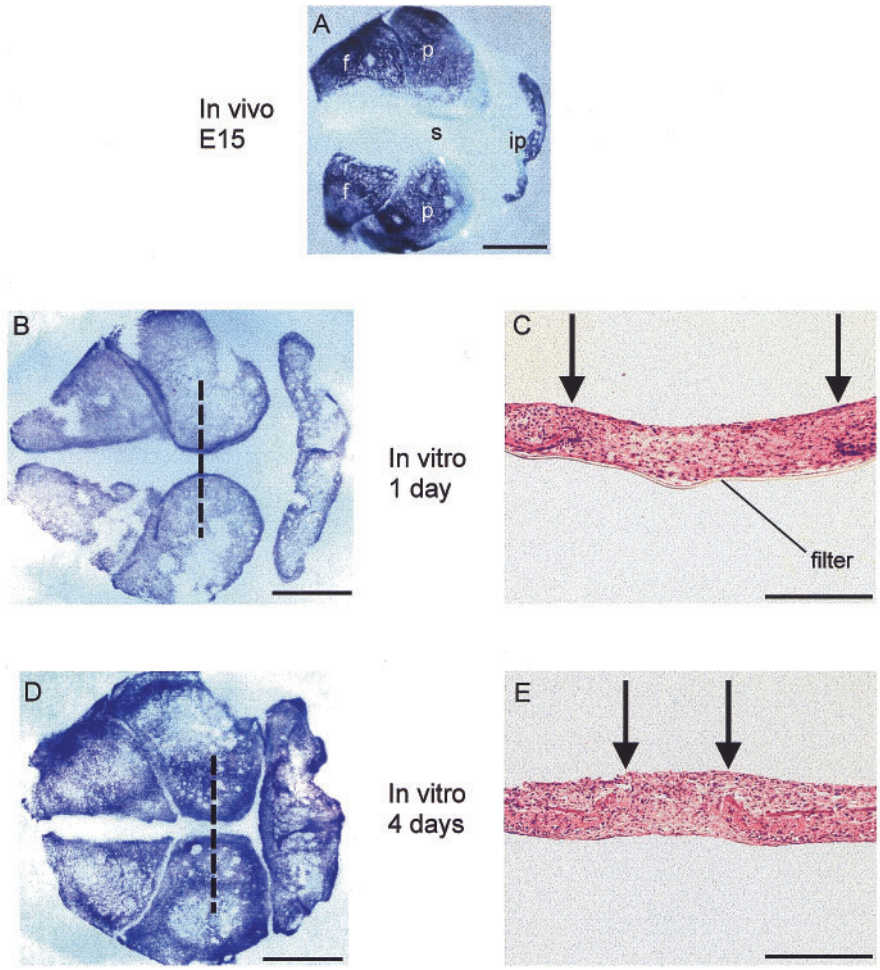

Figure 5 *In vitro* culture of E15 mouse calvaria. (a) Whole mount *in situ* hybridization showing the osteoblast marker, *Bsp* expression in E15 calvaria. The calvaria has been dissected free from the overlying skin and the underlying brain. (b and d) Whole mount *in situ* hybridization showing *Bsp* mRNA expression in E15 calvaria cultured for 1 and 4 days. Not only do explants remain viable with osteoblasts continuing to produce mRNA for *Bsp*, but also the calvarial bones expand into the mesenchymal tissue where they meet to form sutures. (c and e) Haematoxylin and eosin-stained sections of 1 and 4 day calvarial explants. Sections are cut at similar planes to the dotted lines seen in (b) and (d). The osteogenic fronts (arrows) of the calvarial bones approximate but do not fuse, thus mimicking the *in vivo* situation. Not only do the calvarial bones come closer together but there is also an increase in the thickness of the calvarial bones (white dotted lines). f, frontal bone; ip, interparietal bone; p, parietal bone; s, sagittal suture. Scale bars: whole mounts, 1 mm; sections, 200 μm.

morphology with the two, more or less flat-ended, bones confronting each other. In culture, instead of the osteogenic fronts approximating each other they may occasionally over shoot, resulting in an overlapping suture similar to the coronal suture, formed by the parietal bone overlapping frontal bone. This is not entirely surprising, as the *in vitro* system does not have the same mechanical pressures on the developing tissues as would be the case in the developing foetus, due to the removal of the rapidly expanding underlying brain and the overlying skin.

Functional studies

Based on the expression patterns outlined above and by Kim *et al*. (1998), functional experiments were performed *in vitro* attempting to elucidate the signalling pathways in which these genes may lie. Beads soaked in FGF or BMP (also known to be expressed in the calvarial bone osteogenic fronts) proteins were placed on E15 calvarial explants. When FGF4 beads were placed on the osteogenic fronts sutural closure was accelerated (Kim *et al*., 1998). This appears to mimic the pathogenesis of human craniosynostosis syndromes in which mutations in the *FGF receptor* genes apparently cause constitutive activation of the receptors. FGF4 also induced the expression of the *Msx1* gene in sutural tissue, while BMP4 induced both *Msx1* and *Msx2* (Kim *et al*., 1998). In addition it is reported here, for the first time, that BMP2 beads induce *Msx2* mRNA expression (Figure 6c). As the expression patterns of *Fgf2* and *Twist* were discovered to be overlapping, the effects of FGF2

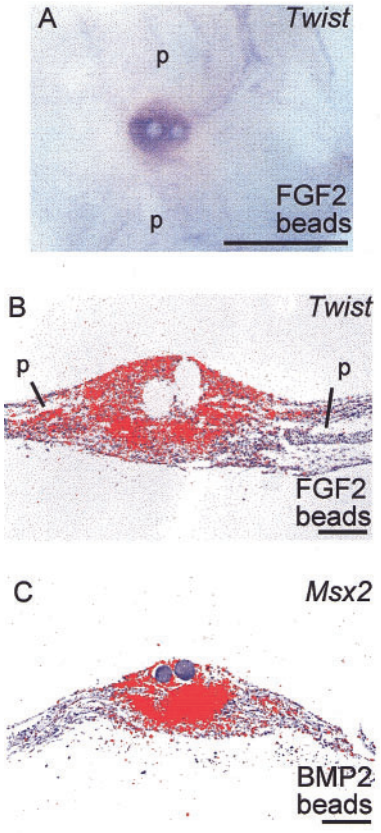

Figure 6 Bead induction experiments. (a and b) FGF2 beads upregulate *Twist* gene expression in both whole mount (a) and sectioned tissue (b). (c) BMP2 upregulates *Msx2* in the calvarial mesenchyme. Gene induction is seen as a blue colour around the beads in whole mount tissue and as a red colour in tissue sections. p, parietal bone. Scale bars: whole mounts, 1 mm; sections, 200 μm. (a and b) Printed with permission of The Company of Biologists.

on Twist was also investigated *in vitro*, and beads soaked in FGF2 were found to induce *Twist* mRNA (Figure 6a,b) (Rice *et al*., 2000). This is in accordance with an earlier report by McDougall *et al*. (1997), who found a similar upregulation in *Twist* mRNA by FGF2 osteoblastic cell cultures.

Discussion

The calvaria as a model system to study osteoblast differentiation

The bones of the calvaria form directly from mesenchymal tissue by intramembraneous ossification, unlike most other bones in which a cartilage anlagen is laid down prior to osteoblast differentiation. In this report it is shown that, in the mouse calvaria, the frontal and parietal bones start to form on the lateral side of the head at E12. In a sandwich between the underlying brain and overlying skin, the bones grow upward towards the apex of the cranium. Indeed, there is evidence that signalling from both these tissues co-ordinates bone development (Dunlop and Hall 1995; Opperman *et al*., 1995). Although craniofacial development would at first sight appear to be complex, the developing calvaria provides us with a relatively uncomplicated picture of bone initiation, growth, and later modelling. At the bone ends or osteogenic fronts one can see all the stages of osteoblastic differentiation. Thus, as osteoblasts differentiate from mesenchymal cells, to osteoprogenitor cells, to preosteoblasts, to mature osteoblasts, their gene expression profile changes: firstly expressing Twist and *Msx2*, and then later *Fgfr2c* and *Msx2*. This is in agreement with expression studies in osteoblastic cell culture, where Twist is expressed very early in osteoblast differentiation but decreases with increasing cellular maturity (Murray *et al*., 1992). Osteoprogenitor cells, preosteoblasts, and mature osteoblasts all express Runx2. Using the developing calvaria as a model system the present and other investigations have identified the first steps in understanding the signalling pathways underpinning both normal osteoblast differentiation and the molecular basis of craniosynostosis (Iseki *et al*., 1997, Kim *et al*., 1998; Rice *et al*., 2000).

The molecular basis of craniosynostosis

Studying the gene expression patterns not only gives an indication of the stage at which these genes (known to cause craniosynostosis) are expressed during osteoblast differentiation, and suture development, but also allows one to hypothesize how these genes may interact. In an attempt to look for upstream mediators and downstream targets of BMPs and FGFs, a culture system was developed and induction experiments performed using beads coated in BMP2 and –4 and FGF2. In line with studies performed in developing tooth germs and osteoblastic cell cultures, in E15 calvarial cultures BMP2 and –4 were found to upregulate Msx2, and FGF2 to upregulate Twist (Vainio *et al*., 1993; McDougall *et al*., 1997), thus giving an indication of signalling networks in which these genes may lie. These findings, from tissue culture experiments, are set into a background of information now emerging from analysis of human mutations, transgenic and natural mutant mice, as well as the culture of osteoblastic cell lines.

FGFRs

Mutations in *FGFR1*, *-2*, and *-3* have been described as causing both syndromic and non-syndromic forms of craniosynostosis. The majority of these are missense mutations, with a smaller number of in-frame insertions and deletions (Wilkie, 1997). Interestingly, the same mutation in a different gene can cause a similar but distinct phenotype. For example, Pro→Arg substitution

in the linker region between the second and third immunoglobulin-like loops can cause Pfeiffer syndrome in *FGFR1*, Apert syndrome in *FGFR2*, and Muenke craniosynostosis in *FGFR3*. Furthermore, the same syndrome may be caused by mutations in different genes. This is exemplified by Pfeiffer syndrome, in which mutations in both the *FGFR1* and *FGFR2* genes have been detected.

There is evidence that mutations in the *FGFR* genes cause craniosynostosis by a ligand-independent constitutive activation of these receptors (Neilson and Friesel, 1995; Galvin *et al*., 1996; Mangasarian *et al*., 1997). These mutations can result in reduced ligand dissociation, altered covalent cross linking and transmembrane hydrogen bonding, increased affinity for FGF ligands, ectopic expression of the *FGFR2b* isoform, and increased calvarial cell differentiation and bone matrix formation (reviewed by Wilkie, 1997; Lomri *et al*., 1998; Wilkie *et al*., 2001). In addition, osteoblastic cell lines carrying either Apert or Crouzon mutations exhibit an inhibition of cellular differentiation and an induction of apoptosis (Mansukhani *et al*., 2000).

Several genetically engineered mouse models affecting *Fgfr*s have been generated. Knocking out the *Fgfr1* gene as well as deleting the third immunoglobulin-like loop of Fgfr2 have been of little use in studying craniosynostosis pathogenesis, as they both result in death before the start of skeletal development (Deng *et al*., 1994; Yamaguchi *et al*., 1994; Xu *et al*., 1998). However, mice carrying the Pfeiffer syndrome mutation (Pro250Arg substitution) in Fgfr1 exhibit premature fusion of calvarial sutures (Zhou *et al*., 2000). The Bulgy-eye or Bey mouse is generated by retroviral insertional mutagenesis in embryonic stem cells. The vector is inserted between the *Fgf3/Fgf4* locus causing an upregulation of both *Fgf3* and *Fgf4*. Heterozygous *Bey* mice exhibit facial shortening and premature closure of several calvarial sutures (Carlton *et al*., 1998). More recently mice having a partial loss of *Fgfr2c* exhibit coronal synostosis. Interestingly, these mice also had shallow orbits apparently caused by premature fusion of the non-calvarial bones making up the lower orbital rim, namely the maxillary, zygomatic, and temporal bones. These fusions caused orbital proptosis, which is a feature common to both Apert and Pfeiffer syndromes (Hajihosseini *et al*., 2001). At first sight this phenotype would seem to contradict the gain of function mutations found in syndromic craniosynostosis patients. However, the partial loss of *Fgfr2c* confers a gain of function in the alternative splice form of the receptor, Fgfr2b. This upregulation of *Fgfr2b* in the sutures apparently causes the craniosynostotic phenotype. This hypothesis is supported by Oldridge *et al*. (1999), who demonstrated ectopic expression of *FGFR2b* in a fibroblastic cell line derived from a patient with a mutation in the IIIc immunoglobulin-like loop of FGFR2.

MSX2

Like the *FGFR* mutations, mutations in the *MSX2* gene appear to cause craniosynostosis by a gain of function mechanism. Boston-type craniosynostosis, observed in a single family, is caused by a Pro148His substitution in the *MXS2* gene (Jabs *et al*., 1993). This mutation confers enhanced DNA binding with a reduction in ligand dissociation, and this occurs without altering target specificity (Ma *et al*., 1996). Msx2-deficient mice exhibit defective proliferation of osteoprogenitors in the developing calvaria and consequently have defects of skull ossification and persistent interparietal foramen (Satokata *et al*., 2000). This phenotype resembles that associated with human MSX2 haploinsufficiency, which causes foramina in the parietal bones (Wilkie *et al*., 2000). Transgenic mice overexpressing the *MSX2* mutation appear to have different phenotypes depending on which promoter is used. One strain exhibits precocious bone formation and accelerated suture closure (Liu *et al*., 1995), while another shows other craniofacial defects, including aplasia of the interparietal bone (Winograd *et al*., 1997). It has been proposed that these differences are MSX2 dosage dependent, with a mild elevation in *MSX2* causing an enhancement of calvarial bone formation (Wilkie, 1997). More recently, transgenic mice overexpressing *Msx2* under the control of the murine *Msx2* promoter have been shown to exhibit an enhancement of calvarial bone growth and a narrowing of the calvarial sutures. These animals also display an increase in the proliferative rate of osteogenic precursors in the osteogenic front (Liu *et al*., 1999). Thus, the precocious bone growth appears to be due to an increase in the pool of potential osteoblasts, this increase ultimately leading to an increase in osteogenesis.

TWIST

Mutations in TWIST cause Saethre–Chotzen syndrome, which is characterized by craniosynostosis and limb defects (El Ghouzzi *et al*., 1997; Howard *et al*., 1997; Paznekas *et al*., 1998). In contrast to the *FGFR* and MSX2 mutations, these are largely deletions or nonsense mutations. This, together with the knowledge that *Twist* heterozygote mice exhibit limb and calvarial phenotypes reminiscent of Saethre–Chotzen syndrome (Bourgeois *et al*., 1998), indicates that Saethre–Chotzen syndrome is caused by a reduction of TWIST function. *Twist* knockout mice die at E11.5, before osteogenesis has started, with a failure of the cranial neural folds to fuse and defects in head mesenchyme (Chen and Behringer, 1995).

Drosophila Twist is thought to be upstream of DFR1, a *Drosophila* FGF-receptor homologue (Shishido *et al*., 1993). Whether this signalling pathway is conserved in the mouse was investigated by analysing the protein

distribution of Fgfrs in a Twist heterozygote background. It was found that Fgfr2 protein was distributed at an ectopic location in the central region of the sutural mesenchyme. *Fgfr2c* mRNA is normally intensely expressed by differentiating osteoblasts at the osteogenic front and at much lower levels at this same location. Interestingly, this is where *Twist* mRNA is also normally expressed. It would therefore appear that as Twist levels decrease, Fgfr distribution is altered (Rice *et al*., 2000). Taken together, it seems reasonable to propose that Twist and Fgfr lie in the same pathway. In addition, the results of this investigation also show that exogenous FGF2 will induce *Twist.* It would therefore appear that FGF signalling may lie both up- and downstream of Twist.

Conclusions

In this study it has been demonstrated that the expression patterns of *Twist*, *Msx2*, *Fgfr1*, *-2*, *-3* and *Fgf2* in the developing mouse calvaria are overlapping and spatiotemporally regulated. A calvarial culture system in which molecular interactions can be studied has been established. Using this system, it has been shown that FGF2 induces *Twist* mRNA expression in sutural mesenchymal tissue; furthermore, that BMP2 and –4 upregulate the expression of the *Msx2* gene. These molecules have been implicated in osteoblastic cell proliferation and differentiation (Iseki *et al.*, 1999; Liu *et al*., 1999). Understanding the processes of osteoblastic cell proliferation and differentiation is fundamental to calvarial development as well as clarifying the pathogenesis of human craniosynostosis. The calvarial culture system can be used to test possible genetic interactions involved in these processes.

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