Integration of FGF and TWIST in calvarial bone and suture development

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SUMMARY

Mutations in the FGFR1-FGFR3 and TWIST genes are known to cause craniosynostosis, the former by constitutive activation and the latter by haploinsufficiency. Although clinically achieving the same end result, the premature fusion of the calvarial bones, it is not known whether these genes lie in the same or independent pathways during calvarial bone development and later in suture closure. We have previously shown that Fgfr2c is expressed at the osteogenic fronts of the developing calvarial bones and that, when FGF is applied via beads to the osteogenic fronts, suture closure is accelerated (Kim, H.-J., Rice, D. P. C., Kettunen, P. J. and Thesleff, I. (1998) Development 125, 1241-1251). In order to investigate further the role of FGF signalling during mouse calvarial bone and suture development, we have performed detailed expression analysis of the splicing variants of Fgfr1-Fgfr3 and Fgfr4, as well as their potential ligand Fgf2. The IIIc splice variants of Fgfr1-Fgfr3 as well as the IIIb variant of Fgfr2 being expressed by differentiating osteoblasts at the osteogenic fronts (E15). In comparison to Fgf9, Fgf2 showed a more restricted expression pattern being primarily expressed in the sutural mesenchyme between the osteogenic fronts. We also carried out a detailed expression analysis of the helix-loop-helix factors (HLH) Twist and Id1 during calvaria and suture development (E10-P6). Twist and Id1 were expressed by early preosteoblasts, in patterns that overlapped those of the FGF ligands, but as these cells differentiated their expression dramatically decreased. Signalling pathways were further studied in vitro, in E15 mouse calvarial explants. Beads soaked in FGF2 induced Twist and inhibited Bsp, a marker of functioning osteoblasts. Meanwhile, BMP2 upregulated Id1. Id1 is a dominant negative HLH thought to inhibit basic HLH such as Twist. In Drosophila, the FGF receptor FR1 is known to be downstream of Twist. We demonstrated that in Twist+/− mice, FGFR2 protein expression was altered. We propose a model of osteoblast differentiation integrating Twist and FGF in the same pathway, in which FGF acts both at early and late stages. Disruption of this pathway may lead to craniosynostosis.

Key words: TWIST, FGF, Craniosynostosis, Suture, ID, FGFR, BMP, Intramembranous bone formation, Mouse, Human

INTRODUCTION

Except for the supraoccipital bone, the majority of the calvarial bones form by intramembranous ossification, directly from mesenchyme without a cartilage preformer. Ossification begins in centres of condensing mesenchymal cells, in which osteoblasts subsequently differentiate. These centres then expand and where two bones confront each other a joint called a suture is formed. Thus a suture consists of two membrane bone ends and interposed mesenchymal tissue. Later in development the sutures are the primary sites of osteoblast differentiation and bone formation. Signalling mechanisms governing this process, as in many other systems in the body, involve conserved growth factors such as FGFs and members of the TGFβ superfamily as well as transcription factors such as MSXs and CBFA1 (Wilkie, 1997 review; Karsenty, 1998 review; Kim et al., 1998). There is evidence that tissue interaction between the underlying brain and the calvarial mesenchyme may be involved (Opperman et al., 1993, 1995, 1998).

Mutations in the genes encoding TWIST and fibroblast growth factor receptors (FGFR) 1-3 as well as MSX2 are known to cause craniosynostosis, premature fusion of the cranial sutures leading to skull deformity (El Ghouzzi et al., 1997; Howard et al., 1997; Paznekas et al., 1998; Muenke et al., 1994; Wilkie et al., 1995; Bellus et al., 1996; Jabs et al., 1993, 1994). Fgfrs are expressed at numerous locations during early mouse development including the craniofacial area (Orr-Urtreger et al., 1991; Wilke et al., 1997) and, although Fgfrs have been detected in developing bones and sutures (Orr-Urtreger et al., 1991; Iseki et al., 1997; Delezoide et al., 1998; Kim et al., 1998), little is known about their detailed expression during calvarial bone development.

Twist proteins are conserved basic helix-loop-helix transcription factors (bHLH) and Inhibitors of differentiation (Ids) are conserved dominant negative helix-loop-helix
proteins (dnHLH). Both have been implicated as regulators of
desmoderin development and myogenesis in both *Drosophila*
and vertebrate development (Benezra et al., 1990; Bate et al.,
1991) though, in contrast to *Drosophila* Twist, murine Twist is
thought to act as a suppressor rather than an activator of
myogenesis (Hebrok et al., 1994). Although, Id1 lacks a DNA-
binding domain, it inhibits bHLH’s function by suppressing
their heterodimerization through direct protein-protein
interactions (Pesce and Benezra 1993). Early osteoblastic cell
cultures have been shown to express both Twist and Id,
with expression decreasing as maturity increases (Ogata and Noda
1991; Murray et al., 1992). We therefore postulate that they
may perform similar regulatory functions in osteoblast
differentiation as in the myoblast lineage.

Individuals with mutations in either FGFR1-FGFR3 or
TWIST have similar craniosynostotic phenotypes. Although
most patients with the Saethre-Chotzen syndrome have TWIST
mutations, some patients with an overlapping phenotype have
a mutation in either FGFR3 or FGFR2 (Paznekas et al., 1998).
Interestingly, *Drosophila* Twist is thought to inhibit DFR1, a
*Drosophila* FGF-receptor homologue (Shishido et al., 1993).
The question then begs whether Twist and Fgf are in the same
or parallel signalling pathways in osteoblast differentiation and
calvarial bone development. To this end, we have performed
detailed in situ hybridisation analysis to detect the splicing
alternatives of Fgfr1-Fgfr3, Fgf2, *Bone sialoprotein* (*Bsp*),
Twist and Id during calvarial bone development. We show that
these genes are developmentally regulated. In addition, we
have performed in vitro experiments and show that FGF2
upregulates Twist and that BMP2 upregulates Id. We also
demonstrate that FGF2 has different effects on Bsp depending
on the stage of osteoblast differentiation and that, in Twist−/−
mice, FGFR2 protein expression is altered.

We thus present evidence of Twist−, Id− and Fgf-mediated
signalling in osteoblast differentiation and calvarial bone
formation, and propose a possible model that integrates Twist
and Fgf in this signalling network.

**MATERIALS AND METHODS**

**Preparation of tissues**

Whole heads of mouse embryos (CBA × NMRI) aged between E10
and E15 and calvaria from mice aged between E15 and P6 were
processed for both sectional and whole-mount in situ hybridisation
according to Kim et al. (1998). 7 μm frontal, serial sections were cut
of both whole heads and calvaria dissected free from the underlying
brain. Calvarial whole mounts were separated from the overlying skin
and underlying brain. We therefore studied stages prior to bone
initiation until suture morphology has been established.

**In vitro experiments**

Calvarial explants were prepared from mice aged E15 according to
Kim et al. (1998). Affi-Gel agarose beads (Biorad) were incubated in
100 ng/μl recombinant human BMP2 protein (Genetics Institute, MA,
USA), heparin-binding acrylic beads (Sigma) were incubated in 25
ng/μl recombinant human FGF2 protein (R&D Systems Ltd, UK),
or bovine serum albumin (BSA) (same concentrations as for BMP2 and
FGF2) for 1 hour at 37°C. Beads were then placed either between the
two parietal bones on the mid-sutural mesenchyme or on the centre
of the parietal bone of freshly prepared calvarial explants. These were
cultured for 48 hours then processed for either paraffin sections or
whole mounts, as previously described by Kim et al. (1998).

**Detection of Twist, Id1, Bsp, Fgfr1b, Fgfr1c, Fgfr2b,
Fgfr2c, Fgfr3b, Fgfr3c, Fgfr4 and Fgf2 mRNA by in situ
hybridisation**

Preparation of Fgf and Bsp mRNA probes has previously been
described (Kettunen et al., 1998; Kim et al., 1998). The Twist
probes were prepared from a 400 bp fragment of murine
Twist cDNA in pT7T3 19U, which was digested with XhoI and EcoRI.
Id1 probes were prepared from a 900 bp fragment of murine Id1
cDNA in pBS-SK, digested with BamHI and HindIII. Murine Fgf2
cDNA in pGEMZ2 was digested with Smal and EcoRI producing 579
bp fragments.

In situ hybridisation on tissue sections was performed using
[35S]UTP-labelled riboprobes as described previously (Vainio et al.,
1991; Kim et al., 1998). Following in situ hybridisation the sections
were stained with Delsefield’s haematoxylin and mounted with DePeX
(BDG). Images were taken using a Cohu 4912-5000 CCD (Cohu,
SanDiego, CA, USA) camera and a Scion LG-3 Frame Grabber card
(Scion, Frederick, MD, USA) with an Olympus BX50 microscope and
a Macintosh PCC computer. Images were then processed with the NIH
Image 1.61 program (US National Institute of Health, available from
the Internet from zippy.nimh.nih.gov) and Adobe Photoshop 4.0
(Adobe Systems, San Jose, CA, USA) software. The silver grains in
the dark-field image were selected, coloured red then superimposed
onto the bright-field image.

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 (Kim
et al., 1998).

**Detection of FGFR by immunostaining**

Whole heads were fixed and sectioned as described (Liu et al., 1999).
Primary FGFR2 antibody (Santa Cruz Biotechnology) was allowed to
react with tissue sections. A biotinylated, affinity-purified secondary
antibody (Zymed Laboratories, San Francisco) was bound to the
primary antibody (Santa Cruz Biotechnology), of the same concentration as the primary antibody,
was used as a control.

**RESULTS**

**Expression of Twist, Id and Bone sialoprotein during
calvarial bone and sagittal suture development**

To address the roles of *Twist* and *Id* in calvarial bone
development, we examined their mRNA expression patterns in
both calvarial whole mounts and serial sections and compared
them to *Bone sialoprotein* (*Bsp*), a marker of mature
osteoblasts. *Bsp* was first expressed at E12 just lateral to the
temporal cartilages, in a strip medial and superior to the eye
extending occipitally (Fig. 2G). From these ossification centres
in the frontal and parietal bones, the expression spread toward
the apex of the cranium where the osteogenic fronts
approximate to form a suture (E15), two osteogenic fronts and
intervening mesenchyme (Fig. 1). Until E17 *Bsp* was expressed
throughout the calvarial bones, most notably on their outer
surfaces (Figs 2J, 3A,H, 4A,C,E). In contrast, osteoclasts are
found mainly on the endocranial surfaces (Rice et al., 1997) and so, as the calvaria expands, there is an intimate balance between bone apposition and resorption, thus maintaining bone thickness and shape. After E17, transcripts became more restricted to areas of high activity, notably the sutures (Figs 3J, 4G). Whole mounts confirmed the findings of sectioned tissue, with Bsp expression clearly demarcating the developing calvarial bones and illustrating the approximation of their osteogenic fronts (Fig. 4A,C,E).

At E10, Twist was intensely expressed in mesenchyme throughout the first and second branchial arches, as well as in the mesenchyme surrounding the developing eye and cranial mesenchyme just beneath the epithelium (Fig. 2B). Expression then became more restricted so that, by E14, transcripts were seen bordering areas of condensing calvarial mesenchyme (Figs 2E,H,K, 4B). These condensations consist of osteoprogenitors that differentiate into functioning osteoblasts, the temporal, frontal and parietal bones being thereby initiated. Twist mRNA was also detected close to developing cartilages. From E15 to P1, Twist continued to be expressed in the

Fig. 1. Schematic diagram to illustrate calvarial bone growth and suture formation. Ossification of the frontal and parietal bones starts on the lateral side of the head. Bsp expression is first seen at E12 in osteoblasts just lateral to the temporal cartilages, in a strip medial and superior to the eye extending occipitally (Fig. 2G). From these centres, the process spreads upward toward the apex of the cranium to meet in the midline where a suture is formed (E15), i.e., two osteogenic fronts with intervening mesenchyme (Figs 3A, 4C). Osteoblasts are active mainly on the ectocranial and sutral surfaces, whereas osteoclastic activity is mainly on the endocranial surface (Rice et al., 1997). Growth of the calvarial bones is co-ordinated between the underlying brain and overlying skin.

Fig. 2. Bsp, Twist, and Id expression during calvarial bone and sagittal suture development E10-14. (A,D,G,J) Bsp is expressed by functioning osteoblasts. Unlike long bones, the calvarial bones form directly from mesenchymal condensations, without a cartilage preformer. Osteoblasts differentiate directly from mesenchyme, and matrix is secreted that then calcifies. In the calvarial bones, these condensations start to appear at E11, so that by E12 (G) Bsp transcripts are seen sandwiched between the developing eye and brain (arrow). As the calvarial bones develop Bsp expression extends rostrally within the calvarial mesenchyme, between the overlying skin and the underlying meninges (J). (B,E,H,K) Twist is intensely expressed in head mesenchyme. At E10 transcripts are seen widely distributed in the first and second branchial arches, the surroundings of the developing eye and cranial mesenchyme below the epithelium, including that around Rathkes pouch (rp) (B). In the calvaria, this becomes confined to mesenchyme from which differentiate osteoblasts (K, arrows). (C,F,I,L) Id has a similar expression pattern to Twist but is also expressed in epithelia including the neuroepithelium of the developing hippocampus (F, dotted arrow), and in the developing retina (F, arrow), meninges (F, arrowhead) and cartilage (L, arrow). rp, Rathkes pouch. Scale bars, 200 μm, images of the same age are the same scale.
calvarial mesenchyme and, as in earlier stages, not in mature osteoblasts (Figs 3B,E,H, 4D,F). Postnatally its intensity had decreased (Figs 3K, 4H). These findings were again corroborated by whole mounts with Twist being expressed in the calvarial mesenchyme between the calvarial bones and most intensely close to the osteogenic fronts (Fig. 4B,D,F). Later, transcripts were confined to areas of high activity, delineating the calvarial bones (Fig. 4H).

Id1, like Twist, was initially expressed in the mesenchyme of the first and second branchial arches, but not in such a widespread manner. Id1 was most notable in the rostral half of the mandibular arch and the lateral section of the maxillary arch (Fig. 2C). Unlike Twist, Id1 was also expressed in epithelia adjacent to these mesenchymal areas and, at E10, in the epithelium of Rathkes pouch, whereas Twist was expressed in the surrounding mesenchyme. From E11, transcripts were also seen in the developing hippocampus and retina (Fig. 2F). Similar to the expression of Fgfr1c, Fgfr2b and Fgfr3c, Id1 was expressed in developing cartilage (compare to Fig. 6E), which mainly does not directly contribute to the calvarial bone. Interestingly, in E11-14 embryos, Id1 mRNA was detected in the calvarial mesenchyme just prior to and during the condensation of early osteoblasts (Fig. 2F,I,L). By E15, this Id1 activity in calvarial mesenchyme had reduced (Fig. 3C,F). Id1 transcripts were also notably detected in the deeper layers of the skin and in the meninges (Figs 2F,I,L, 3C,F,I).

Expression of Fgfr1b, Fgfr1c, Fgfr2b, Fgfr2c, Fgfr3b, Fgfr3c and Fgfr4 during calvarial bone and sagittal suture development

The FGFRs are high-affinity tyrosine kinase receptors, which together with cofactors mediate the effects of FGFs. They are transmembrane glycoproteins with two or three extracellular immunoglobulin domains. These binding domains differ between alternative splice variants, which are of particular interest as they possess different ligand-binding specificities (Ornitz et al., 1996) as well as exhibiting unique temporospatial expression patterns suggesting unique functions (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993; Kettunen et al., 1998). In addition, many of the human mutations in FGFR1-FGFR3 causing disorders in bone development are found in or close to the third immunoglobulin (III) domain (Wilkie, 1997). We have previously detected the IIIc splice variant of Fgfr2 (bek) (Kim et al., 1998) and Iseki et al. (1997) showed the full-length Fgfr2 in the developing calvaria. Here we have investigated the expression patterns of the IIIb and IIIc splice variants of Fgfr1-Fgfr3, as well as Fgfr4.

The expression pattern of Fgfr1b was generally weak compared to the other Fgfrs and transcripts were not seen in either the developing calvarial bone or sutures (Fig. 5A-C). Developing teeth were used as positive controls, where Fgfr1b mRNA was intensely expressed in the inner enamel epithelium of E16 molar teeth (data not shown; Kettunen et al., 1998).
Very low levels of Fgfr1c expression were detected in the calvarial bones, most notably in the osteogenic fronts between E15 and 17, major sites of osteoblastic condensation and differentiation (Fig. 5D-F). Fgfr1c was also expressed in many cartilages in the developing head.

Fgfr2b (kgfr) was expressed in the osteogenic fronts of the parietal bones (E15-17). In addition, transcripts were located in developing epithelia, notably the skin including hair follicles (Fig. 5G-I). Fgfr2c (bek) was found in similar locations to Fgfr2b but at generally much stronger intensity (Fig. 5J-L). Transcripts were generally detected in perichondria and periostea, as well as in the superficial layers of skin. Postnatally, expression was at the same locations but diminished in intensity (Fig. 5L). In developing epithelia, transcripts were detected at low intensity, in comparison to Fgfr2b.

Fgfr3b was weakly and Fgfr3c strongly expressed in many cartilages of the head. The majority of this cartilage does not

**Fig. 4.** Bsp and Twist expression during calvarial bone and suture development, detected by whole-mount in situ hybridisation. (A,B) Whole heads; (C-H) calvariae dissected free from the underlying brain and overlying skin. (A,C,E,G) Bsp starts in ossification centres on the lateral and posterior surfaces of the calvaria, these expand so that by E17 they have begun to assume their characteristic bone shapes (E). Thereafter, osteoblast activity and therefore Bsp expression is largely concentrated in the sutures (G). (B,D,F,H) Twist mRNA is confined to the mesenchyme in between the forming bones, with its strongest expression directly neighbouring the osteogenic fronts (arrows). Twist is also expressed in the developing digits (B dotted arrow). c coronal suture; f, frontal bone; fs, frontal suture; ip, interparietal bone; l, lambdoidal suture; p, parietal bone; s, sagittal suture. Scale bars, 1 mm.

**Fig. 5.** Fgfr1b, Fgfr1c, Fgfr2b and Fgfr2c expression during calvarial bone and sagittal suture development. Frontal sections showing the two parietal bones (p), their osteogenic fronts (of) and the mid-sutural mesenchyme (mes). Transcripts of Fgfr1b, are not detected in the developing calvaria studied. At E15, Fgfr1c, Fgfr2b, Fgfr2c and Fgfr3c (Fig. 6D) are all expressed in the sutural osteogenic front (arrows), Fgfr1c and Fgfr2b being expressed with the lowest intensity, Fgfr2c with the strongest (compare to Fig. 3D-F). After E15, the intensity decreases with only Fgfr2c being expressed postnatally (L). Fgfr1c, Fgfr2b and Fgfr2c mRNAs are also detected in epithelia including hair follicles, with expression commencing at E15; in this regard, Fgfr2b shows the strongest expression (G-I), of, osteogenic fronts; mes, mid-sutural mesenchyme; p, parietal bone. Scale bars, 200 μm. Scale in A applies to A,B,D,E,G,H and J; scale in C, applies to C,F,I and L.
Fig. 6. Fgfr3b, Fgfr3c, Fgfr4 and Fgf2 expression during calvarial bone and sagittal suture development. Fgfr3b and Fgfr3c, as well as Fgfr1c, are expressed in many cartilages in the developing embryonic skeleton of the head. These include those of the cranial base, the condyle of the mandible (C) and cartilages that form on the lateral aspect of the cranial vault, endocranial to the forming intramembranous bones. Cartilage rods are also seen in the sutural mesenchyme; their appearance is transient, around the time of birth and maybe a tissue reaction to mechanical irritation. This cartilage does not contribute to the calvarial bones that form, in the main by intramembranous and not endochondral ossification. Fgfr1c, Fgfr3b and Fgfr3c are expressed in all these cartilages. Fgfr3c mRNA is detected most strongly (E, dotted arrow, compare to Fig. 3I). At E15, Fgfr3c is also weakly expressed in the developing calvarial bones (D dotted arrow), notably at the sutural osteogenic front (D, arrow). (G,H) Fgfr4 mRNA is not detected in the developing calvarial bones or mesenchyme (G, E15; H, P1). Transcripts are found in the developing muscle, on the lateral aspects of the cranium between the epithelium and the calvarial bones (G arrows). (I-K) Fgf2 transcripts are intensely expressed in the mid-sutural mesenchyme (I, arrow), and weakly in the calvarial bones and underlying meninges. This expression is most notable at E15. of, osteogenic fronts; mes, mid-sutural mesenchyme; p, parietal bone. Scale bars, 200 μm. Scale in A refers to A-E,G,I,J; scale in F refers to F,H,K.

Differential regulation of Twist and Id by FGF2 and BMP2

To test the effect of FGF2 on Twist expression, we placed FGF2 beads on the mid-sutural mesenchyme of E15 calvaria. FGF2 stimulated the expression of Twist in explants cultured for 48 hours (11 out of 14 explants). This was detected by in situ hybridisation in whole mounts as a halo of expression around the beads, which was confirmed by in situ hybridisation in tissue sections (Fig. 7A,B). BSA beads had no apparent effect (n=7). The effect of BMP on Twist expression was also tested. BMP2 did not induce Twist. Conversely, one can speculate that BMP2 might inhibit Twist, in a similar manner to that demonstrated by RT-PCR in cell culture (Tamura and Noda, 1999); however, conclusions regarding inhibition could not be drawn from our experiment.

Bmps are known to be expressed in the OFs of the developing calvarial bones (Kim et al., 1998; Rice et al., 1999). Also, BMP2 has previously been found, in cell culture, to stimulate Id in osteoblast-like cells, in pluripotent fibroblastic cells and transiently in myoblast cells that convert to osteoblasts with BMP2 treatment (Ogata et al., 1993; Katagiri et al., 1994). In addition to stimulating Id, Katagiri et al. (1994) found that BMP2 induced alkaline phosphatase activity osteocalcin production, markers of osteoblast maturation, as well as inhibiting MyoD. We therefore set out to investigate the effects of BMP on Id in the developing calvaria. Beads releasing BMP2 stimulated the expression of Id in the mesenchyme of E15 calvarial explants cultured for 48 hours (8 out of 8). This was demonstrated by in situ hybridisation in both whole mounts and sectioned tissue (Fig. 7G,H), while beads releasing BSA had no apparent effect (n=4).
**Fgfr and Twist are in the same signalling network**

To test whether FGFR1 and FGFR2 protein in the calvaria of mice with a Twist+/− background. Twist−/− mice were not used as they die before calvarial development has progressed sufficiently. The distribution pattern of FGFR2 (Fig. 8) was altered in the sagittal suture of Twist+/− mice, as compared to wild type. FGFR2 protein was detected in a discrete central area of the mid-sutural mesenchyme. This area contains undifferentiated mesenchymal cells that will differentiate in osteoblasts, osteoprogenitors. FGFR1 protein was detected in the osteoblasts of the calvarial bones (Fig. 9), in the mid-sutural mesenchyme, as well as in the meninges. No difference in FGFR1 distribution could be distinguished between the wild-type and Twist+/− mice. Peptide competition studies, the use of appropriate negative and positive controls confirmed antibody specificity and validity (not shown).

**Dual effect of FGF2 on Bsp expression**

At E15, Bsp is highly expressed by mature osteoblasts throughout the developing parietal bones (Fig. 3A) and this expression continues when the calvaria are cultured in vitro (Fig. 7E). However, when FGF2-releasing beads were placed on the parietal bone and cultured for 48 hours, this expression was inhibited (Fig. 7C,D) (n=13 out of 14). This effect was seen in whole mounts as a well-defined clear area of reduced Bsp expression around the beads and in sections as an area of reduced expression in the calvarial bone. Interestingly, in the osteogenic front, activity was greatest was maintained (Fig. 7D). An intriguing finding was that, although Bsp expression was reduced in cells already differentiated, in other cells in close proximity to the bead Bsp expression was induced. This expression was barely evident in whole mounts but verified in sections with Bsp being expressed in cells around the beads. These were presumably mesenchymal osteoprogenitor cells (Fig. 7D). BSA beads had no such effects (Fig. 7E,F; n=7).

**DISCUSSION**

In this study, we have analysed the association of Twist and Id with FGF and BMP signalling during cranial bone and suture development. Based on previous data from several laboratories, including our own, and the findings of this study, we have proposed a model of how they may interrelate (Fig. 10).

**Twist and Id in calvarial osteogenesis**

Our detailed comparison of the expression patterns showed that Bsp, which encodes a bone matrix protein was highly expressed by osteoblasts (Bianco et al., 1991; Chen et al., 1991), whereas Twist and Id were expressed by mesenchymal and osteoprogenitor cells. In the developing calvarial bones, Twist and Id are expressed by mesenchymal cells, which directly neighbour Bsp-expressing osteoblasts lining the bone matrix. This is consistent with the finding that, in cultured osteoblastic cells, Twist and Id are expressed very early in osteoblast differentiation and that their expression decreases with maturity (Kawaguchi et al., 1992; Murray et al., 1992). Interestingly, osteoblastic cells overexpressing Twist stay in an undifferentiated state, with an increased proliferation rate and change from osteoblast to fibroblast in morphology (Glackin et al., 1997). Our results demonstrate for the first time in vivo that Twist and Id expression correlate well with early osteoprogenitors. Generally, the expression patterns in the craniofacial region are in accord with previously published data (Füchtbauer et al., 1995; Bourgeois et al., 1998; Jen et al., 1996).

**Twist−/− homozygotic mice die at E11.5 with a failure of the cranial neural folds to fuse and defects in head mesenchyme,**

![E15 Calvarial Explants](image)

**Fig. 7.** In vitro effects of FGF2 and BMP beads on E15 calvarial explants. (A,C,E,G) Digoxigenin-UTP labelled in situ hybridisation of whole mounts. (B,D,F,H) [35S]UTP-labelled in situ hybridisation on tissue sections. (A,B) FGF2 beads induce Twist in calvarial mesenchyme, (C,D) FGF2 beads inhibits Bsp in osteoblasts (arrows) but also stimulates Bsp mRNA production in calvarial mesenchyme (osteoprogenitors; dotted arrow), when placed on the parietal bones (p). (E,F) BSA beads have no effect on the normal Bsp expression. (G,H) BMP2 beads induce Id when placed on calvarial mesenchyme. f, frontal bone; ip, interparietal bone; of, osteogenic front; p, parietal bone. Scale bar in sections 200 μm, all sections same scale. Scale bar in whole mounts 1 mm.
somites and limb buds (Chen and Behringer, 1995). Twist+/−
mice exhibit limb and calvarial phenotypes reminiscent of
Saethre-Chotzen syndrome which is caused by TWIST
mutations (El Ghouzzi et al., 1997; Bourgeois et al., 1998).
This possibly suggests haploinsufficiency in Saethre-Chotzen
patients rather than a dominant negative gene mutation (El
Ghouzzi et al., 1997) and is in line with Twist being a negative
regulator of osteoblast differentiation.

Others and we have previously shown Msx1 and Msx2 to be
expressed in the calvarial mesenchyme (Mackenzie et al.,
1992; Kim et al., 1998) and we note that their expression
patterns overlap with that of Twist and at early stages with Id.
Both MSX1 and MSX2 are known to be intimately involved
in osteoblast differentiation (Hodgkinson et al., 1993;
Hoffmann et al., 1996) and in the pathogenesis of
calvarial bone disorders (Jabs et al., 1993;
Satokata and Maas 1994; Liu et al., 1999). Also
the osteocalcin promoter contains binding sites
for both HLH and MSX proteins (Tamura and
Noda 1994; Heinrichs et al., 1993). One can
therefore speculate, whether HLHs and MSXs
could be either in the same or in parallel
signalling pathways.

Role of FGF in calvarial osteogenesis
The expression of Fgfr1-Fgfr3 in the
developing calvarial suture is consistent with
mutations in the human FGFR1-FGFR3 genes
causing craniosynostosis syndromes. The IIIc
splicing variants of Fgfr1-Fgfr3 and IIIB
variant of Fgfr2 are all, to some degree,
expressed by differentiating osteoblasts at the
calvarial bone ends, the osteogenic fronts.
Craniosynostosis syndromes, are characterised
by early fusion of these bone ends, and this is
presumably based on an increase in osteoblast
activity possibly as result of precocious
osteoblast differentiation.

Interestingly, Fgfr1-Fgfr3 are also intensely
expressed in the cartilages of the cranial base
(unpublished observations). Mutations in these
genes could therefore lead to a growth defect in
the cranial base, which could have the
secondary effect of bringing the calvarial bones
closer together and therefore increasing the
likelihood of premature fusion.

There is evidence indicating that mutations in
FGFR1-FGFR3 cause craniosynostosis syndromes by ligand-independent constitutive
activation of receptors (Neillson and Friesel 1995;
Galvin et al., 1996; Mangasarian et al., 1997). In
addition, analysis of the Bulgy-eye mouse, which
is generated by an insertional mutation at the
Fgf3/Fgf4 locus, indicates that ligand
overexpression may also cause a craniosynostotic
phenotype (Carlton et al., 1998). However, mice
ubiquitously overexpressing FGF2 exhibit
chondrodysplasia, with shortened long bones.
Interestingly, their occipital bone, a bone formed
partly intramembranously and partly from
endochondral ossification, appeared to be
enlarged (Coffin et al., 1995). Expression of Fgf2, although
overlapping, differs from the Fgfrs; Fgf2 is expressed primarily
in the sutural mesenchyme and Fgfrs in the OFs, suggesting
paracrine interactions. We have found earlier that FGF4, if placed
in vitro on the embryonic osteogenic fronts, will accelerate suture
closure, as well as stimulate cell proliferation (Kim et al., 1998).
Transgenic mouse models have until now yielded limited
information with regard to FGF signalling in intramembranous
bone formation and suture fusion. Both knocking out Fgfr1 and
deletion of Fgfr2’s third Ig loop results in death before start of
skeletal development (Deng et al., 1994; Yamaguchi et al., 1994;
Xu et al., 1998). Knocking out Fgfr3 results in long bone
overgrowth indicating that Fgfr3 is a negative regulator of
endochondral ossification (Colvin et al., 1996; Deng et al., 1996).

![Fig. 8.](image-url) Immunohistochemical staining for FGFR2 in the, newborn, sagittal suture of
wild-type (A,C,E) and Twist+/− mice (B,D,F). (C,E) High magnifications of the
sections shown in A; (D,F) high magnifications of the sections shown in B. In
wild-type mice, FGFR2 protein is detected in the osteoblasts at the osteogenic front
(C dotted arrow) as well as weakly and diffusely in the mesenchyme (E arrows).
FGFR2 distribution is altered in the Twist mutant, being localised more discretely in
the mid-sutural mesenchyme (D,F arrows). mes, mid-sutural mesenchyme; p, parietal
bone. Scale bars, 100 μm.
Fgf2 knockout mice are morphologically normal with no apparent skeletal phenotype (Zhou et al., 1998). FGF2 is known to stimulate osteoblast proliferation and inhibit osteoblast differentiation markers (Canalis and Raisz 1980). The effects on bone matrix formation are more confusing, with data suggesting both stimulation and inhibition. Indeed, the effects of FGF2 may be differentiation stage specific with FGF2 causing an increase in matrix mineralization and osteocalcin production in more mature cells (Debiais et al., 1998). Our finding that FGF2 both downregulates Bsp mRNA in mature osteoblasts and also induces Bsp expression in calvarial mesenchymal cells supports the hypothesis that FGF2 may indeed have different effects at different levels of cell maturity. Interestingly, it has been shown that Bsp expression is biphasic, detectable in some early, alkaline phosphatase-negative osteoblastic cells, as well as again later in more mature cells (Malaval et al., 1999).

**FGF is associated with Twist signalling**

Fgf2 and Twist exhibit overlapping expression patterns, both being intensely expressed in the mid-sutural mesenchyme between the calvarial bones, (compare Figs 3B and 6I). We have previously described the expression pattern Fgf9, which is also detected at similar locations (Kim et al., 1998). This suggests that FGF and Twist maybe involved in a common signalling network. We therefore studied the effect of FGF on Twist and found that FGF2 upregulates Twist expression in calvarial mesenchyme. This is in accordance with an earlier report by McDougall et al. (1997), who found a similar upregulation in osteoblast cell cultures.

Furthermore, we show that in Twist+/- mice FGFR distribution is altered, being localised in an ectopic location in the mid-sutural mesenchyme. We have also demonstrated that, in the wild-type suture, Twist is expressed at this location, while Fgfr2 is found here with only weak intensity. Conversely, Fgfr is intensely expressed at the osteogenic front, in differentiating osteoblasts, where Twist is not; i.e., when Twist is reduced, FGFR alters. These findings, coupled with the knowledge that the TWIST craniosynostosis phenotype is thought to be due to a loss-of-function, and the FGFR craniosynostosis phenotype due to a gain-of-function mechanism, lead us to speculate that Twist’s role is to inhibit the terminal differentiation of osteoprogenitors into osteoblasts.

**Integration of Twist and Id in FGF-mediated signalling during osteogenesis**

In this paper, we have demonstrated, in vivo, that Twist and Id are expressed by osteoprogenitors but not by mature osteoblasts. We propose that FGFs have functions at several stages of osteoblast differentiation. FGF2 has both inhibitory and stimulatory effects on osteoblast activity and we present evidence that the inhibitory effects may be via a Twist regulated pathway. In line with Twist having a negative regulatory effect on osteoblast differentiation, the TWIST mutation causing craniosynostosis is thought to be a loss-of-function mutation (El Ghouzzi et al., 1997). Also, we show that Twist regulates FGFR, with Twist+/- mice exhibiting altered...
FGFR protein expression. Thus, Twist would appear to be upstream of FGF/FGF signalling, though whether it is inhibitory or stimulatory cannot yet be definitively concluded. FGF may also act at a later stage in osteoblast differentiation, with both excess FGF and overactivation of FGF receptors causing an acceleration of suture closure (Carlton et al., 1998; Kim et al., 1998; Neilson and Friesel, 1995).

It is known that Id inhibits bHLH factors such as Twist (Pesce and Beneazzi, 1993), also that BMP2 induces osteoblast maturation (Katagiri et al., 1994). Here we show that BMP2 stimulates Id and we therefore postulate that the effects of BMP2 on osteoblast differentiation may be via Id’s inhibition of Twist, thereby promoting cell differentiation instead of proliferation. However, it is known that overexpression of Id decreases the activity of the osteocalcin promoter (Tamura and Id, 1995). Abnormal bone growth and axial organization. Dev. Biol. 13, 3045-3057.


