

Msx2 and *Twist* cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault

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Summary

The flat bones of the vertebrate skull vault develop from two migratory mesenchymal cell populations, the cranial neural crest and paraxial mesoderm. At the onset of skull vault development, these mesenchymal cells emigrate from their sites of origin to positions between the ectoderm and the developing cerebral hemispheres. There they combine, proliferate and differentiate along an osteogenic pathway. Anomalies in skull vault development are relatively common in humans. One such anomaly is familial calvarial foramina, persistent unossified areas within the skull vault. Mutations in *MSX2* and *TWIST* are known to cause calvarial foramina in humans. Little is known of the cellular and developmental processes underlying this defect. Neither is it known whether *MSX2* and *TWIST* function in the same or distinct pathways. We trace the origin of the calvarial foramen defect in *Msx2* mutant mice to a group of skeletogenic mesenchyme cells that compose the frontal bone rudiment. We show that this cell

population is reduced not because of apoptosis or deficient migration of neural crest-derived precursor cells, but because of defects in its differentiation and proliferation. We demonstrate, in addition, that heterozygous loss of *Twist* function causes a foramen in the skull vault similar to that caused by loss of *Msx2* function. Both the quantity and proliferation of the frontal bone skeletogenic mesenchyme are reduced in *Msx2-Twist* double mutants compared with individual mutants. Thus *Msx2* and *Twist* cooperate in the control of the differentiation and proliferation of skeletogenic mesenchyme. Molecular epistasis analysis suggests that *Msx2* and *Twist* do not act in tandem to control osteoblast differentiation, but function at the same epistatic level.

Key words: Skull vault, Calvarial foramina, *Msx2*, *Twist*, Neural crest, Mouse

Introduction

How migratory mesenchymal cells produce patterned structures is a key question in animal development. A compelling example of such a patterning process is the development of the mammalian skull vault. Comprising the frontal, parietal and squamosal bones, the skull vault develops from mesenchyme of neural crest and mesodermal origin (Couly et al., 1993; Jiang et al., 2002). These cells migrate to sites overlying the cerebral hemispheres where, in response to signals from the underlying neural tissue (Schowing, 1968), they coalesce, proliferate and differentiate along an osteogenic pathway. In later stages, the bones of the skull vault are united by sutures, fibrous joints that serve as growth centers and allow the skull vault to grow in concert with the brain (Wilkie, 1997; Cohen and MacLean, 2000; Wilkie and Morriss-Kay, 2001). The morphogenesis of the skull vault thus occurs in two phases, the first comprising the genesis, migration and initial specification of skeletogenic mesenchymal precursor cells, the second the differentiation of the skeletogenic

mesenchyme and the ensuing appositional growth of the bones.

The development of the skull vault is of interest not only because it provides a model of how mesenchymal populations produce patterned structures, but also because of its relevance to human disease (Wilkie, 1997; Cohen and MacLean, 2000; Wilkie and Morriss-Kay, 2001). Anomalies in skull vault development are common in humans, occurring as frequently as 1 per 2500 live births (Cohen and MacLean, 2000). Among these are craniosynostosis and persistent calvarial foramina. Craniosynostosis is the premature fusion of the calvarial bones at the sutures. Persistent calvarial foramina are defects in the ossification of bones of the skull vault. Several genes responsible for one or both of these defects have been identified (Wilkie, 1997; Wilkie and Morriss-Kay, 2001; Ornitz and Marie, 2002). These include FGF receptors 1, 2 and 3 (Jabs et al., 1994; Muenke et al., 1994; Reardon et al., 1994; Meyers et al., 1995), the basic HLH gene, *Twist* (Wilkie, 1997), and the homeobox genes *Msx2* and *Alx4* (Wilkie and

Morriss-Kay, 2001; Wilkie et al., 2001). A gain-of-function mutation in *Msx2* can cause craniosynostosis (Jabs et al., 1993). Heterozygous loss of *Msx2* function results in persistent foramina in the skull vault (Wilkie et al., 2000; Wuyts et al., 2000b). More recently, haploinsufficiency for *Alx4* has been shown to cause calvarial foramina (Wu et al., 2000; Wuyts et al., 2000a; Mavrogiannis et al., 2001). Intriguingly, heterozygous loss of *Twist* function can cause craniosynostosis, and, in some affected individuals, calvarial foramina (el Ghouzzi et al., 1997; Howard et al., 1997; Thompson et al., 1984; Young and Swift, 1985). Thus, in humans, *Twist* is required to prevent premature suture fusion, and for the normal growth of the calvarial bones. Similarly, *Msx2* is required for calvarial bone growth, and, when carrying a gain-of-function (P146H) mutation, can cause fusion of calvarial bones.

Analysis of transgenic mice and targeted mouse mutants has provided results that parallel findings in humans. *Twist* mutant mice have synostosis of the coronal suture (el Ghouzzi et al., 1997; Howard et al., 1997; Carver et al., 2002). Overexpression of *Msx2* under the control of its own promoter or heterologous promoters causes overgrowth of the bones of the skull vault, a phenotype that may mimic the early stages of synostosis (Liu et al., 1999). Similarly, *Msx1* and *Msx2* mutant mice exhibit calvarial foramina (Satokata and Maas, 1994; Satokata et al., 2000).

Beyond the identification of these genes and basic descriptions of their mutant phenotypes in humans and mice, there is little information about their roles in the cellular processes underlying normal skull vault development or the pathogenesis of craniosynostosis and calvarial foramina. Neither is it clear whether these genes function in the same or distinct developmental pathways, an issue of significance not only for understanding basic developmental mechanisms but also in the search for modifier genes in humans that can influence the penetrance and expressivity of developmental anomalies. We investigate both of these issues, focusing on the role of *Msx2* in skull vault development, and a potential interaction between *Msx2* and *Twist*.

We trace the origin of the calvarial foramen defect in *Msx2* mutants to a group of skeletogenic mesenchyme cells that compose the frontal bone anlagen. We show that this cell population is reduced because of defects in differentiation and proliferation – not because of apoptosis or deficient migration of neural crest-derived precursor cells. We demonstrate, in addition, that heterozygous loss of *Twist* function causes a foramen in the skull vault resembling that resulting from heterozygous loss of *Msx2*. This defect is substantially worse in *Msx2-Twist* double heterozygous embryos than in individual heterozygotes. Underlying this worsening of the skull vault defect are further deficiencies in both the differentiation and proliferation of frontal bone skeletogenic mesenchyme. Finally, both genetic and molecular data show that *Msx2* and *Twist* do not function in a simple, linear pathway but rather act in parallel. We suggest that *Msx2*, a target of the BMP pathway (Vainio et al., 1993), and *Twist*, a target of the FGF pathway (Rice et al., 2000), integrate inputs from these two pathways to cooperatively control the differentiation and proliferation of neural crest-derived skeletogenic mesenchyme and thus the patterning of the frontal bone.

Materials and methods

Mouse mutants and genotyping

The *Msx2* mutant was a kind gift of Dr Richard Maas. The mutant was obtained on a BALB/c background and, over the course of the present study, crossed into a C57Bl/6 background. The *Twist* mutant, a kind gift of Dr Richard Behringer, was obtained in a 129 background and, as with the *Msx2* mutant, was crossed into a C57Bl/6 background. Genotyping of *Msx2* and *Twist* mutants was as described (Chen and Behringer, 1995; Satokata et al., 2000).

Histology and immunostaining

To visualize mineralized bone, skullcaps of postnatal day 4 mice were dissected and stained with a solution of Alizarin Red S (50 mg/l in 0.2% KOH) for 48 hours. Skulls were then cleared with glycerol.

For whole-mount histochemical staining for alkaline phosphatase, embryos were fixed in 4% paraformaldehyde in PBS. E12.5 and E14.5 embryo heads were bisected midsagittally, and the brain and associated dura were removed, leaving intact the presumptive calvarial bones and epithelium. In the case of E16.5 embryos, the skin was also removed. The specimens were washed with NTMT (0.1 M NaCl, 0.1 M Tris-HCl pH 9.5, 50 mM MgCl₂, 0.1% Tween20), then stained with NBT and BCIP (Roche). Detection of alkaline phosphatase in tissue sections was carried out as described previously (Liu et al., 1999). Apoptotic cells were detected by means of the TUNEL assay using an In Situ Cell Death Detection Kit (Roche). FITC signals were visualized by confocal microscopy. Osteoclast activity was detected by staining for tartrate-resistant acid phosphatase (TRAP) using the Sigma Acid Phosphatase, Leukocyte Kit (Rajapurohitam et al., 1997).

Analysis of *Wnt1-Cre/R26R* reporter gene expression was carried out largely as described previously (Jiang et al., 2002). β -galactosidase staining was performed on embryo whole mounts or 10 μ m cryosections. In some experiments, whole mount-stained embryos were embedded in paraffin wax, sectioned (6 μ m) and counterstained with Nuclear Fast Red.

For immunostaining of frozen sections, embryos were fixed with 4% paraformaldehyde, embedded in HistoPrep (Fisher Scientific) and sectioned in a cryostat (10 μ m). Immunohistochemistry was performed using Zymed Histostain-SP anti-rabbit and phosphorylated Histone H3 polyclonal antibody (Upstate, 1:200 dilution) according to the manufacturer's instructions.

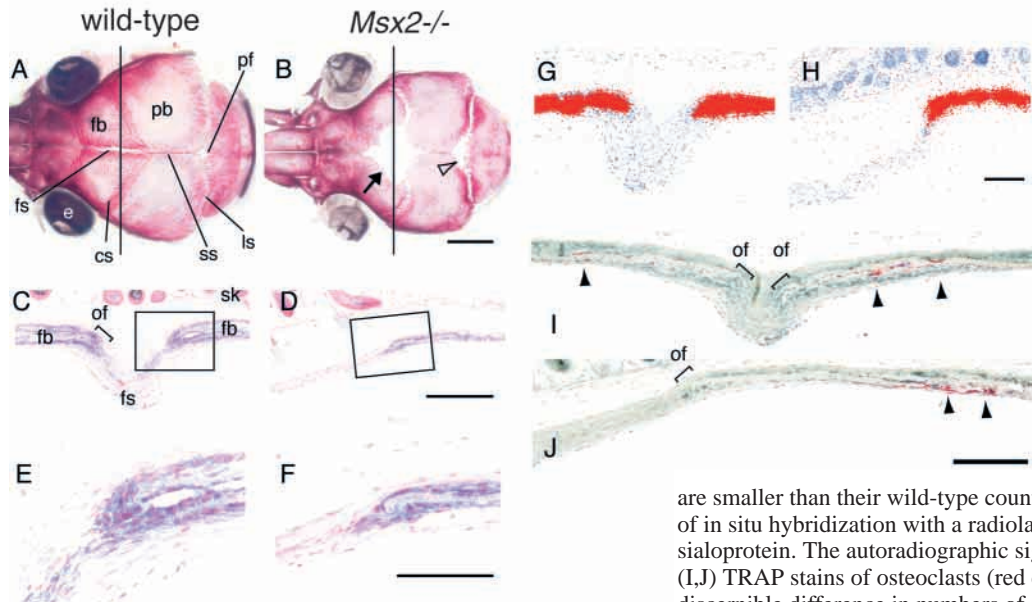
In situ hybridization

A *Twist* 5' cDNA fragment excluding bHLH domain was obtained by *Bam*HI and *Pst*I digestion of CMV-*M-Twist* (Hamamori et al., 1997). This fragment was subcloned into pBluescriptSKII(+) and used as a template for synthesis of riboprobes. The *Msx2* probe, consisting of the entire first exon, was amplified from a full-length mouse cDNA by PCR and cloned into the *Eco*RI and *Sal*I sites of pBSKII(+). In situ hybridization probes for *Runx2* and *Bsp* were as described (Ducy et al., 1997; Rice et al., 1999). Radioactive in situ hybridization was performed as described (Kim et al., 1998; Rice et al., 2000). Whole-mount in situ hybridization was performed as described by Hogan et al. (Hogan et al., 1994).

Results

Restricted domains of osteogenic marker gene expression but normal levels of apoptosis in developing frontal bones of *Msx2* mutant mice

Msx2 mutant mice exhibit an ossification defect in the frontal bone (Satokata et al., 2000) (Fig. 1A,B). To investigate the cellular basis of this defect, we carried out a molecular marker analysis (Figs 1, 2). We assessed the activity of the osteoblast differentiation marker, alkaline phosphatase (ALP), the expression of which commences in preosteoblasts and



wild type (I). cs, coronal suture; e, eye; fb, frontal bone; fs, frontal suture; ls, lambdoid suture; of, osteogenic front; pb, parietal bone; pf, parietal foramen; sk, skin; ss, sagittal suture. Scale bars: 2 mm in B; 200 μ m in D,J; 100 μ m in F; 80 μ m in H.

continues in post-proliferative osteoblasts (Aubin et al., 1995). We also examined the expression of the *bone sialoprotein* (*Bsp*) gene, which is expressed predominantly in osteoblasts (Aubin et al., 1995). Cross-sections at the level of the defect showed that the frontal bones of P4 *Msx2*^{-/-} mice were thinner than those of wild-type, littermate controls and osteogenic fronts were smaller (Fig. 1C-F). *Bsp* was expressed in the appropriate spatial domains coincident with mineralizing bone (Fig. 1G,H). An area in which neither marker was expressed was evident along the dorsal midline (Fig. 1D,H), corresponding to the site of the calvarial defect. We note that this unossified region did not contain a population of cells that expressed ALP and not *Bsp*. This finding suggested that a block in the terminal differentiation of osteoblasts was not a likely explanation for the defect. TRAP stains showed no differences in osteoclast activity in the region of the foramen, making it unlikely that a local increase in bone resorption is responsible for the defect (Fig. 1I,J).

To trace the embryological origins of this deficiency in osteogenic cells, we examined marker gene expression in the frontal bone anlagen at successively earlier stages in development (Fig. 2). In whole mounts of E16.5 embryos, the domain of ALP expression was reduced in the prospective frontal bone (Fig. 2A-D; broken lines). Such a reduction was also evident at E14.5 (Fig. 2E-H) and E12.5 (Fig. 2I-L), the earliest stage that ALP expression could be detected. From cross-sections of E12.5 embryos, it was evident that the area of the ALP staining was reduced in frontal bone primordia of mutant embryos relative to littermate controls (Fig. 2M-O). Immunostaining for osteopontin, which, like ALP, is a marker of early-stage osteoblasts, also revealed a reduced domain of expression in the mutant compared with the wild type (data not shown). We also assessed the expression of the osteoblast determinant, *Runx2*, which is required for the transition from skeletogenic precursor cells to osteoblasts and is the earliest known marker of osteoblast differentiation (Ducy et al., 1997;

Fig. 1. Osteoblast and osteoclast marker expression in postnatal *Msx2* mutant mice.

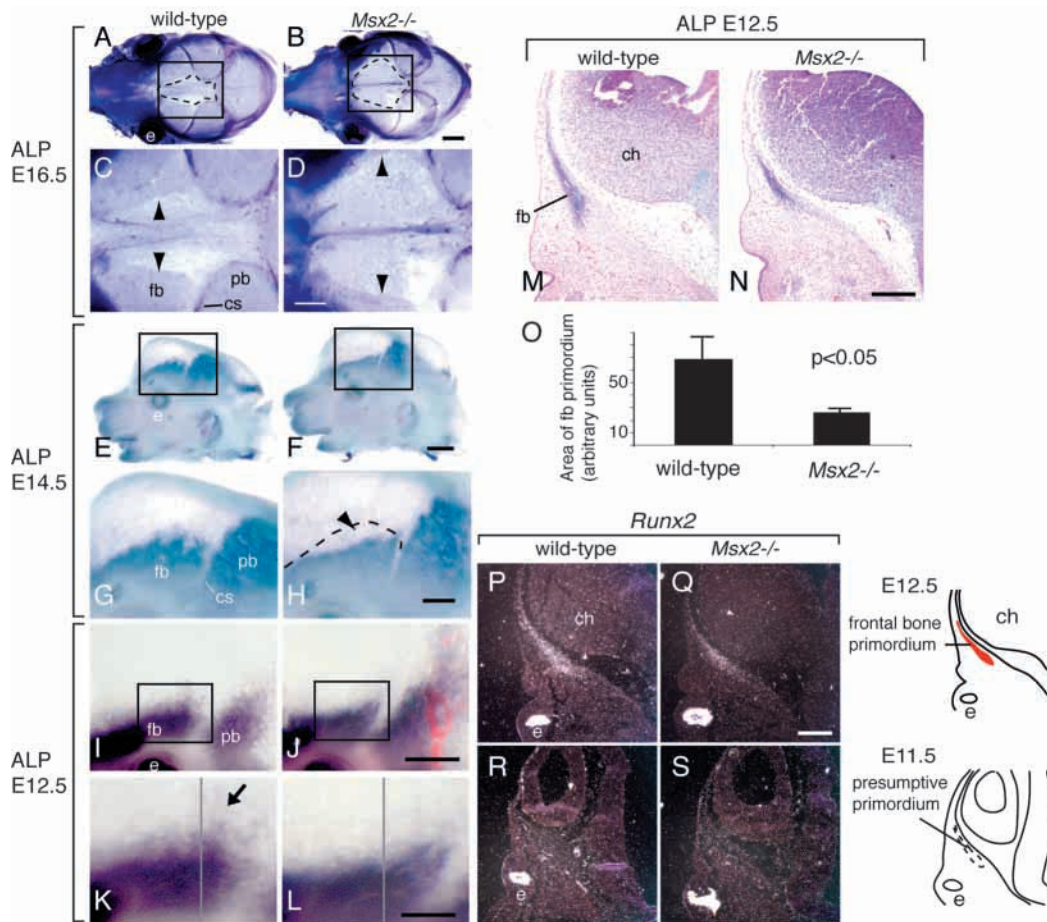
(A,B) Skull vaults of postnatal day 4 mice were stained with Alizarin Red S. Note the large foramen in the frontal bone (fb) of the *Msx2*^{-/-} skull (arrow) and increased parietal foramen (pf, open arrowhead). (C-F) Cross sections, indicated by lines in A and B, at the level of the defect, stained for alkaline phosphatase (ALP) activity. The rectangles in C and D demarcate areas shown at higher magnification in E and F. The osteogenic fronts of *Msx2*^{-/-} mutants (F) are smaller than their wild-type counterparts (E). (G,H) Autoradiograms of in situ hybridization with a radiolabeled (³⁵S) probe against bone sialoprotein. The autoradiographic signal has been transformed to red. (I,J) TRAP stains of osteoclasts (red color, arrowheads). There was no discernible difference in numbers of osteoclasts between mutant (J) and

Komori et al., 1997; Otto et al., 1997; Karsenty, 2001). In situ hybridization revealed that the onset of *Runx2* expression in the frontal bone primordium was between E11.5 and E12.5 (Fig. 2P-S). As was the case for ALP, the domain of *Runx2* expression was consistently reduced in *Msx2* mutants (Fig. 2P,Q).

We next sought to understand why the frontal bone primordia are reduced in size in *Msx2* mutants. One possibility is that cells that compose the primordia undergo apoptosis at an increased rate. We carried out TUNEL assays for apoptotic cells to assess survival of skeletogenic mesenchyme cells in *Msx2* mutants. Examples of such stains are shown in Fig. 3. Relatively few positive cells were evident in frontal bone rudiments of wild-type embryos at E12.5, and no difference was apparent between *Msx2* mutants and wild-type controls. Similar results were obtained E13.5 and postnatal day 4 (data not shown). These data suggest that apoptosis does not contribute significantly to the deficiency of frontal bone osteogenic cells in *Msx2* mutants.

Distribution of neural crest cells in skull vaults of *Msx2* mutants

Beginning at ~E8.5, the frontonasal neural crest migrates from the midbrain and forebrain to a position in the ventral aspect of the developing cerebral hemispheres (Jiang et al., 2002). Subsequently, neural crest cells migrate dorsally, where they form the dura and the skeletogenic mesenchyme of the frontal bone (Jiang et al., 2002). One explanation for the reduction of osteogenic cells in *Msx2* mutants is an anomaly in the neural crest cell population that forms the frontal bone. Neural crest migration, for example, could be impaired in *Msx2* mutants, resulting in fewer cells in the frontal bone rudiment, and thus reduced domains of skeletogenic marker gene expression. We therefore asked whether loss of *Msx2* function influences the contribution of neural crest cells to the frontal bone rudiment. To address this question, we made use of the *Wnt1-Cre/R26R*



(P,R) and *Msx2* mutant (Q,S) embryos. Tissue sections through the prospective frontal bone of E12.5 (P,Q) and E11.5 (R,S) embryos were incubated with a ³³P-labeled *Runx2* antisense probe. The sections were subjected to autoradiography and photographed under dark field. Schematics depicting key anatomical features of the sections are shown on the right. Note that *Runx2* expression is first detectable between E11.5 and E12.5. Note also that the hybridization signal is reduced in the frontal bone rudiment of the *Msx2* mutant compared with the wild type. ch, cerebral hemisphere; cs, coronal suture; e, eye; fb, frontal bone rudiment; pb, parietal bone rudiment. Scale bars: 1 mm in B,F; 500 μ m in D,H,J; 200 μ m in L,N,Q.

neural crest marker system (Soriano, 1999; Chai et al., 2000; Jiang et al., 2000). We produced embryos with the genotype *Msx2*^{-/-}; *Wnt1-Cre*⁺; *R26R*⁺. These embryos were stained for β -galactosidase activity either in whole mount or after they were sectioned (Fig. 4). Control in situ hybridization experiments showed that *Wnt1* expression was not altered in the dorsal neural tubes of E9.5 *Msx2*^{-/-} embryos (data not shown). Thus, any changes in the distribution of *lacZ*-positive cells in the *Msx2*^{-/-}; *Wnt1-Cre*; *R26R* mice are not likely to be due to effects on *Wnt1* promoter activity.

As illustrated in Fig. 4, the distribution of *lacZ*-positive cells was not detectably altered in E9.5 (Fig. 4A,B) or E11.5 (Fig. 4C,D) embryos. Moreover, at E12.5, when the deficiency in ALP-positive cells in the frontal bone rudiment was clearly evident, there was no apparent difference in the neural crest population composing the frontal bone rudiment (Fig. 4E,F). In some embryos, there was a transient deficiency in *lacZ*-positive cells populating the dorsal aspect of the cerebral hemispheres (Fig. 4G-J). However, by E16.5, this deficiency was not detectable (Fig. 4K-N). At the newborn stage, a normal complement of *lacZ*-positive cells was evident within the area of the foramen (data not shown). No deficiency in neural crest

cells composing the dura was apparent at any stage (data not shown). These data argue against gross effects on neural crest distribution as the cause of the deficiency in frontal bone osteogenic cells.

Reduced proliferation of osteogenic cells in the frontal bone rudiment of *Msx2* mutants

Data presented thus far suggest that *Msx2* mutants have a defect in the transition of undifferentiated neural crest-derived mesenchyme to *Runx2*-expressing skeletogenic mesenchyme. Previous studies established that forced expression of *Mx* genes can promote cell proliferation (Dodig et al., 1999; Hu et al., 2001), and that inactivation of *Msx2* results in reduced proliferation of osteoblastic cells in metopic sutures of postnatal *Msx2* mutant mice (Satokata et al., 2000). These studies raise the possibility that, in addition to defects in the differentiation of the frontal bone skeletogenic mesenchyme, *Msx2* mutants may also have defects in the proliferation of this cell population. To test this idea, we monitored a phosphorylated form of histone H3, a marker of M phase. Phosphorylation of histone H3 at serine 10 accompanies and is required for chromosome condensation during mitosis

Fig. 2. Restricted domains of alkaline phosphatase and *Runx2* expression in *Msx2* mutant embryos. We examined alkaline phosphatase (ALP) activity and *Runx2* expression in the developing frontal bones of *Msx2* mutant and control embryos. (A-L) Whole-mount ALP stains of heads at the indicated stages. (A-D) Dorsal views; (E-L) Lateral views. Boxed areas are shown at higher magnification in the panels below. Note the reduced area of ALP staining in mutant embryos (C,D arrowheads; H, arrowhead, broken line). The arrow in K indicates a halo of ALP-positive cells which is not present in the *Msx2* mutant (L). (M,N) Cross-sections at the levels indicated by lines in K and L. The area of ALP staining in three mutant and three wild-type individuals was measured by quantitating pixels (O). Error bars indicate one standard deviation. The wild-type and *Msx2*^{-/-} data sets were compared using Student's *t*-test. (P-S) In situ hybridization analysis of *Runx2* expression in wild type

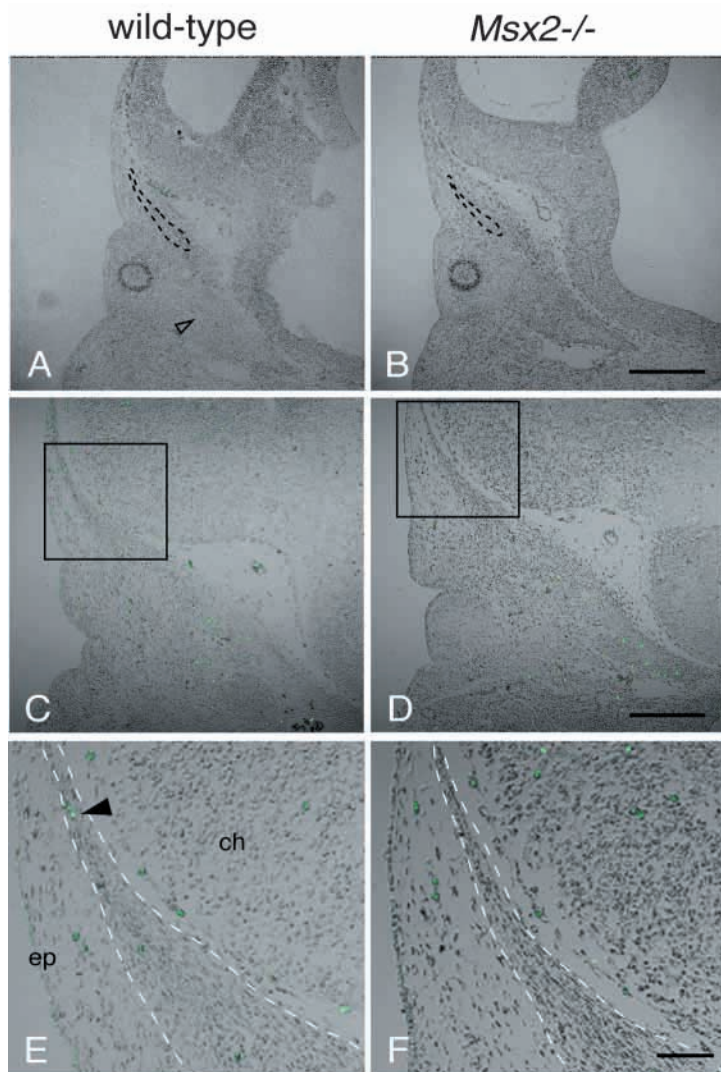


Fig. 3. Normal levels of apoptosis in frontal bone rudiments of *Msx2* mutant embryos. We performed TUNEL stains on cross-sections through the presumptive frontal bone of *Msx2*^{-/-} and wild-type littermate embryos at E11.5 (A,B) and E12.5 (C-F). The broken lines indicate the approximate area occupied by the frontal bone skeletogenic mesenchyme. The squares in C,D demarcate areas shown at higher magnification in E and F. Note that at E11.5, no TUNEL-positive cells (green) were evident in the presumptive frontal bones of either mutant or wild-type embryos. A few positive cells were evident elsewhere in the section (e.g. arrowhead). At E12.5, there were near-equal numbers of apoptotic cells in frontal bone mesenchyme (stained green, arrowheads) of mutant and wild-type embryos. ep, epithelium; ch, cerebral hemisphere. Scale bars: 200 μ m in B,D; 50 μ m in F.

Msx2 and *Twist* cooperate in frontal bone development

We next sought to identify genes that interact with *Msx2* to cause the calvarial foramen phenotype. Our approach was to take advantage of human genetic studies that have led to the discovery of genes which, when mutated, produce phenotypes similar to those elicited by *Msx2* mutations. One such gene is *Twist*. Haploinsufficiency of *Twist* causes Saethre-Chotzen syndrome (el Ghouzzi et al., 1997; Howard et al., 1997), characterized by craniosynostosis, and, in some affected individuals, calvarial foramina (Thompson et al., 1984; Young and Swift, 1985).

We examined *Twist* mutant mice to determine whether they exhibited a defect in frontal bone development similar to that of *Msx2* mutant mice and individuals with Saethre-Chotzen syndrome. Mice homozygous for a null mutation in *Twist* die at E11 with vascular and neural tube defects (Chen and Behringer, 1995). *Twist*^{+/-} mice are viable and, like individuals with Saethre-Chotzen syndrome, have craniosynostosis (el Ghouzzi et al., 1997; Carver et al., 2002). Such mice also have anterior digit duplications (Bourgeois et al., 1998). Alizarin Red staining of skulls of P4 *Twist*^{+/-} mice revealed a subtle but highly penetrant defect in the posterior region of the frontal bone (Fig. 6A,D).

The similarity of the *Twist* foramen phenotype to that of *Msx2* mutant mice, together with the finding that *Twist* and *Msx2* are coexpressed in the developing calvarial bones and sutures (Kim et al., 1998; Rice et al., 1999; Rice et al., 2000), suggested that *Twist* and *Msx2* might cooperate in frontal bone development. If this were the case, then the frontal foramen phenotype should become worse in *Msx2-Twist* double mutants compared with individual mutants. Consistent with this expectation, the frontal bone defect was approximately threefold larger in double heterozygous *Msx2*^{+/-}; *Twist*^{+/-} animals than in *Msx2*^{+/-} or *Twist*^{+/-} mice (Fig. 6G). Intriguingly, the frequency of coronal synostosis in *Twist*^{+/-} mice appeared to be reduced, an observation that will be the subject of another report (data not shown).

Msx2 and *Twist* are also co-expressed in developing limb (Coelho et al., 1991; Davidson et al., 1991; Robert et al., 1991; Fuchtbauer, 1995; Stoetzel et al., 1995), prompting us to ask whether the *Msx2* genotype affected the penetrance of the anterior digit duplication characteristic of *Twist*^{+/-} mice (Bourgeois et al., 1998). The incidence of the digit duplication phenotype in *Twist*^{+/-} mice (34%, *n*=58) was identical to that in *Msx2*^{+/-};

(Henzel et al., 1997). Using an antibody specific for serine 10-phosphorylated H3, we assessed proliferation within the population of ALP-expressing cells composing the frontal bone rudiment. We focused on an interval spanning the stage at which the deficiency in the differentiation of skeletogenic mesenchyme was first evident (Fig. 5). This interval was prior to the appearance of a morphologically distinct osteogenic front, defined as a discreet population of proliferative osteogenic cells located at the edge of the growing bone. At E12.5, we did not detect a difference between mutant and wild-type mice in the number of 10-phosphorylated H3 positive cells per unit area of ALP expression (Fig. 5A-E). However, by E14.5, a significant difference was evident (Fig. 5F-J). These results suggest (1) that *Msx2* mutants have a defect in the proliferation of the skeletogenic mesenchyme, and (2) that this proliferation defect occurs after the defect in the differentiation of the frontal bone skeletogenic mesenchyme. Together, our results show that loss of *Msx2* function results in a decrease in the proportion of proliferative skeletogenic mesenchyme cells within the frontal bone rudiment between E12.5 and E14.5. *Msx2* therefore is required for the differentiation and subsequent proliferation of the skeletogenic mesenchyme.

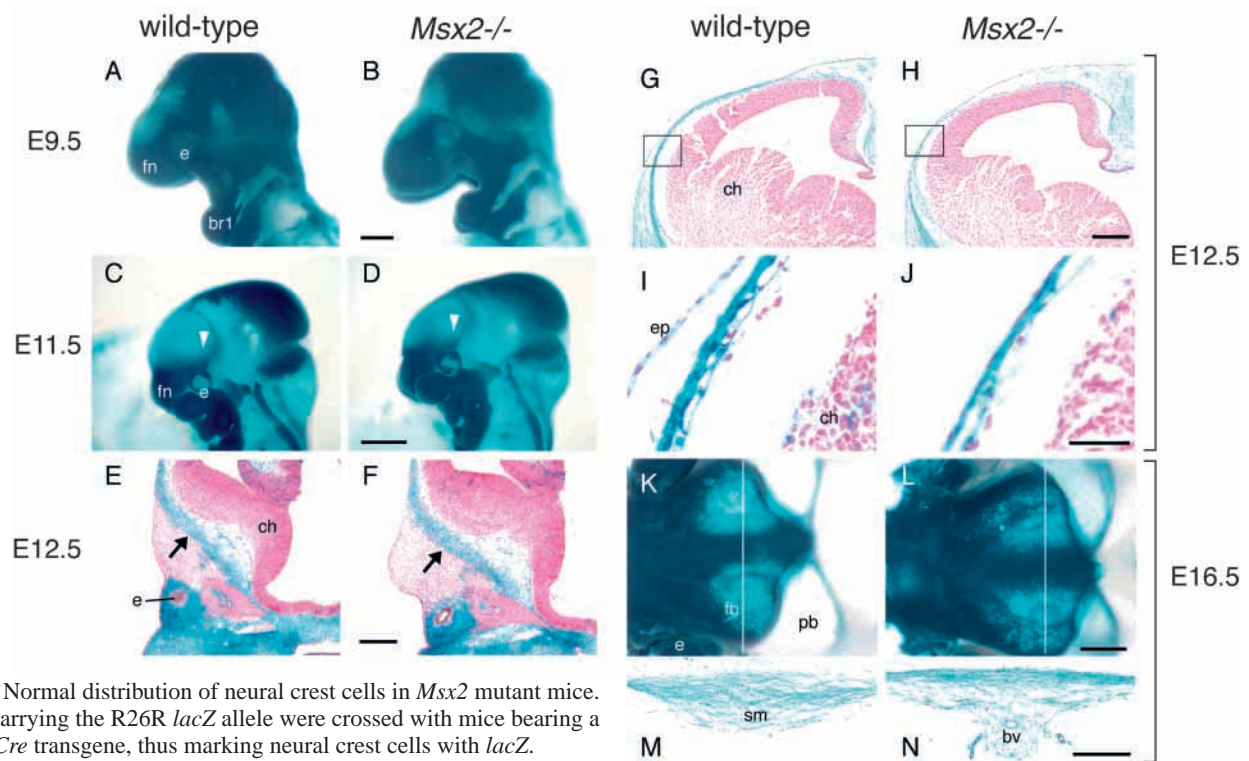


Fig. 4. Normal distribution of neural crest cells in *Msx2* mutant mice. Mice carrying the R26R *lacZ* allele were crossed with mice bearing a *Wnt1-Cre* transgene, thus marking neural crest cells with *lacZ*. Embryos at the indicated developmental stages were stained for β -galactosidase activity and the stain visualized in wholemount or cross section. (E,F) Frozen sections, (G-J,M,N) paraffin sections. Enlarged views of boxed areas in G and H are shown in I and J. Lines in K and L indicate the level of sections shown in M and N. No significant difference in neural crest distribution was evident at E9.5 (A,B). At both E11.5 and E12.5, there was no difference in the density or distribution of *lacZ*-positive cells in the area of the frontal bone rudiment (C,D, arrowheads; E,F, arrows). In some E12.5 embryos there was a delay in the migration of neural crest dorsally (compare G and I with H and J). By E16.5, no difference was apparent (compare K and M with L and N). br1, first branchial arch; bv, blood vessel; ch, cerebral hemisphere; e, eye; ep, epithelium; fb, frontal bone; fn, fronto-nasal process; pb, parietal bone; sm, sutural mesenchyme. Scale bars: 200 μ m in B,F,H,N; 1 mm in D,L; 50 μ m in J.

Twist^{+/-} mice (34%, *n*=109). Thus, *Msx2* and *Twist* did not interact genetically in the developing limb, despite being co-expressed there.

***Msx2* and *Twist* cooperatively control the differentiation and proliferation of the frontal bone skeletogenic mesenchyme**

We knew from our analysis of *Msx2* mutant embryos that defects in the differentiation and proliferation of the skeletogenic mesenchyme were associated with the frontal foramen. We sought to determine whether a decrease in *Twist* dose specifically made these defects more severe. As is evident in Fig. 7, the *Msx2*^{+/-}; *Twist*^{+/-} genotype resulted in further reductions over *Msx2* individual mutants in the domains of ALP staining and *Runx2* expression in the frontal bone rudiment of E12.5 embryos (Fig. 7A-D). *Wnt1-Cre/R26R* analysis at E12.5 showed no difference in neural crest distribution in *Msx2-Twist* compound mutants compared with individual *Msx2* mutants (Fig. 7E,F). Nor was there an increase in numbers of apoptotic cells in *Msx2-Twist* double mutants (data not shown).

We next asked whether *Msx2* and *Twist* cooperatively influence proliferation. Immunostaining of phosphorylated H3 showed that the number of proliferative cells within the frontal bone rudiment was reduced significantly in double heterozygotes compared with *Msx2*^{+/-} (Fig. 7G-K). Taken together, our results thus suggest that the increased severity of

the frontal bone phenotype in *Msx2-Twist* double mutants compared with *Msx2* individual mutants is caused specifically by a cooperative interaction between *Msx2* and *Twist* in the differentiation and proliferation of the osteogenic cell population composing the frontal bone rudiment.

We sought to understand the molecular mechanism by which *Msx2* and *Twist* cooperate in the control of the development of the skeletogenic mesenchyme (Fig. 8). An initial question was whether their function in a simple linear pathway, in which one controls the other's expression. In situ hybridization showed that at E10.5, *Msx2* and *Twist* are co-expressed in presumptive frontal bone mesenchyme (Fig. 8A,G). By E11.5, *Twist* is expressed broadly in the frontonasal neural crest, while *Msx2* is expressed in a band of cells representing the primordia of the frontal and parietal bones (Fig. 8C,I). At E12.5, *Msx2* and *Twist* are co-expressed in the preosteogenic and osteogenic mesenchyme of the frontal bone (Fig. 8E,K). Thus, in principle either gene could regulate the expression of the other. However, in situ hybridization experiments showed that at E10.5, E11.5 and E12.5, the distribution of *Msx2* transcripts was not significantly different in *Twist*^{+/-} mice than in controls (Fig. 8A-F; data not shown). Similarly, analysis of *Twist* expression in *Msx2* mutant embryos showed no apparent change in E10.5, E11.5 and E12.5 embryos (Fig. 8G-L). These data suggest that, within the limits of resolution of in situ hybridization, *Msx2* and

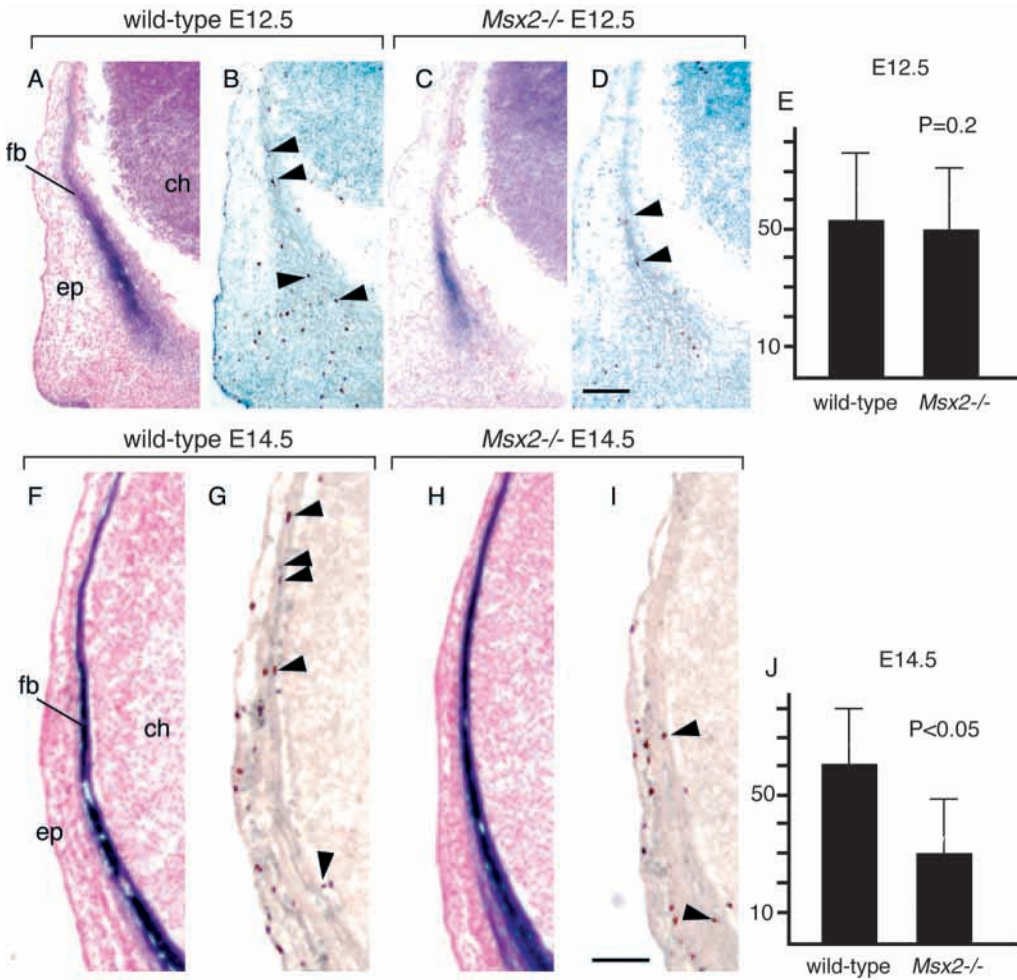
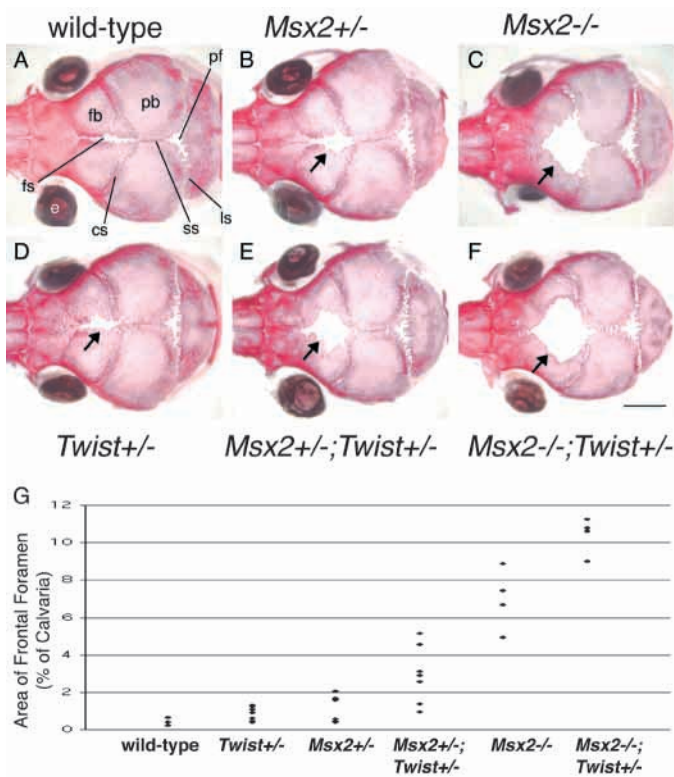


Fig. 5. Reduced proliferation of osteogenic cells in the frontal bone rudiment of *Msx2* mutant mice. An antibody against serine 10-phosphorylated histone H3 (P-H3) was used to stain mitotic cells in sections of embryos in the area of the frontal bone rudiment at E12.5 (A-D) and E14.5 (F-I). Adjacent sections were stained for alkaline phosphatase activity. (A,C,F,H) Alkaline phosphatase stain; (B,D,G,I) P-H3 stain. P-H3-positive cells within the ALP stained area (arrowheads) were counted, and the counts normalized to the area of the ALP-stained region. (E,J) Plots of these normalized values. Error bars are standard deviations derived from three independent experiments. Note that at E12.5, P-H3 labeling indices of mutant and wild-type embryos were similar. At E14.5, a statistically significant (Student's *t*-test) reduction of labeling index was evident in mutant embryos. ch, cerebral hemisphere; fb, frontal bone; ep, epithelium. Scale bars: 100 μ m.



Twist do not regulate each other's activity at the level of mRNA abundance.

Discussion

Transcription factors often act in combination to produce specific biological effects. That humans with loss-of-function mutations in *Msx2* exhibit skull vault ossification defects, and that similar defects occur, with reduced penetrance, in individuals with haploinsufficiency for *Twist* prompted us to ask whether *Msx2* and *Twist* cooperate in skull vault development. Here, using mice with targeted mutations in *Msx2* and *Twist*, we show that this is indeed the case. We demonstrated previously that *Msx2* mutant mice have a defect in skull vault development that resembles a defect in humans

Fig. 6. *Msx2* and *Twist* cooperatively control the patterning of the frontal bone. (A-F) Skulls of mice with the indicated genotypes were taken at postnatal day 4. Mineralized bone was stained with Alizarin Red S. (G) Measurements of the areas of frontal foramina obtained from several individuals of each genotype. Note that the defect (arrows, B-F) is more severe in *Msx2*-*Twist* double heterozygotes than in either individual heterozygote, indicating a cooperative interaction between *Msx2* and *Twist* in the control of frontal bone development. cs, coronal suture; fb, frontal bone; fs, frontal suture; ls, lambdoid suture; pb, parietal bone; pf, parietal foramen; ss, sagittal suture. Scale bar: 2 mm.

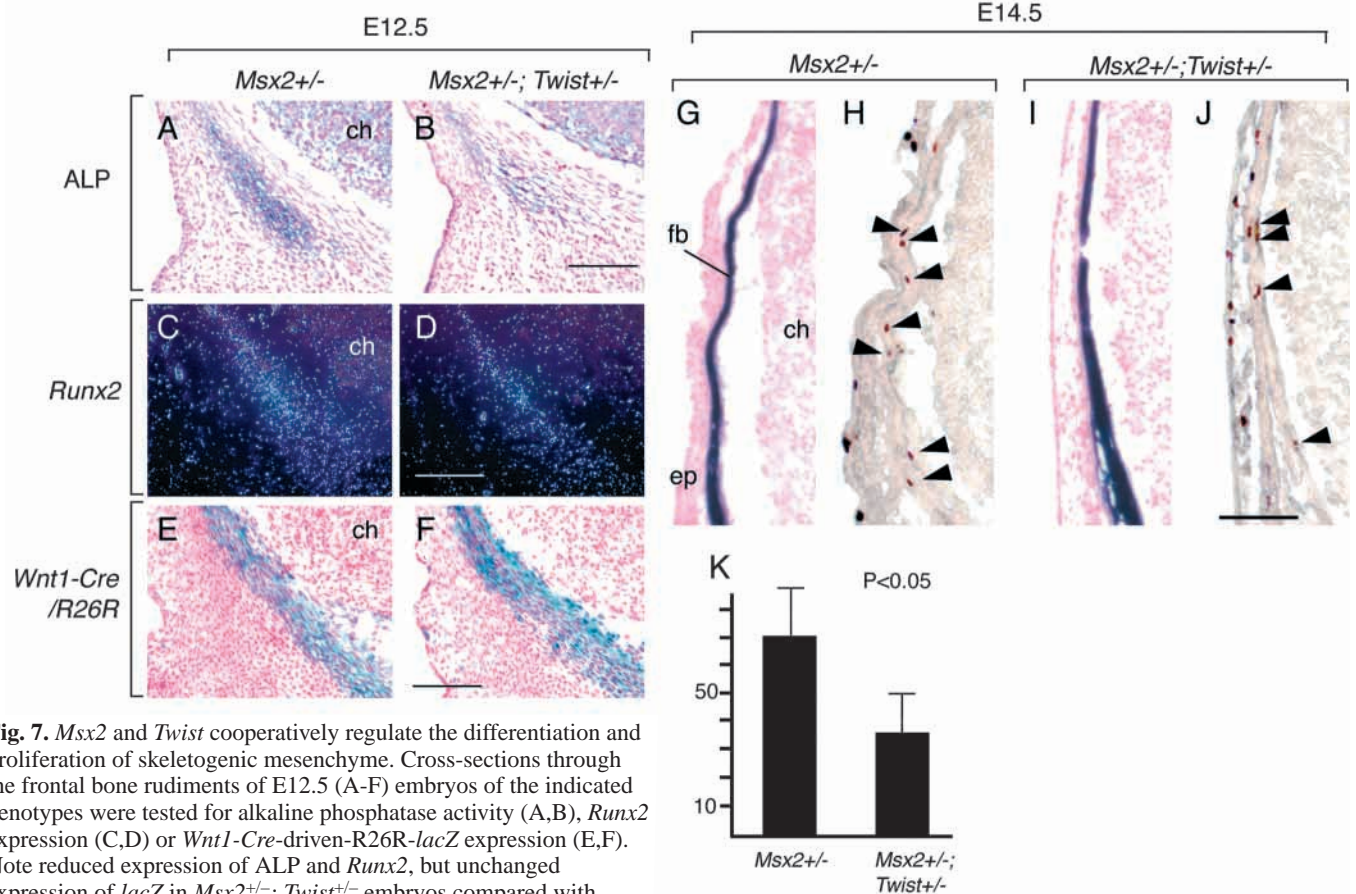


Fig. 7. *Msx2* and *Twist* cooperatively regulate the differentiation and proliferation of skeletogenic mesenchyme. Cross-sections through the frontal bone rudiments of E12.5 (A-F) embryos of the indicated genotypes were tested for alkaline phosphatase activity (A,B), *Runx2* expression (C,D) or *Wnt1-Cre*-driven-R26R-*lacZ* expression (E,F). Note reduced expression of ALP and *Runx2*, but unchanged expression of *lacZ* in *Msx2*^{+/-}; *Twist*^{+/-} embryos compared with *Msx2*^{+/-} embryos. (G-J) Cross-sections of E14.5 frontal bones stained for ALP activity (G,I) and adjacent sections stained for 10-phosphorylated histone H3 (H,J) to mark proliferating cells. P-H3-positive cells (arrowheads) were counted in the regions of developing frontal bones shown in H and J. The resultant counts were normalized to the area of ALP-staining shown in G and I, and are plotted in K. Error bars represent standard deviations derived from three independent experiments. Note the statistically significant (Student's *t*-test) reduction of labeling index in *Msx2*^{+/-}; *Twist*^{+/-} mutant embryos compared with *Msx2*^{+/-} embryos. ch, cerebral hemisphere; ep, epithelium; fb, frontal bone. Scale bars: 100 μ m.

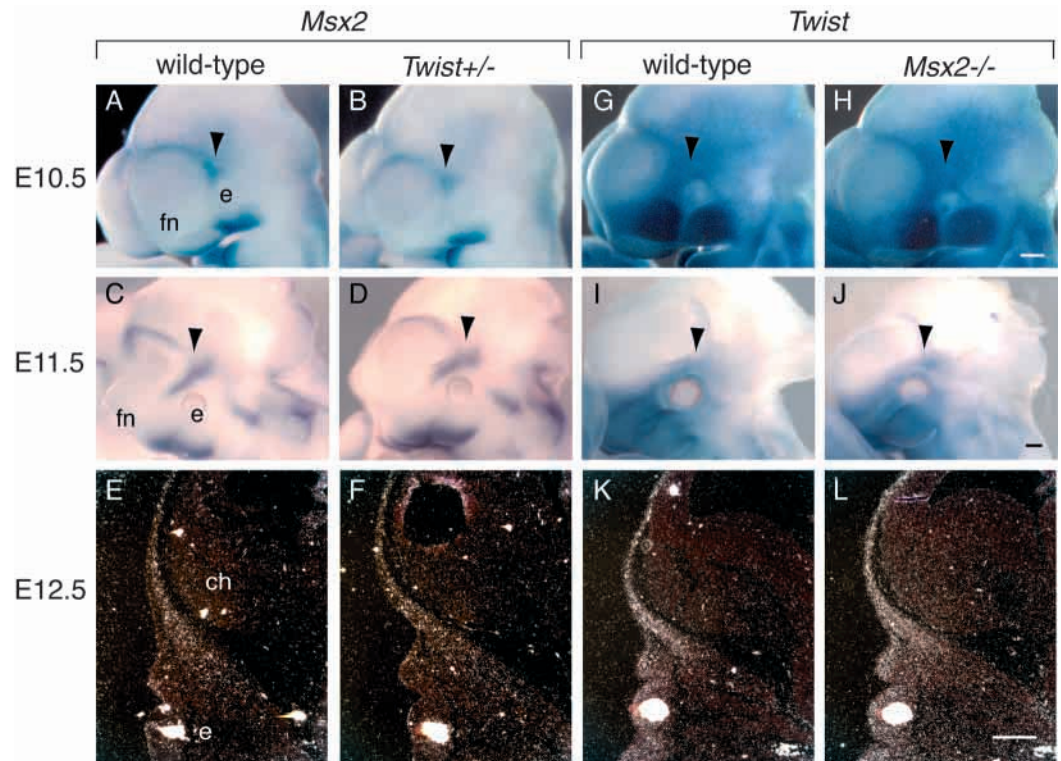
with *Msx2* mutations. Tracing this defect back in embryogenesis, we show that its earliest detectable manifestation is in a population neural crest-derived skeletogenic mesenchyme that composes the frontal bone anlagen in the E12.5 embryo. Although neural crest precursor cells are present in normal numbers in the frontal bone anlagen, fewer such cells express osteoblastic markers, and by E14.5, proliferation is decreased within this population of cells. A reduction in *Twist* gene dosage in combination with the *Msx2* mutation causes this defect to become substantially more severe, demonstrating that *Msx2* and *Twist* cooperatively control the differentiation and proliferation of the skeletogenic mesenchyme. Cross-regulatory influences on *Msx2* and *Twist* transcript levels were not evident at E12.5, suggesting that *Msx2* and *Twist* do not act in tandem to control the frontal bone osteoblast differentiation, but function at the same epistatic level.

***Msx2* is required at or before E12.5 for the development of the frontal bone skeletogenic mesenchyme from neural crest precursors**

Several studies have linked *Msx* genes to the control of the timing of cellular differentiation and proliferation (Woloshin et al., 1995; Liu et al., 1999; Odelberg et al., 2000; Hu et al.,

2001). Virally mediated overexpression of *Msx2* in chick primary calvarial osteoblasts caused an increase in proliferation and slowed differentiation (Dodig et al., 1999). Reciprocally, expression of antisense *Msx2* mRNA caused more rapid differentiation and a reduction of proliferation (Dodig et al., 1999). Abate-Shen and colleagues found that overexpression of *Msx1* or *Msx2* in several different cell lines inhibits differentiation and causes upregulation of cyclin D1 (Hu et al., 2001). We showed, in addition, that transgenic overexpression of *Msx2* causes an increase in the number of proliferative osteoblasts in the osteogenic front of postnatal mice (Liu et al., 1999). These studies have led to the view that a normal function of *Msx2* is to maintain cells in an undifferentiated proliferative state. Accordingly, we would expect calvarial osteogenic cells of *Msx2* mutant mice to exhibit both premature differentiation and reduced proliferation. Our findings differ from this expectation in that we did not see any evidence of premature differentiation at any stage. Furthermore, although we did see an effect on proliferation at later stages (E14.5 (Fig. 5) and newborn (Satokata et al., 2000)), no such effect was evident at E12.5. Thus, the proliferation of osteogenic cells was sensitive to *Msx2* dosage only at later stages.

Fig. 8. Analysis of regulatory interactions between *Msx2* and *Twist* in the developing frontal bone. In situ hybridization was used to assess *Msx2* expression in *Twist*^{+/-} mutant embryos and *Twist* expression in *Msx2*^{-/-} embryos. (A-F) *Msx2* probe hybridized with wild-type and *Twist* mutant heads. (G-L) *Twist* probe hybridized with wild-type and *Msx2* mutant heads. (A-D,G-J) Embryo wholemounts at E10.5 and E11.5 hybridized with digoxigenin-labeled probes. Arrowheads point to the site of the frontal bone primordium. (E,F,K,L) In situ hybridization of radiolabeled (³³P) probes with tissue sections through frontal bone rudiments at E12.5. Note apparent lack of change in *Msx2* expression in *Twist* mutant, and *Twist* expression in *Msx2* mutant at E10.5, E11.5 and E12.5. ch, cerebral hemisphere; e, eye; fn, frontonasal process. Scale bars: 200 μm.



That knockdown of *Msx2* activity causes premature differentiation of cultured osteoblasts, although targeted inactivation of *Msx2* in the mouse has no such effect may be due to a difference in the behavior of osteogenic cells derived from late-stage embryos versus early embryos used in our study. Alternatively, this discrepancy could be a consequence of a selection during the isolation of calvarial osteoblasts. *Msx2* may function differently in the subpopulation of osteogenic cells placed in culture than in osteogenic cells *in vivo*.

In addition to its effect on the proliferation of the skeletogenic mesenchyme, *Msx2*, in principle, could influence the number of skeletogenic mesenchyme cells through effects on the survival of such cells or their neural crest precursors. However, TUNEL assays at several developmental stages from E12.5 to postnatal day 4 failed to provide evidence for increased apoptosis in *Msx2* mutants, arguing against the idea that effect on cell survival contributes significantly to the frontal bone defect.

In *Msx2* mutants, a reduction in the expression domain of *Runx2* is evident as early as E12.5, the earliest that *Runx2* expression could be detected. Changes in ALP activity, another early marker of osteoblasts, are also apparent at E12.5. These differences in the number of cells expressing early osteoblast marker genes could result from a defect in either the specification or differentiation of the skeletogenic mesenchyme. They could also result from defective migration of the subpopulation of neural crest precursor cells that ultimately give rise to the frontal bone anlagen. As markers specific for this precursor cell population are not available, we could not follow the development of cells allocated to the skeletogenic mesenchyme prior to E12.5. However, we were able to examine the influence of *Msx2* genotype on neural crest in general by means of the *Wnt1-Cre/R26R* system.

Analysis of the distribution of neural crest in embryos from E9.5 to newborn led to two important conclusions. First, at E12.5 neural crest cells are present in frontal bone anlagen of *Msx2* mutants in normal numbers. Second, by E16.5, prospective frontal bone and frontal suture neural crest lineage cells are distributed normally in *Msx2* mutants. These data argue against gross defects in neural crest as a cause of the frontal bone defect. That there is a normal number of neural crest cells in mutants at E12.5, but a reduction in ALP expressing cells at that same stage, is consistent with a defect in the differentiation of osteogenic cells from neural crest precursors. One caveat in this interpretation is that because the *Wnt1-Cre/R26R* system marks all neural crest cells, our data do not exclude mis-migration or mis-specification events. Such events might result, for example, in a mixed population of neural crest cells in the frontal bone rudiment, including some not competent to differentiate into osteoblasts. Our data also cannot exclude an effect on the proliferation of a small subpopulation of neural crest cells as the cause of the reduction in the number of ALP-positive cells at E12.5.

Despite these caveats, it is interesting that the apparent requirement for *Msx2* in the differentiation of the frontal bone skeletogenic mesenchyme parallels findings on the function of the *msh* gene of *Drosophila*. Isshiki, Nose and colleagues (Isshiki et al., 1997; Nose et al., 1998) have shown that *msh* is required for the development subsets of dorsal neuroblasts and muscle progenitors. *msh* does not participate in the initial specification of these cells, but in the realization of the differentiated phenotype. *Msx2* may play an analogous role in the development of the frontal bone skeletogenic mesenchyme, controlling its differentiation into osteogenic cells. We note, however, that *Msx1/Msx2* double mutant embryos have profound defects in neural crest-derived craniofacial structures,

leaving open the possibility that *Msx1* and *Msx2* may be required together for earlier events in neural crest specification or migration (Satokata et al., 2000) (M.I. and R.E.M., unpublished).

Msx2 is likely to have a role in osteogenic cell populations outside the frontal bone skeletogenic mesenchyme. Satokata et al. (Satokata et al., 2000) documented a deficiency in long bone osteoblasts in *Msx2* mutants. In addition, *Msx2* mutant mice have a subtle defect in the parietal bone (Figs 1, 6; data not shown). It is interesting that calvarial defects in humans with heterozygous loss of *Msx2* function are usually located in the parietal bone (Wilkie et al., 2000). This difference between mice and humans in the relative severity of the parietal and frontal bone defects may reflect a species difference in the *Msx* gene dosage requirements for the development of different calvarial bones.

***Msx2* and *Twist* cooperatively control the differentiation and proliferation of the frontal bone skeletogenic mesenchyme**

To understand the biological significance of the genetic interaction between *Msx2* and *Twist*, it was important to know whether the two genes affect frontal bone patterning through the same development processes. That reduction of *Msx2* or *Twist* gene dosage specifically exacerbated the defects in the differentiation and proliferation of skeletogenic mesenchyme cells composing the frontal bone rudiment is significant: it narrows the possible cooperative functions of *Msx2* and *Twist* to the same development interval and the same set of processes. Although our data do not address the molecular pathways through which *Msx2* and *Twist* exert their effects, they do enable us to conclude that *Msx2* and *Twist* function cooperatively in the processes of differentiation and proliferation.

We note that at E12.5, reduced *Twist* dosage, with or without an *Msx2* mutation, did not affect levels of apoptosis. This is perhaps surprising in light of findings that *Twist* can protect against Myc-induced apoptosis in Rat1 cells (Maestro et al., 1999), and that cultured calvarial osteoblasts derived from the fused coronal suture of an individual with Saethre-Chotzen syndrome with a heterozygous loss of function in *Twist* exhibit increased sensitivity to Tnf-induced apoptosis (Yousfi et al., 2002). This difference in the apoptotic behavior of cultured osteogenic cells compared with frontal bone skeletogenic mesenchyme, *in vivo*, may reflect differences between conditions in cell culture versus those in the whole animal, or the potentially distinct embryological origins of coronal suture osteoblasts from individuals with Saethre-Chotzen syndrome (mixed mesoderm and neural crest) versus frontal bone osteogenic cells (exclusively neural crest).

Tam and colleagues (Soo et al., 2002) have shown that *Twist*^{-/-} embryos exhibit defects in cranial neural crest migration. Transplantation experiments demonstrated that *Twist* is required both in neural crest cells and in paraxial mesoderm for the guidance of migrating neural crest cells. In addition, *Twist* is required for neural crest differentiation, whereas in wild-type embryos, *Sox10* expression is downregulated progressively in migratory neural crest cells, in mutant embryos it is maintained at a high level, suggesting that neural crest cells may be arrested at an early stage of differentiation (Soo et al., 2002).

Our analysis shows that in both *Twist*^{+/-} and in *Msx2-Twist* double mutant embryos, cranial neural crest cells are distributed normally, at least as assessed by the *Wnt1-Cre/R26R* marker. That Runx2/ALP-expressing cells are reduced in *Msx2-Twist* mutants, while neural crest cells populate the frontal bone anlagen in normal numbers suggests that *Msx2* and *Twist* cooperate in the differentiation of the skeletogenic mesenchyme from neural crest precursors. Whether the differentiation of neural crest cells is arrested at an early stage in *Msx2-Twist* double mutants as it apparently is in *Twist*^{-/-} embryos remains to be established. Our data also demonstrate that presumptive frontal bone cells of E14.5 *Msx2-Twist* double heterozygous embryos proliferate at a lower rate than corresponding cells of *Msx2*^{+/-} embryos. This result is consistent with findings linking forced expression of *Twist* to uncontrolled proliferation (Maestro et al., 1999; Gullaud et al., 2003; Pajer et al., 2003).

Genetic and molecular data provide some insight into the nature of the cooperation between *Msx2* and *Twist*. That the foramen was significantly larger in *Msx2*^{-/-};*Twist*^{+/-} mice than in *Msx2*^{-/-} mice argues against a linear pathway in which one gene is an obligate downstream effector of the other. This view is also supported by *in situ* hybridization data on E12.5 embryos showing no evidence of crossregulatory interactions between *Msx2* and *Twist* at the mRNA level (Fig. 8). *Msx2* and *Twist* may thus control proliferation and differentiation of skeletogenic mesenchyme through parallel pathways. It remains possible, however, that *Msx2* and *Twist* interact on a level other than transcription. Consistent with such a possibility are observations that *Msx2* and *Twist* proteins are capable of interacting both *in vitro* and in intact cells (Y. Hamamori and R.E.M., unpublished), and that such interactions have been documented for other homeodomain and bHLH proteins (Knoepfler et al., 1999; Poulin et al., 2000). Although we do not know whether *Msx2* and *Twist* interact in developing embryos, or, if so, whether this interaction is significant functionally, the idea of a direct, cooperative protein-protein interaction does provide a molecular-level hypothesis to explain the cooperativity between *Msx2* and *Twist* in skull vault development. We note that *Twist* has been shown to inhibit acetyltransferase activities of p300 and PCAF through interactions with their HAT domains (Hamamori et al., 1999). In addition, *Msx* proteins can serve as transcriptional repressors (Zhang et al., 1996; Newberry et al., 1997), and, through interactions with MINT/SHARP, may recruit histone deacetylases (Newberry et al., 1999; Shi et al., 2001; Oswald et al., 2002). It is thus possible that *Msx2* and *Twist* cooperatively repress one or more genes whose downregulation is required for the differentiation of frontal bone skeletogenic mesenchyme.

Msx2 and *Twist* are both targets of morphogen pathways. During suture development, *Twist* integrates inputs from the BMP and FGF pathways (Rice et al., 2000). *Msx2* is an immediate-early gene in the BMP2/4 pathway (Hollnagel et al., 1999). We suggest that in the frontal bone anlagen, *Msx2* and *Twist* act as a nexus for BMP and FGF signaling, and participate in the control of the identity and/or proliferation of the frontal bone skeletogenic mesenchyme. We envisage a model similar to a regulatory network documented in *Drosophila*, in which *msh*, together with *ladybird* and *even skipped*, regulate the identity of cardiac muscle progenitor cells

(Jagla et al., 2002). Such a combinatorial interaction could maintain stringent control over the differentiation and proliferation of the skeletogenic mesenchyme, and thus serve as part of the mechanism that coordinates the growth of skull with that of the brain. Finally, our results have implications for the pathophysiology of familial parietal foramina, and possibly craniosynostosis. These results predict that in humans, *Twist* activity may influence the penetrance of calvarial defects caused by haploid loss of *Msx2* function. Reciprocally, *Msx2* activity may influence the penetrance of defects resulting from *Twist* mutations. The clinical manifestations in individuals affected with familial parietal foramina and Saethre-Chotzen syndrome may thus depend on the sum of the activity of these two genes.

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