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

Mamoru Ishii1, Amy E. Merrill1, Yan-Shun Chan1, Inna Gitelman2, David P. C. Rice3, Henry M. Sucov1,4 and Robert E. Maxson, Jr1,*

1Department of Biochemistry and Molecular Biology, USC/Norris Comprehensive Cancer Center and Hospital, Keck School of Medicine, University of Southern California, 1441 Eastlake Avenue, Los Angeles, CA 90089-9176, USA
2Department of Morphology, Faculty of Medicine, Ben Gurion University of the Negev, Beer Sheva, Israel
3Department of Craniofacial Development, King’s College, London, UK
4Institute for Genetic Medicine, Keck School of Medicine, University of Southern California, 1441 Eastlake Avenue, Los Angeles, CA 90089-9176, USA

*Author for correspondence (e-mail: maxson@hsc.usc.edu)

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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
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, skulls 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 epiphelium. 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 μm cryosections. In some experiments, whole mount-stained embryos were embedded in paraffin wax, sectioned (6 μm) 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 μm). 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 BamHI and PstI 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 EcoRI and SalI 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
continue 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 unclassified 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; 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
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 Msx 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.
Development and disease

**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\(^{+/−}\);
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 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 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 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 sought to understand the molecular mechanism by which Msx2 and Twist cooperate in the control of the development 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.

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development and disease

Msx2 and Twist in skull vault development

Development and disease

Msx2 and 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. 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 μm.

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.
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.
Mrx2 and Twist in skull vault development

Fig. 8. Analysis of regulatory interactions between Mrx2 and Twist in the developing frontal bone. In situ hybridization was used to assess Mrx2 expression in Twist+/– mutant embryos and Twist expression in Mrx2+/– embryos. (A-F) Mrx2 probe hybridized with wild-type and Twist mutant heads. (G-L) Twist probe hybridized with wild-type and Mrx2 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 Mrx2 expression in Twist mutant, and Twist expression in Mrx2 mutant at E10.5, E11.5 and E12.5. ch, cerebral hemisphere; e, eye; fn, frontonasal process. Scale bars: 200 μm.

That knockdown of Mrx2 activity causes premature differentiation of cultured osteoblasts, although targeted inactivation of Mrx2 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. Mrx2 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, Mrx2, 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 Mrx2 mutants, arguing against the idea that effect on cell survival contributes significantly to the frontal bone defect.

In Mrx2 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 Mrx2 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 Mrx2 mutants in normal numbers. Second, by E16.5, prospective frontal bone and frontal suture neural crest lineage cells are distributed normally in Mrx2 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 Mrx2 in the differentiation of the frontal bone skeletogenic mesenchyme parallels findings on the function of the msh gene of Drosophila. Ishikawa, 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. Mrx2 may play an analogous role in the development of the frontal bone skeletogenic mesenchyme, controlling its differentiation into osteogenic cells. We note, however, that Mrx1/Mrx2 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 crosstalk between 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. In addition, 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.
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(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, we 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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References


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