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Premature suture closure and ectopic cranial bone in mice expressing *Msx2* transgenes in the developing skull

(craniosynostosis/skull development/homeobox/transgenic mice)

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ABSTRACT **The coordinate growth of the brain and skull is achieved through a series of interactions between the developing brain, the growing bones of the skull, and the fibrous joints, or sutures, that unite the bones. These interactions couple the expansion of the brain to the growth of the bony plates at the sutures. Craniosynostosis, the premature fusion of the bones of the skull, is a common birth defect (1 in 3000 live births) that disrupts coordinate growth and often results in profoundly abnormal skull shape. Individuals affected with Boston-type craniosynostosis, an autosomal dominant disorder, bear a mutated copy of *MSX2*, a homeobox gene thought to function in tissue interactions. Here we show that expression of the mouse counterpart of this mutant gene in the developing skulls of transgenic mice causes craniosynostosis and ectopic cranial bone. These mice provide a transgenic model of craniosynostosis as well as a point of entry into the molecular mechanisms that coordinate the growth of the brain and skull.**

Inductive tissue interactions play a central role in vertebrate development. Such interactions are mediated by intercellular signaling molecules, their receptors, and a cascade of signal transducers that modulate the activity of transcription factors (1). Among the transcription factors that are likely to have important roles in inductive tissue interactions are those encoded by the highly conserved Msx-class homeobox genes (2, 3). Extensive analyses of the embryonic expression patterns of *Msx2*, one of three related genes in the mammalian genome, have documented transcripts in various structures whose development is mediated by tissue interactions (4–9); these structures include the developing cranium and the underlying neural tissue that induces it (ref. 10; unpublished data).

Suggesting a direct role for *Msx2* in the tissue interactions that pattern the cranium is our finding that a mutation in the human *MSX2* gene is associated with Boston-type craniosynostosis, an autosomal dominant disorder of skull development (10). Growth of the flat bones at the top of the skull (calvaria) consists of two phases. These bones first grow outward from centers of osteogenesis, eventually uniting in fibrous joints, or sutures; then the calvarial bones grow at the sutures in concert with the expanding brain (11–13). After the growth of the brain is complete, the calvarial bones merge and ultimately fuse. Craniosynostosis, the premature fusion of the calvarial bones at the sutures, is a relatively common birth defect (1 in 3000 live births) with environmental and genetic causes (11–13). It often results in highly abnormal skull shape, sometimes with neurological consequences. Since the initiation and maintenance of sutures depend on interactions with underlying tissues (14, 15), it is likely that the developmental defect that produces Boston-type craniosynostosis lies in the series of interactions that pattern the sutures and maintain them during the growth of the brain.

The Boston-type craniosynostosis mutation is a single base change resulting in the substitution of a histidine for a proline in the N-terminal arm of the *Msx2* homeodomain (10), a region that has been implicated in protein–DNA and protein–protein interactions (16, 17). Although Boston-type craniosynostosis and the associated mutation in *MSX2* have so far been described only in a single family (18), several lines of evidence converge on the view that the *MSX2* mutation is the cause of this disorder. (*i*) *MSX2* is tightly linked to the Boston-type craniosynostosis gene; (*ii*) the mutation is present only in affected individuals; (*iii*) molecular modeling analyses suggest that the substitution of a histidine for the highly conserved proline residue is likely to have significant functional consequences; and (*iv*) *Msx2* is expressed in mesenchymal tissue of presumptive sutures and in the underlying neural tissue that has been shown to regulate cranial suture morphogenesis (10).

To investigate the function of *Msx2* in tissue interactions and in vertebrate craniofacial development, and to further test the hypothesis that a mutation in *Msx2* is the cause of Boston-type craniosynostosis, we engineered the Pro-7 → His (Pro7His) mutation in the mouse *Msx2* gene and expressed the mutant gene in the developing skulls of transgenic mice. We show here that these mice exhibit precocious fusion of cranial bones, thus confirming the link between *Msx2* and Boston-type craniosynostosis and providing a model with which to examine the role of *Msx2* in cranial development.

MATERIALS AND METHODS

***Msx2* Transgene Constructs and the Creation of Transgenic Mice.** To generate the TIMP1-*Msx2*Pro7His transgene, we excised the tissue inhibitor of metalloproteinase 1 (TIMP1) promoter fragment (1.8 kb) from TIMP-lacZ plasmid (the kind gift of B. R. G. Williams, University of Toronto) and inserted it in the *Sal* I/*Hin*dIII site of the pBluescript II SK− (Stratagene) containing a 671-bp *Bam*HI/*Pst* I fragment from pKSV10 (Pharmacia) to provide a poly(A) signal. Into the *Hin*dIII/*Eco*RI sites of this construct, we inserted a 1.1-kb fragment of the mouse *Msx2* gene containing 0.3 kb of the 3.5-kb intron and a PCR-mutagenized version of the second exon bearing the Pro7His mutation. The 3.7-kb transgene fragment was freed from vector sequences by digesting with *Sal* I and *Bst*XI and was gel purified and microinjected into mouse zygotes derived from B6CBA F_1 donors (19). To create cytomegalovirus (CMV) versions of the transgenes, a fragment of immediate early promoter of the human CMV was excised from pRc/CMV (Invitrogen) as a *Sal* I/*Hin*dIII fragment and ligated to compatible sites on pBluescript II SK−. A 671-bp

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Abbreviations: CMV, cytomegalovirus; TIMP1, tissue inhibitor of metalloproteinase 1; FGF, fibroblast growth factor.

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*Bam*HI/*Pst* I fragment from pKSV10 (Pharmacia) was also inserted to supply a poly(A) signal and a transgene-specific probe. A 1.1-kb fragment of the mouse *Msx2* gene with a deletion of intronic sequences (0.3 kb of the 3.5-kb intron remaining) was inserted into the *Hin*dIII/*Eco*RI site to create pCMVMsx2WT. pCMVMsx2Pro7His was created by replacing the second exon of pCMVMsx2WT with a PCR-mutagenized version of the second exon bearing the Pro7His mutation. The 2.5-kb transgene fragments were freed from the vector sequences by digesting the plasmids with *Nru* I and *Bam*HI and were gel purified prior to microinjection into mouse zygotes (19).

***In Situ* Hybridization Analysis of *Msx2* Transgene Expression.** A heterozygous CMVPro7His transgenic F_1 male and a nontransgenic B6CBAF_1/J female were crossed and resultant neonates were genotyped by Southern blot analysis of tail DNA. Heads were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Sections (5 μm) of transgenic and nontransgenic littermates were mounted on the same poly(lysine)-coated slide for hybridization (20). The probe was a 671-bp simian virus 40 sequence that we engineered into the transgene. We generated the hybridization probe by transcription with SP6 RNA polymerase [Ambion (Austin, TX) Megascript kit] in the presence of digoxigenin-labeled UTP. Sections were deparafinized, hydrated, treated with 5 μg of proteinase K per ml, and acetylated. Hybridization was carried out overnight at 50°C at a probe concentration of 1 μg/ml. A final stringent wash was performed in 0.1× SSC at 45°C for 20 min. To detect hybrids formed between the *Msx2* transgene mRNA and the probe, we blocked tissue sections by incubating with 10% heat-inactivated sheep serum for 1 hr. This was followed by overnight incubation at 4°C with alkaline-phosphatase-conjugated anti-digoxigenin antibody in 1% heat-inactivated sheep serum. The color was developed in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 1–5 hr. Sections were counterstained with neutral red.

Analysis of Cranial Phenotypes in *Msx2* Transgenic Mice. Heads were skinned, fixed, and stained in alcian staining/fixing solution (75% ethanol/3.5 M acetic acid/0.01% alcian blue 8GX). They were then destained, rehydrated through an ethanol series, and cleared in 1% KOH in the presence of alizarin red S. Stained specimens were stored in glycerin (21). For histology, alcian/alizarin red-stained skulls were washed with H_2O overnight to remove glycerin and were dehydrated through graded ethanol, infiltrated with xylene and paraffin, and embedded in paraffin. Sections 8 μm thick were cut and stained with hematoxylin/eosin.

RESULTS

The dominant *Msx2*Pro7His mutation could act by eliminating the function of the mutated allele, thus creating a condition of haploinsufficiency (10). Alternatively, it could function as a dominant-interfering mutation, or one that leads to a gain of function as a consequence of either ectopic expression or hypermorphic activity of the mutant protein. If the mutation acts by a dominant-interfering or gain-of-function mechanism, then expressing the mutant *Msx2* gene in transgenic mice should create the mutant phenotype. Accordingly, we introduced the Pro7His mutation into a cloned mouse *Msx2* gene (2) and fused this construct with promoters that would express *Msx2* in mouse embryos in tissues that participate in the development of sutures. Since at the outset we knew of no promoters that would specifically target *Msx2* to such tissues, we chose to use two general promoters, the TIMP1 promoter and the CMV immediate early promoter (Fig. 1), whose domains of expression are known to or are likely to include neural crest-derived mesenchyme that contributes to the calvarial bones and the sutures as well as the underlying central nervous system tissue that presumably induces these structures (22). *In situ* hybridization analysis of TIMP1 expression in embryonic day 14.5 mouse embryos revealed a ubiquitous distribution of transcripts, with particularly high concentrations in osteogenic tissues, including those of the developing calvarium (23). The CMV immediate early promoter is known to be active in various cultured cell types (24, 25), though its activity has not been examined systematically in developing mouse embryos. We confirmed the suitability of this promoter by using a transgene-specific probe to assess CMV-transgene expression in late embryonic and newborn mice. Specific hybridization of this probe was evident in the connective tissue of sutural spaces, in the periosteum, dura mater, intramembranous bone, and brain—tissues that contribute to or interact with developing sutures and adjacent osteogenic tissues (Fig. 2*A*; unpublished observations).

The developmental status of calvarial sutures in control and transgenic mice was first evaluated by staining the skeletal elements of skulls with alizarin red S, providing a simple and accurate means of assessing the degree to which the calvarial bones had invaded the sutural spaces. In a survey of nontransgenic B6CBAF_1/J mice (n = 47), we found that the sutural spaces were filled with bone (i.e., the sutures were closed) between 13 and 17 days postnatal (unpublished observations), with sagittal suture closed at day 13 postnatal and anterior lambdoidal suture closed at day 15 postnatal; accordingly, we defined premature suture closure as obliteration of the sutural space prior to that interval. Analysis of the skulls of 10 independent TIMP1-*Msx2*Pro7His transgenic mice revealed 3

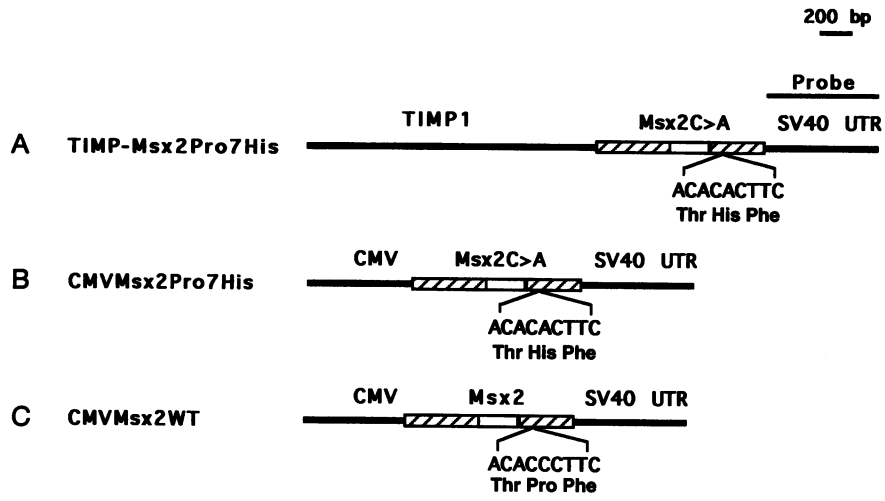

FIG. 1. Structure of *Msx2* transgenes. The *Msx2*Pro7His gene was fused with the TIMP1 promoter to produce TIMP1-*Msx2*Pro7His (*A*) or with the CMV immediate early promoter to produce CMV-*Msx2*Pro7His (*B*). Wild-type *Msx2* was fused with the CMV promoter to generate CMV-*Msx2*WT (*C*). SV40 UTR, simian virus 40 untranslated region.

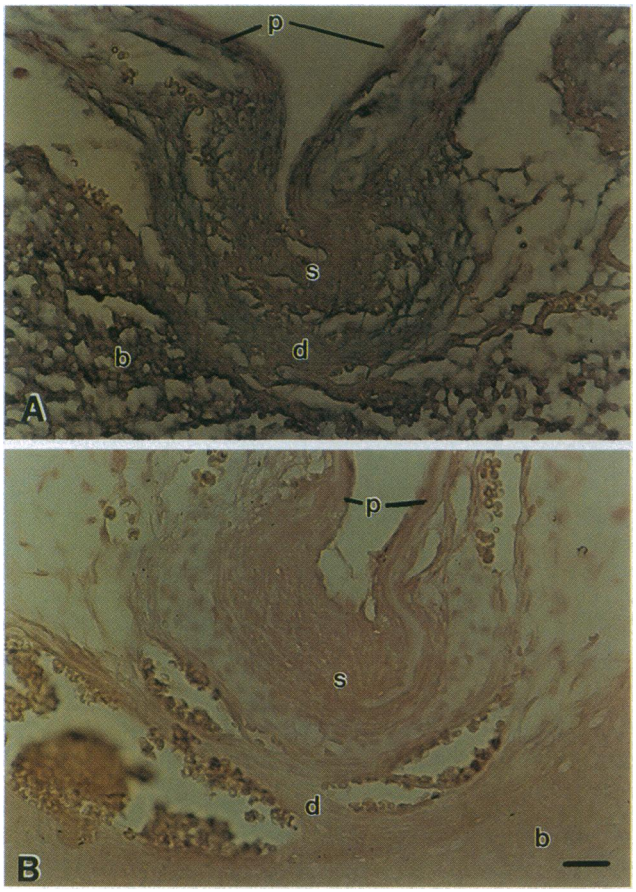

FIG. 2. Expression of the CMV-*Msx2*Pro7His transgene in the calvaria of neonatal mice. (*A*) Hybridization of transgene-specific probe with frontal section through sagittal suture of a neonatal transgenic animal. Note hybridization signal (purple stain) in the periosteum, in the mesenchyme of the sutural space, in the dura, and in neuronal tissue of the brain. (*B*) Control hybridization of probe with frontal section through sagittal suture of a neonatal, nontransgenic littermate. The probe was a 671-bp digoxigenin-labeled antisense RNA derived from sequences of the simian virus 40 untranslated region (SV40 UTR). (Bar = 25 μm.) b, Brain; d, dura mater; p, periosteum; s, sagittal suture.

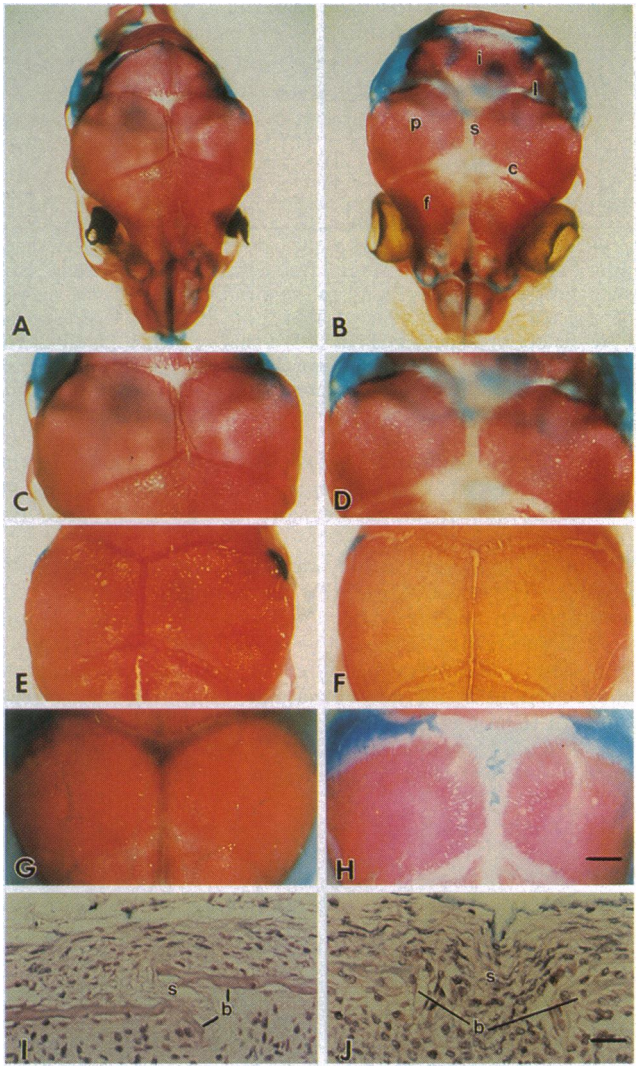

FIG. 3. Premature closure of calvarial sutures in *Msx2* transgenic animals. (*A*) Skull of TIMP1*Msx2*Pro7His F_0 transgenic animal 1 day postnatal. The skull was cleared and stained with alcian blue to reveal cartilage (blue color) and with alizarin red S to reveal mineralized bone (red color). The coronal suture and the sagittal suture were closed, and the lambdoidal suture was partially closed. (*B*) Skull of nontransgenic littermate of animal shown in *A*, 1 day postnatal. Note that all sutures were open. (*C*) Higher magnification of the skull shown in *A*. Note that the gaps (sutural spaces) among bony plates were obliterated by mineralized bone. (*D*) Higher magnification of the skull in *B*. (*E*) Skull of CMV-*Msx2*Pro7His transgenic animal 12 days postnatal. All sutures except the metopic were closed. (*F*) Skull of a nontransgenic littermate 12 days postnatal. All sutures were open, as indicated by narrow gaps among bony plates. (*G*) Skull of CMV-*Msx2*WT transgenic animal. Four days after birth, when this F_0 animal died, all sutures except the metopic were closed. (*H*) Skull of nontransgenic littermate 4 days postnatal. All sutures were open. (*I*) Cross section of the sagittal suture of the skull in *G*. Note the overlap of the parietal bones, obliterating the sutural space. (*J*) Histological section of the sagittal suture of the skull in *H*. Note that the opposing parietal bones were separated by a mesenchymal blastema. (Bar for *A* and *B* = 1 mm, *C* and *D* = 1.5 mm, *E* and *F* = 1.3 mm, *G* and *H* = 0.85 mm, and *I* and *J* = 25 μm.) b, Bone; c, coronal suture; f, frontal bone; i, interparietal bone; l, lambdoidal suture; p, parietal bone; s, sagittal suture.

whose calvarial sutures had closed prior to postnatal day 3 (Fig. 3 *A–D*; Table 1). Moreover, 3 of 11 transgenic lines bearing the CMV-*Msx2*Pro7His transgene exhibited suture closure on or before day 12 (Fig. 3 *E* and *F*; Table 1). These results indicate that overexpression or ectopic expression of the Pro7His mutant gene can create craniosynostosis in mice and thus strongly support the hypothesis that the Pro7His mutation is the cause of Boston-type craniosynostosis.

These data are consistent with either a dominant-interfering or a dominant, gain-of-function mechanism. If the Pro7His mutation acts by a dominant-interfering mechanism, then expression of the wild-type *Msx2* gene should produce no phenotype or a different phenotype from that caused by the mutant gene. If, on the other hand, the Pro7His mutation acts by a dominant, gain-of-function mechanism, then CMV-directed expression of the wild-type *Msx2* gene should mimic the effect of the mutation and produce the craniosynostosis phenotype. Analysis of seven transgenic lines bearing the CMV-*Msx2*WT transgene showed that five exhibited a suture-closure phenotype very similar to that caused by the mutant *Msx2* gene while two had no phenotype (Fig. 3 *G* and *H*; Table 1). Histological sections across the sagittal sutures of these mice revealed that the mesenchymal blastema of the suture was largely absent, and the parietal bones had overlapped, confirming suture closure (Fig. 3 *I* and *J*). Closure of sutures was also confirmed histologically for selected TIMP1-*Msx2*-Pro7His transgenic mice (data not shown). Taken together with our finding that the *MSX2*Pro7His mutation is associated with Boston-type craniosynostosis in humans, these data are consistent with the view that in mice and in humans, *Msx2*Pro7His is a dominant, gain-of-function mutation. Our

Table 1. Summary of transgenic mice with craniosynostosis phenotype

Transgene	No. of independent transgenics	No. with craniosynostosis	% with craniosynostosis
TIMP1-*Msx2*Pro7His	10	3	30
CMV-*Msx2*Pro7His	11	3	27
CMV-*Msx2*WT	7	5	71

We defined craniosynostosis as the obliteration of the sutural space prior to postnatal day 14. Sutural fusion was scored by alizarin red S staining of whole skulls (Fig. 3) and confirmed by histological analysis of selected skulls. All affected TIMP1-*Msx2*Pro7His F_0 transgenic mice died prior to postnatal day 3. We analyzed CMV-*Msx2*Pro7His and CMV-*Msx2*WT transgenic animals directly or as F_1 progeny of outcrosses between F_0 transgenic animals and nontransgenic B6CBAF_1/J mice.

results do not distinguish between enhanced activity of the *Msx2* gene product versus ectopic expression of the *Msx2* gene as mechanisms by which the *Msx2* transgenes produce the craniosynostosis phenotype.

In addition to prematurely closed sutures, skulls of CMV-*Msx2*Pro7His and CMV-*Msx2*WT exhibited a small patch of ectopic bone centered above the posterior portion of the sagittal suture just anterior to the lambdoid suture (Fig. 4). This bone was normal histologically and had mesenchymal condensations at the lateral margins, indicating that it grew outward from the midline.

The *Msx2* transgenic mice also exhibited several noncranial phenotypes, including anomalies in the skin, eye, and ear

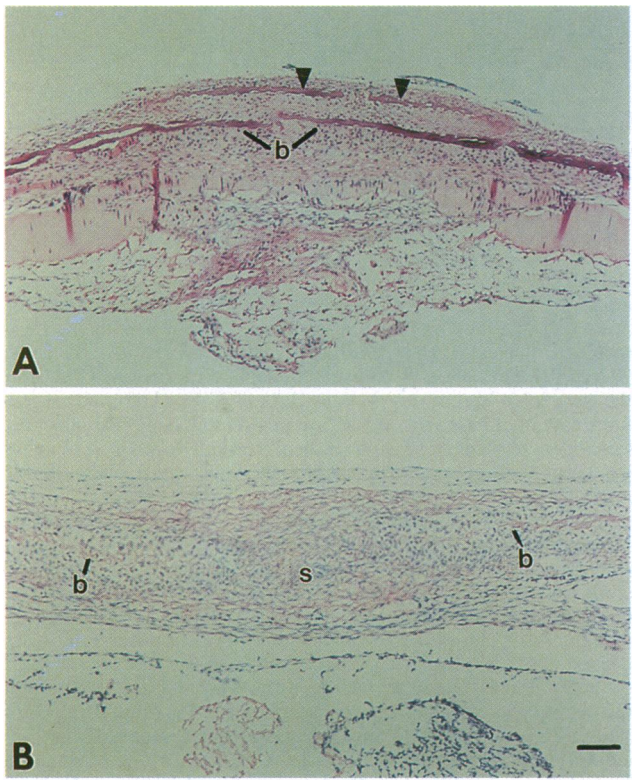

FIG. 4. Ectopic cranial bone in *Msx2* transgenic animals. (*A* and *B*) Cross section of sagittal suture in CMV-*Msx2*WT transgenic mouse (*A*) and age-matched control mouse (*B*) 4 days postnatal. Skulls were stained with alcian blue and alizarin red S as in the legend to Fig. 3. Histological sections were subsequently prepared and stained with hematoxylin/eosin. Note the advanced state of cranial bone growth and the overlapping parietal bones in the CMV-*Msx2* transgenic skull shown in *A* compared to the control (*B*). Note also a segment of ectopic bone centered above the sagittal suture in *A* (arrowheads). (Bar = 100 μm.) b, Parietal bone; s, sagittal suture.

(unpublished observations), each of which is a site of endogenous *Msx2* expression (26, 27). Overexpression of *Msx2* thus creates a spectrum of developmental defects in tissues whose development depends on epithelial–mesenchymal interactions.

DISCUSSION

The shape of the skull is critically dependent on the rate and geometry of cranial bone growth and on the timing of suture closure (11–13); skull shape may also be influenced by biomechanical forces generated by the expanding brain as it pushes against the skull (11). Changes in the relative timing of these morphogenetic processes can have dramatic structural consequences. For example, craniosynostosis, the premature fusion of sutures, often results in profoundly abnormal skull shape (11). We demonstrated previously that individuals affected with an autosomal dominant form of craniosynostosis known as Boston-type carry a Pro7His mutation in the *MSX2* gene (10); here we have shown that the expression of the Pro7His mutant *Msx2* gene in the developing mouse cranium causes premature suture closure. Taken together, these data provide strong support for the view that the mutation in human *MSX2* is the cause of Boston-type craniosynostosis and suggest that this gene has a role in the temporal regulation of suture closure.

That the *Msx2*Pro7His transgene causes the craniosynostosis phenotype demonstrates that the Pro7His mutation does not inactivate Msx2 but probably modifies its activity in some way. The mutation could produce a dominant-interfering activity, though our finding that the wild-type *Msx2* gene is able to elicit premature suture closure in transgenic mice virtually as efficiently as the Pro7His mutant *Msx2* gene argues against this possibility. Alternatively, the mutation could cause a gain of function. For example, it could stabilize the Msx2 protein, leading to overexpression or ectopic expression, or it could enhance the regulatory activity of Msx2 in some way. We stress, however, that our data do not provide positive evidence that the Pro7His mutation causes a gain of function in Msx2; confirmation of such a mechanism must await a demonstration of the craniosynostosis phenotype in a mouse bearing a targeted Pro7His mutation in one copy of the endogenous *Msx2* gene.

In humans, the craniosynostosis trait is present in all individuals that carry the Pro7His mutation and is thus fully penetrant (28). In the *Msx2* transgenic mice, in contrast, only about 30% of the *Msx2*Pro7His transgenic lines exhibited this phenotype. A likely explanation for this difference in incidence between humans and mice is that the majority of transgenic mouse lines express the *Msx2* transgene at a level below a critical threshold value and consequently do not show the craniosynostosis phenotype. However, we have not yet measured levels of transgene expression in different mouse lines; therefore, we cannot distinguish this explanation from one that postulates a difference between humans and mice in the molecular-developmental processes of suture development and a consequent difference in the penetrance of the craniosynostosis trait.

The developmental processes affected in the *Msx2* transgenic mice and in individuals with Boston-type craniosynostosis may include the migration of cranial neural crest cells that form the calvarial bones and the mesenchyme of the sutural spaces (29), the initial specification of these cells through interactions with the underlying neural tissue (22), or later influences on their growth and differentiation within the developing suture (15). It is interesting that tissue recombination and organ culture experiments have shown that a soluble, heparin-binding factor emitted by the dura mater, a thin layer of tissue between the brain and the skull, prevents the calvarial bones from growing into the sutural space (30).

Expression of *Msx2* transgenes in mice or expression of *MSX2*Pro7His in humans affected with Boston-type craniosynostosis may abrogate this negative signal and thus permit the inappropriate growth of the calvarial bones.

Our finding that ectopic bone is present above the fused sagittal suture in the *Msx2* transgenic mice demonstrates clearly that the expression of *Msx2* transgenes can cause inappropriate calvarial bone growth. Such ectopic bone can be elicited by CMV- and TIMP1-induced expression of *Msx2* and by mutant and wild-type *Msx2* genes. Although information on the pathogenesis of skull defects in Boston-type craniosynostosis is limited (18), ectopic bone is not a typical finding in craniosynostotic syndromes in general (11–13). This apparent difference between the human syndrome and the *Msx2* transgenic mice may be a consequence of the focal expression of the endogenous human *MSX2* gene in cranial sutures as compared with the more general expression patterns of the TIMP1 and CMV promoters that were used to direct expression of the *Msx2* transgenes in mice (23, 31). Arguing against this hypothesis is the result of a recent experiment in which we made use of the *Msx2* promoter (32) to direct expression of *Msx2* transgenes in mice. This promoter construct, driving either the wild-type or the Pro7His *Msx2* gene, produced an ectopic bone phenotype virtually identical to the one obtained with the CMV and TIMP1 promoters (unpublished data). Like the endogenous *Msx2* gene, this construct is expressed in a highly localized manner in the developing cranium. Thus, generalized, ectopic expression of the *Msx2* transgenes seems less likely than overexpression as an explanation of the ectopic bone phenotype.

What are the molecular mechanisms by which the human *MSX2*Pro7His and the mouse *Msx2* transgenes affect cranial development? Two sets of results provide indirect support for the view that growth factor-mediated signaling pathways may be involved. (*i*) *Msx2* expression in the oral epithelium and in the hindbrain can be modulated by the implantation of beads soaked in BMP-4 (33, 34). BMP-4 is a transforming growth factor β-like signaling ligand with functions in various processes, including bone growth (35). (*ii*) Crouzon syndrome, Jackson–Weiss syndrome, Apert syndrome, and Pfeiffer syndrome, 4 of $\approx$100 genetic syndromes in which craniosynostosis is a feature (11, 36), are caused by mutations in the extracellular domain of the fibroblast growth factor (FGF) receptor 2 gene (37–40). FGF receptor 2 is coexpressed with *Msx2* in various sites in the developing mouse embryo (41, 42), and a direct role for FGF receptors in determining the competence of a tissue to respond to a signaling molecule has been established by experiments in *Xenopus* showing that a dominant negative mutant FGF receptor interferes with inductive processes that determine posterior mesodermal fate (43). We suggest that FGF receptor 2 and *Msx2* may function together, perhaps in the same epistatic pathway, to regulate the temporal sequence of suture development and thus coordinate the growth of the skull with that of the brain. The *Msx2*-expressing transgenic mice that we describe here will provide a useful tool with which to address this hypothesis.

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