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Proliferative Osteogenic Cells in Growth Centers of the Developing Murine Skull: A Possible Mechanism for *MSX2*-Mediated Craniosynostosis in Humans

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Throughout its complex morphogenesis, the vertebrate skull must at once protect the brain and expand to accommodate its growth. A key structural adaptation that allows this dual role is the separation of the bony plates of the skull with sutures, fibrous joints that serve as growth centers and allow the calvarial bones to expand as the brain enlarges. Craniosynostosis, the premature fusion of one or more calvarial bones with consequent abnormalities in skull shape, is a common developmental anomaly that disrupts this process. We found previously that a single amino acid substitution in the homeodomain of the human *MSX2* gene is associated with the autosomal dominant disorder craniosynostosis, Boston type. This mutation enhances the affinity of *Msx2* for its target sequence, suggesting that the mutation acts by a dominant positive mechanism. Consistent with this prediction, we showed that general overexpression of *Msx2* under the control of the broadly expressed CMV promoter causes the calvarial bones to invade the sagittal suture. Here we use tissue-specific overexpression of *Msx2* within the calvarial sutures to address the developmental mechanisms of craniosynostosis and skull morphogenesis. We demonstrate that a segment of the *Msx2* promoter directs reporter gene expression to subsets of cells within the sutures. In late embryonic and neonatal stages, this promoter is expressed in undifferentiated mesenchymal cells medial to the growing bone. By P4, promoter activity is reduced in the suture, exhibiting a punctate pattern in undifferentiated osteoblastic cells in the outer margin of the osteogenic front. Overexpression of *Msx2* under the control of this promoter is sufficient to enhance parietal bone growth into the sagittal suture by P6. This phenotype is preceded by an increase in both the number and the BrdU labeling of osteoblastic cells in the osteogenic fronts of the calvarial bones. These findings suggest that an important early event in *MSX2*-mediated craniosynostosis in humans is a transient retardation of osteogenic cell differentiation in the suture and a consequent increase in the pool of osteogenic cells.

INTRODUCTION

The vertebrate skull is a complex structure that must function as a rigid, protective barrier and at the same time adjust to the expansion of the developing brain. These dual requirements are met by the separation of the bony plates of the skull with sutures, fibrous joints that serve as growth centers and allow the calvarial bones to expand as the brain enlarges (Cohen, 1993). The growth of the calvarial bones begins as mesenchymal precursor cells of neural crest origin condense at specific sites on the dorsal surface of the head and synthesize bone matrix (Cohen, 1993; Couly *et al.*, 1993). These condensates extend radially outward, eventu-

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ally meeting at the presumptive sutures. Growth then occurs at osteogenic fronts, aggregations of osteoblastic cells located at the lateral margins of the sutures, in concert with the growth of the brain. Ablation and transplantation experiments have shown that the bones and sutures of the brain case are patterned by the underlying neural tissue (Benoit and Schowing, 1970). In the final stages of skull morphogenesis, the dura mater, a three-layered membrane of neural crest origin lying between the brain and the skull, can inhibit the growth of bone into the sutures (Opperman *et al.*, 1993, 1995).

The molecular mechanisms of skull patterning and morphogenesis are poorly understood. A variety of signaling molecules are known to be expressed in the tissues involved in the patterning of the calvarial bones (Davidson, 1995; Wilkie, 1997), though the developmental functions of these genes remain unclear. *Fgf9* is expressed in the developing suture, as is the bek isoform of *Fgfr2* (Kim *et al.*, 1998). *Bmp2* and *Bmp4* are expressed in the osteogenic front and *Bmp4* in the dura mater and sutural mesenchyme (Kim *et al.*, 1998). Manipulation of Fgf ligand concentrations in the murine suture by implantation of Fgf2- or Fgf4-impregnated beads can cause both proliferation and differentiation of osteogenic cells (Iseki *et al.*, 1997; Kim *et al.*, 1998). Implantation of Bmp4-impregnated beads boosts the number of sutural cells, but does not affect the proximity of the calvarial bones (Kim *et al.*, 1998).

Insight into the molecular mechanisms underlying calvarial development has also come from human genetics. Mutations in several genes are known to cause craniosynostosis, the premature fusion of calvarial sutures with consequent abnormalities in skull shape (Wilkie, 1997). A feature in more than 100 genetic syndromes, craniosynostosis occurs in about 1 in 3000 live births (Cohen, 1993; Wilkie, 1997). Apert, Jackson-Weiss, Crouzon, and Pfeiffer syndromes are caused by activating mutations in *FGF* receptor genes 1, 2, and 3 (Wilkie, 1997), consistent with findings that application of FGFs to sutural tissue can stimulate proliferation and differentiation of osteogenic cells. Saethre–Chozen syndrome is caused by loss-of-function mutations in the basic HLH protein twist (Howard *et al.*, 1997; el Ghouzzi, *et al.*, 1997). We showed that a mutation in *MSX2* (P148H), a member of the highly conserved *Msx* homeobox gene family, causes craniosynostosis, Boston type (Jabs *et al.*, 1993), an autosomal dominant disorder affecting a large kindred in the Northeastern United States (Warman *et al.*, 1993; Muller *et al.*, 1993).

Despite the identification of specific genetic defects that cause craniosynostosis syndromes, little is known at the cell and tissue level about the nature of craniosynostosis phenotypes or the developmental mechanisms that underlie them. One general model to account for syndromic craniosynostosis is that the various mutations affect the maturation rate of osteogenic cells within the sutures. Evidence in favor of this hypothesis comes from cell culture studies demonstrating that increased numbers of osteoblastic cells can be recovered from sutures of individuals affected with Apert syndrome (Lomri *et al.*, 1998). Additional support is provided by a recent study showing that forced expression of *Msx* genes in calvarial osteogenic cells retards differentiation, maintaining cells in a proliferative state (Dodig *et al.*, 1998). This finding led to the suggestion that a transient retardation of osteogenic cell differentiation within the suture might lead to an increased pool of proliferative osteogenic cells and ultimately to an increase in calvarial bone growth. However, the extent to which these findings apply to calvarial growth and craniosynostosis *in vivo* has not been established.

To evaluate possible effects on osteogenic cell populations *in vivo,* and more broadly to investigate the signaling pathways underlying skull morphogenesis, we have taken a genetic approach focused on *Msx2*. *Msx2* is one of three related genes in the murine genome (Davidson, 1995). It is expressed at several key sites in the developing skull, including the neural crest population that gives rise to the calvarial bones. It is also expressed in the dura mater and in underlying neural tissue, both of which have been implicated in the signaling events that pattern the skull and sutures (Opperman *et al.*, 1993; Kim *et al.*, 1998). Homologous targeting in mice has shown that *Msx1* and *Msx2* have pleiotropic activities in development, functioning in epithelial mesenchymal interactions that underlie the development of the skull, teeth, eyes, skin, mammary glands, and kidneys (Satokata *et al.*, 1994; Chen *et al.*, 1996; Phippard *et al.*, 1996; R. Maas, personal communication). *Msx* genes increasingly have been linked to the *BMP* pathway (Vainio *et al.*, 1993; Ferrari *et al.*, 1998). Epistasis studies have shown that expression of the *Drosophila msh* gene during neurogenesis is regulated by *Dpp* (D'Alessio *et al.*, 1996). The murine *Msx1* gene is required for *BMP4* autoinduction in the oral mesenchyme (Bei *et al.*, 1995), and in *Xenopus*, *Msx1* can rescue phenotypes induced by a dominant negative BMP receptor (Suzuki *et al.*, 1997).

The dominant nature of the Boston-type craniosynostosis mutation prompted us to use transgenic mice to gain insight into the mechanisms of craniosynostosis as well as the function of *Msx2* in calvarial morphogenesis and BMP signaling. Previously we created transgenic mice in which the p148h mutant *Msx2* gene was overexpressed under the control of a CMV (cytomegalovirus) or TIMP1 (tissue inhibitor of metalloprotease) promoter. Such mice exhibited enhanced calvarial bone growth (Liu *et al.*, 1995). Overexpression/misexpression of wild-type *Msx2* also created this phenotype, consistent with the molecular data suggesting that the p148h mutation acts by a dominant positive mechanism. This phenotype was not characterized in detail at a histological level; thus the cell types affected by enhanced *Msx2* activity were not identified. Also this transgenic model was limited by the rather general expression patterns of the CMV and TIMP promoters, which made it difficult a priori to investigate precisely how overexpression/misexpression of *Msx2* caused anomalous bone growth.

Accordingly, we decided to use the murine *Msx2* pro-

moter to overexpress *Msx2* focally and to use such *Msx2* transgenic mice to investigate the cellular and developmental mechanisms of craniosynostosis. Winograd *et al.* (1997) also used a portion of the human *MSX2* promoter to drive expression of the human *MSX2* gene in transgenic mice. Such mice apparently did not exhibit craniosynostosis; however, they died perinatally with neural tube defects, precluding analysis of postnatal stages.

Here we describe the expression pattern of a murine *Msx2* promoter segment in calvarial development. This promoter directs reporter gene expression to the developing sutures, including the osteogenic fronts of the calvarial bones. We show that unlike the *MSX2* transgene driven by the human *MSX2* promoter, this murine transgene does not result in lethality, thus enabling us to recover stable lines and analyze cranial phenotypes. Overexpression of *Msx2* under the control of this promoter results in enhanced calvarial bone growth. Moreover, this phenotype is associated with an increase in the number of proliferative, early-stage osteoblastic cells in the osteogenic fronts. These data, together with findings of Dodig *et al.* (1998) on the effect of forced expression of *Msx2* on osteogenic cell differentiation, are consistent rather with the view that enhanced expression of *Msx2* transiently inhibits osteoblast differentiation, resulting in an increase in the osteoblast pool and ultimately in an increase in bone growth.

MATERIALS AND METHODS

Transgene Constructs

A *Spe*I–*Not*I fragment, which contains part of the first exon and minimal promoter of the *Msx2* gene, from pλB1.6 was subcloned into pBluescript SK II(−) (Stratagene, La Jolla, CA) to generate pSK-SN. Subsequently, a 5-kb *Xho*I–*Spe*I fragment covering most of the upstream regulatory sequences for the *Msx2* gene was subcloned into the *Spe*I/*Xho*I site of pSK-SN to generate pSK-SNNX. The *Not*I–*Xho*I fragment from pSK-SNNX was then moved into *Not*I/*Xho*I sites of pGem11Zf(−) (Promega, Madison, WI) to make pGem11Zf-NX-#3. To create the coding region for the transgene, a *Bss*HII−*Xho*I fragment covering the entire coding region, intron, and 3′ sequences of the *Msx2* gene was cloned into the *Hin*dIII/*Xho*I sites of pSP73 (Promega) to generate pClone17. A *Sal*I–*Nde*I fragment that contains the entire first exon and intron was moved from pClone17 and replaced the *Kpn*I–*Nde*I fragment of pCRIIc-*Msx2*. A *Sal*I site was regenerated as a result. The intron was shortened to approximately 500 bp by removing a 3.1-kb *Kpn*I fragment from this plasmid to generate pCRIIWTΔKpnI. A PCR product containing the 3′ UTR and polyadenylation signal from the human *MSX2* gene was then cloned into the *Eco*RI/*Xho*I site of pCRIIWTΔKpnI to create pCRIIWTΔKpnIhpa. To complete the construction of p*Msx2*WThpa, the *Sal*I–*Not*I fragment in pCRIIWTΔKpnIhpa was replaced by the *Sal*I–*Not*I DNA fragment from pGem11Zf-NX-#3. For microinjection, the transgene was freed from vector sequences by cleaving the plasmid with *Xho*I.

Generation of Transgenic Mice and Analysis of Cranial Phenotypes

Fertilized eggs were obtained from superovulated, 4- to 5-week-old (C57BL/6J × CBA/J)F_1 females impregnated by (C57BL/6J × CBA/J)F_1 adult males (The Jackson Laboratory, Bar Harbor, ME). Pseudopregnant females for embryo transfer were produced by matings between CD1 adult females and vasectomized CD1 adult males (Charles River, Wilmington, MA). Microinjection and oviduct transfer of injected zygotes was performed as described (Hogan *et al.*, 1986). Usually, both procedures were performed on the same day. The concentration of DNA used for injection was 1 μg/ml. Genotype analysis was carried out as previously described (Liu *et al.*, 1994).

Fixation and histological analysis of embryonic heads were as described by Liu *et al.* (1995). To analyze expression of the *Msx2* lacZ transgene, calvariae were dissected free of brain and skin and stained as described in Liu *et al.* (1994).

BrdU Labeling

Conditions for BrdU labeling and detection were adapted from Silvestrini *et al.* (1994). Mice were injected intraperitoneally with 100 μg of BrdU per gram of body weight. After 2 h, the animals were sacrificed and heads were fixed and decalcified in 4% paraformaldehyde + 10% EDTA for 24–48 h, depending on the age of the animal (older animals required longer times). Heads were dehydrated through graded ethanol and embedded in paraffin. Sections were cut (5 μm), deparaffinized, and soaked in 3% hydrogen peroxide in methanol for 10 min. They were washed three times in PBS and treated with proteinase K (100 μg/ml in 50 mM Tris–HCl, 5 mM EDTA, pH 8.0) for 20 min at 37°C. Subsequently, to depurinate DNA, sections were incubated in freshly prepared 2 N HCl for 45 min at room temperature and neutralized in 0.1 M sodium borate (pH 8.5) for 10 min. Sections were then rinsed three times in 1× PBST (1× PBS, 0.1% Tween 20). Immunodetection of BrdU was performed with a Zymed HistoMouse Immunostaining kit according to the manufacturer's instructions.

Histochemical Detection of Alkaline Phosphatase

Deparaffinized sections were washed three times in 1× TBST (10× TBS: 8 g NaCl, 0.2 g KCl, 3g Tris, pH 7.6, in 100 ml; 0.1% Tween 20). This was followed by three washes in 1× NMTT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris–HCl, pH 9.5, 0.1% Tween 20). NBT-BCIP (0.34 mg/ml nitroblue tetrazolium salt, 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) staining solution in 1× NMT was applied to sections until a dark purple color appeared. The reaction was stopped by washing sections with 1× PBS, and sections were counterstained with Nuclear Fast red. Simultaneous detection of lacZ and alkaline phosphatase was carried out as follows. Calvariae, stripped of skin and brain tissue, were stained in X-gal solution lacking NP-40 and sodium deoxycholate at 37°C for 4–16 h. Stained calvariae were then fixed for 1 to 2 h in 4% paraformaldehyde, 10% EDTA and subsequently embedded in paraffin and sectioned. Sections were stained for alkaline phosphatase in the presence of NBT–BCIP as described above.

Immunohistochemistry

The bone sialoprotein rabbit polyclonal antibody was a generous gift from Dr. Larry Fisher of the NIH (NIDR). The Fgfr2 antibody

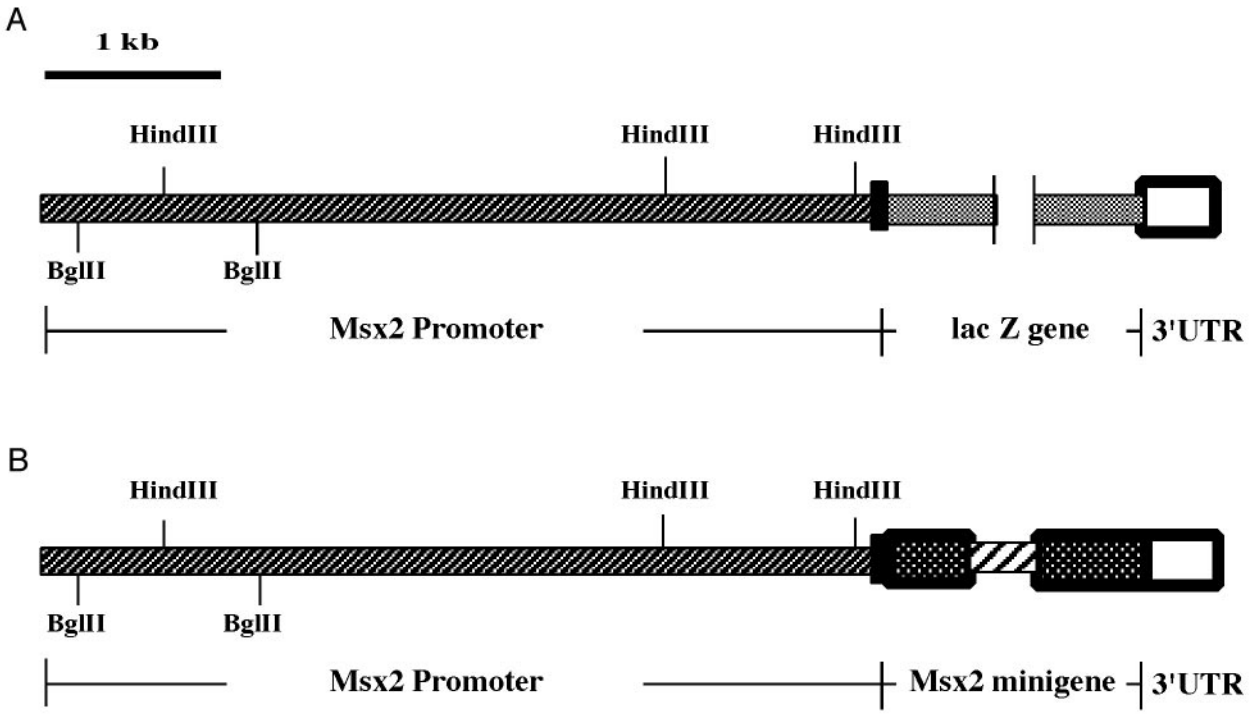


FIG. 1. Schematic maps of *Msx2* transgenes. A 5.2-kb segment of the *Msx2* promoter shown previously to approximate closely the expression pattern of the endogenous *Msx2* gene was fused either to a lacZ reporter (A) or to an *Msx2* minigene (B). The *Msx2* minigene contained the two *Msx2* exons and a portion of the intron (Bell *et al.*, 1993).

was purchased from Santa Cruz Biotechnology. Primary antibody was allowed to react with tissue sections of mouse calvariae. A biotinylated, affinity-purified secondary antibody was then bound to the primary antibody (Zymed Laboratories, San Francisco, CA). An HRP-conjugated streptavidin was added as a signal amplifier. The immunoreactivity was visualized with an AEC chromagen/substrate system that creates a red deposit. Sections were counterstained with hematoxylin.

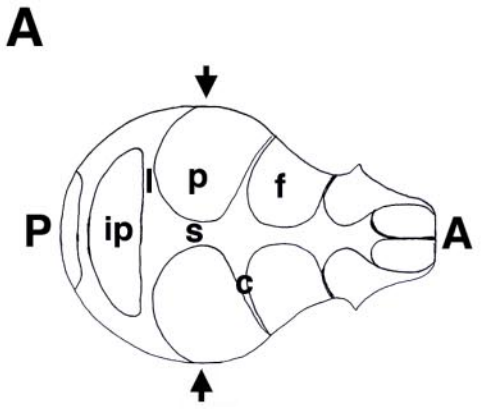
Cell Counts and Statistical Analysis

Histological sections of the midsagittal suture were visualized under Nomarski optics. Osteoblastic (alkaline phosphatase positive) cells in the osteogenic front, which were clearly distinct from other sutural cells, were counted independently by three different observers in at least three different sections. These cell counts were averaged to obtain an overall average for each section and for each individual mouse. A minimum of three mice were analyzed in each experimental group. Our test of the statistical significance of the cell counts took into account the variability between sections and between mice. An analysis of variance was used to test the effect of genotype on cell number. We used a standard *F* test, the denominator of which was a linear combination of the mean square of the individual and the mean square of the tissue section, as determined by the expected mean squares (with the individual and section taken as random effects and treatment taken as a fixed effect). Estimates of the mean effect were based on mouse averages; standard errors were based on the variance components for the individual and section variability. An identical approach was used to test differences in cell numbers between developmental stages.

RESULTS

An Msx2 Promoter Segment That Directs Expression to the Developing Skull and Sutures

We showed previously that a construct containing 5.2 kb of *Msx2* 5′ flanking sequence fused with a lacZ reporter closely approximates the expression of the endogenous *Msx2* gene (Liu *et al.*, 1994). In four distinct transgenic lines, this promoter was expressed in a near-identical pattern, demonstrating that it was not subject to strong position effects. Here we use this promoter segment to test the effects of *Msx2* overexpression on the development of the skull (Fig. 1). We first examined the expression of the −5.2 *Msx2*–lacZ transgene in greater detail in the developing skull (Fig. 2). Whole mounts of lacZ-stained embryos at E18–P4 revealed strong staining in the calvarial sutures (Figs. 2B–2H), delineating the presumptive parietal and interparietal bones. The anterior boundary of lacZ expression coincided with the boundary between the parietal and the frontal bones. At E18 and P0 (newborn) stages, lacZ expression was absent from two spots on the dorsal surface that correspond to the fontanels (Figs. 2B and 2C, arrows).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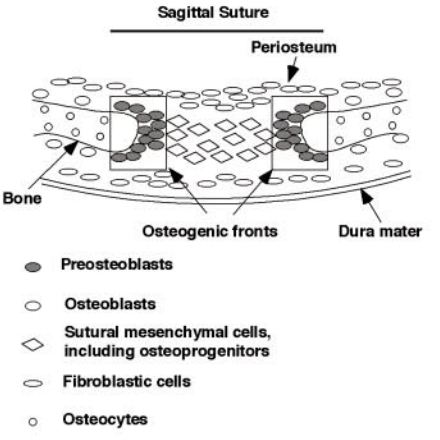
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Bone
Osteogenic fronts
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Sutural mesenchymal cells, including osteoprogenitors
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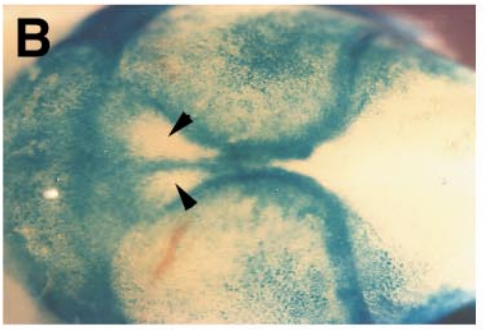
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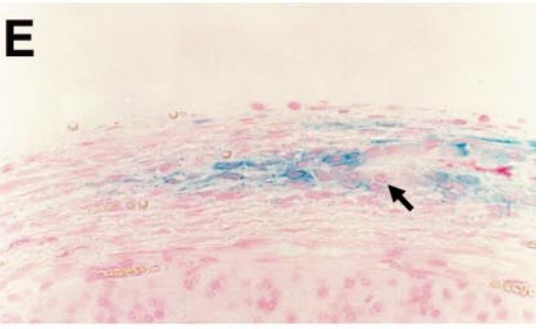
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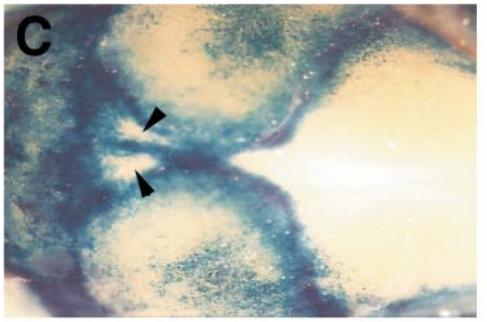
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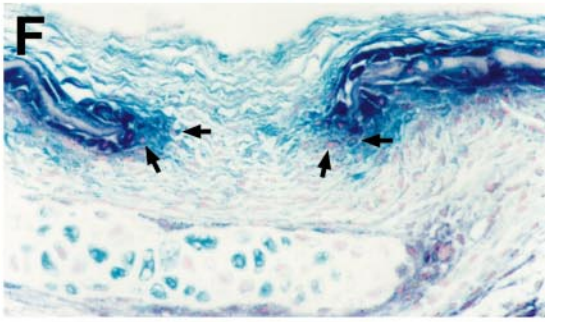
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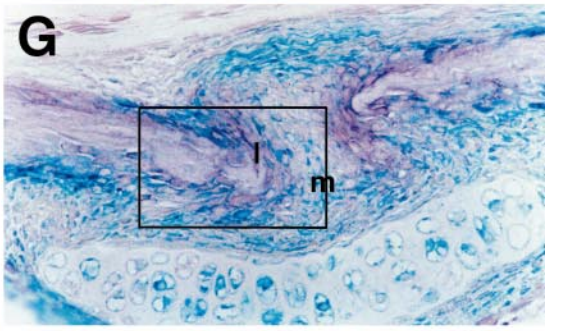
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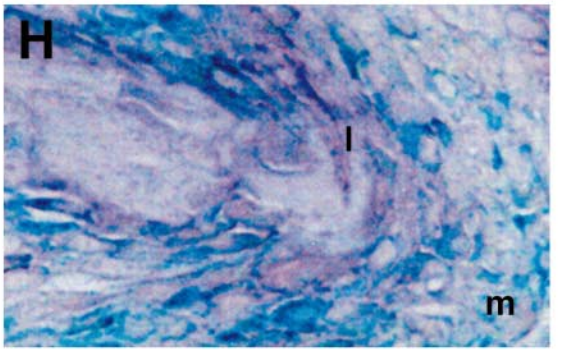
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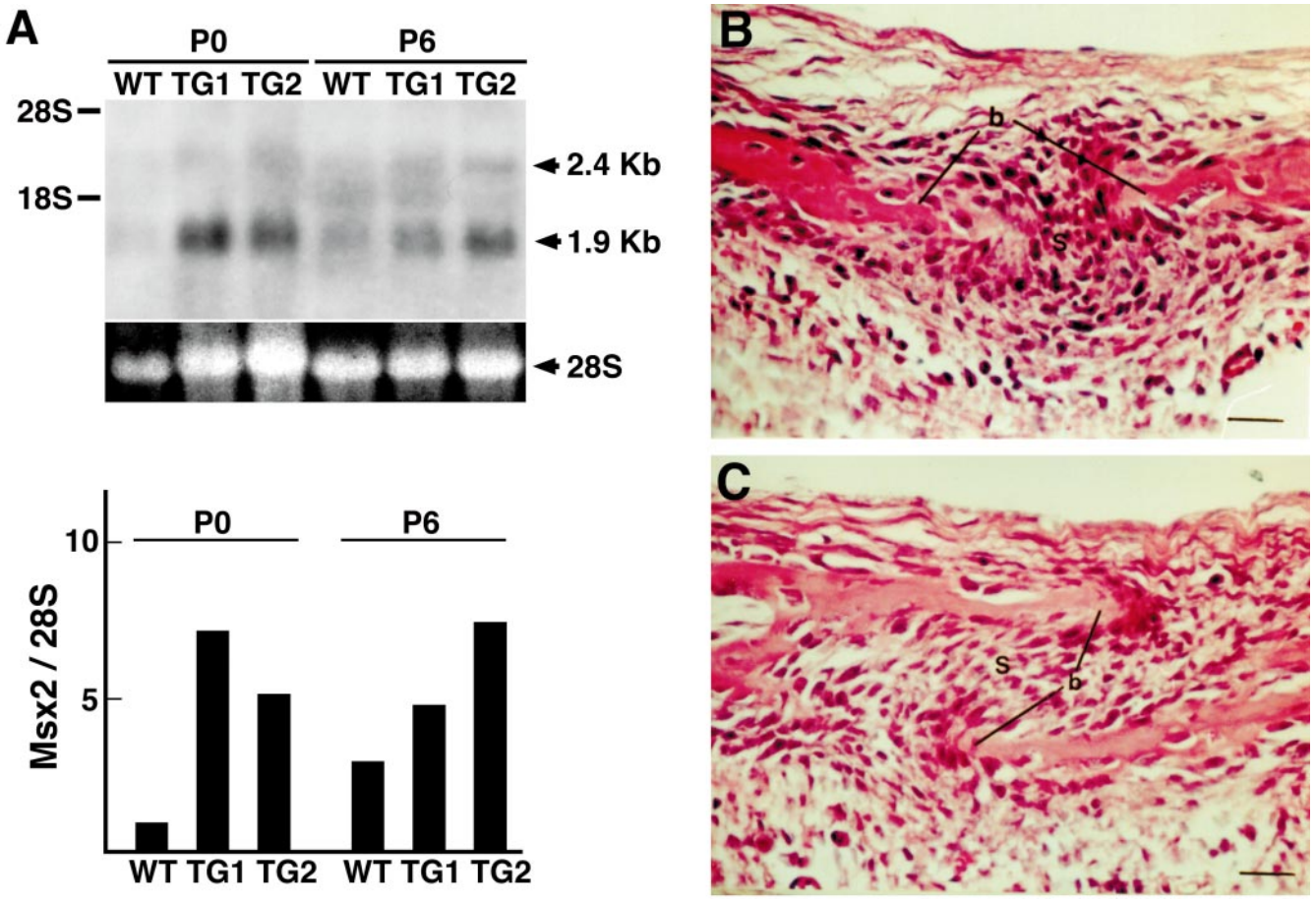


FIG. 3. Overexpression of *Msx2* causes the parietal bones to grow into the sutural space. The 5.2-kb *Msx2* promoter segment was fused to an *Msx2* minigene and injected into mouse zygotes. Two transgenic lines (TG1 and TG2) were analyzed for transgene expression and the growth status of the parietal bones. A representative Northern analysis of transgene expression is shown in A. RNA from whole calvaria of transgenic and littermate control mice was blotted and allowed to hybridize with a probe that recognized both endogenous (1.9 and 2.4 kb) and transgenic (1.9 kb) transcripts. 28S ribosomal RNA is shown below. Densitometry (bar graph) revealed that the 1.9-kb band, representing the transgene transcript and one of two endogenous transcripts, was elevated relative to 28S rRNA in both TG1 and TG2 lines at P0 (newborn) and P6. WT, wild type littermate control; TG, transgenic. B and C show representative images of skulls derived from a hemizygous *Msx2* transgenic and a littermate control at postnatal day 6. Skulls were processed for histology and sectioned coronally through the midsagittal suture. Sections were stained with hematoxylin and eosin. Note that in the control skull (B), the parietal bones were well apart, while in the transgenic skull (C), the bones invaded the sutural space and overgrew one another.

FIG. 2. Expression of the −5.2-kb *Msx2*–lacZ transgene in mouse calvaria. (A) Schematic map of murine skull (left) and cross section of sagittal suture, showing cell populations (right) A, anterior; P, posterior; f, frontal bone; p, parietal bone, ip, interparietal bone; s, sagittal suture; c, coronal suture, l, lambdoidal suture. Arrows indicate the level of sections shown in E–H. (B–H) Stains for β-galactosidase activity at E18.5 (B, E), P0 (newborn) (C, F), and P4 (D, G, H). B, C, and D are whole mounts of skulls in dorsal aspect. Note staining in the sagittal, coronal, and lambdoid sutures. E–H show coronal sections through the midsagittal suture. The sections shown in F, G, and H were stained for alkaline phosphatase as well as β-galactosidase. The image in H is an enlargement of the boxed region in G. At E18.5 (E), β-galactosidase stain is evident in cells at the medial edge of the osteogenic front, as well as in osteogenic cells above and below the bone. Note, however, the zone of reduced lacZ expression just medial to the edge of the parietal bone. At the newborn stage, lacZ is expressed in alkaline phosphatase-positive cells of the osteogenic front (arrows), in fibroblastic cells of the suture, and in periosteal cells. At 4 days postnatal (G, H), lacZ is detectable in some cells at the medial edge of the osteogenic front (m). Little stain is apparent in the cells of the lateral osteogenic front (l).

To identify sutural cell types expressing the *Msx2* lacZ transgene, we sectioned skulls of a series of *Msx2* lacZ transgenic mice at stages E18.5, P0, and P4 (Figs. 2E–2H). Selected sections were also stained for alkaline phosphatase activity (Figs. 2F, 2G, and 2H). Alkaline phosphatase is an osteogenic cell lineage marker that is first detectable in preosteoblasts as they make the transition to osteoblasts, and continues to be expressed through the remainder of osteoblast differentiation (Aubin *et al.,* 1995). At E18.5, lacZ staining was present in periosteal cells and in osteoblastic cells at the margin of the growing parietal bone, as well as above and below the parietal bone (Fig. 2E). These marginal cells compose the osteogenic front, which serves as a growth center for the bone (Markens, 1975; Johansen *et al.,* 1982; Decker and Hall, 1985). Intriguingly, a region of reduced lacZ staining was consistently present in the osteogenic front just medial to the bone (Fig. 2E, arrow). By P0, lacZ staining was detectable in the periosteum, in fibroblastic cells of the suture, and in osteoblastic cells of the osteogenic front and adjacent to the bone (Fig. 2F). At both P0 and P4, costaining for alkaline phosphatase revealed a partial overlap of lacZ and alkaline phosphatase in osteoblastic cells (Figs. 2F, 2G, and 2H). At P4, lacZ staining was reduced in the osteogenic front and was detectable in a punctate pattern in the outer margins of the alkaline phosphatase-expressing cell population of the osteogenic front (Figs. 2G and 2H). The spatial pattern of *Msx2* lacZ expression in the suture is consistent with *in situ* hybridization data on the expression of the endogenous *Msx2* gene (Kim *et al.,* 1998).

Overexpression of Msx2 Enhances Calvarial Bone Growth

We fused the −5.2-kb *Msx2* promoter fragment with the full-length mouse *Msx2* cDNA bearing a human *MSX2* polyadenylation signal (Fig. 1B). This construct was injected into mouse zygotes, resulting in seven F_0 transgenic pups as judged by Southern analysis with an *Msx2* cDNA probe (data not shown). Grossly, these animals were smaller than their nontransgenic littermates; one was anophthalmic and died shortly after birth (not shown). Two F_0 animals displayed circling behavior, consistent with a defect in the vestibular system. One of these died before reaching sexual maturity. Three of the seven F_0 animals reached sexual maturity but did not transmit the transgene, presumably because it was incorporated mosaically in cells of the F_0.

The two remaining F_0 transgenics were crossed with (C57BL6 × CBA)F_1 mice to produce F_1 hemizygotes. Matings between *Msx2* transgenic hemizygotes revealed that none of the transgenic pups were homozygotes, suggesting that homozygosity for the transgene resulted in embryonic lethality. Examination of midgestation embryos supported this possibility. Of 12 homozygous transgenic embryos examined, all 12 exhibited gross abnormalities, including anophthalmia and exencephaly (Y-H. Liu and R. Maxson, unpublished observations). The severity of these phenotypes made it impossible to investigate processes of calvarial morphogenesis in homozygotes; therefore we focused our analysis on hemizygotes.

Northern analysis was used to verify that the *Msx2* transgene was in fact overexpressed in *Msx2* transgenic hemizygotes (Fig. 3A). RNA from whole calvaria of *Msx2* transgenic and littermate control mice at P0 and P6 was size separated and blotted, and the blot was incubated with a probe that recognized both endogenous (1.9 and 2.4 kb) and transgenic (1.9 kb) *Msx2* transcripts. Densitometry showed that relative to 28S ribosomal RNA, the endogenous 1.9-kb transcript increased in amount by approximately 3-fold in control mice between P0 and P6. Since *in situ* hybridization and expression of the *Msx2* lacZ transgene showed that *Msx2* levels in the osteogenic front and sutures decline during this interval (Kim *et al.,* 1998), it is likely that the higher overall level of *Msx2* expression detected by Northern analysis results from increased *Msx2* expression in nonsutural tissues. In two independent transgenic lines (TG1 and TG2), the 1.9-kb transcript was elevated 7-fold and 5-fold, respectively, at P0 and 1.8-fold and 2.8-fold at P6. It is thus clear (i) that *Msx2* was overexpressed in calvarial tissues of *Msx2* transgenic mice and (ii) that the level of overexpression relative to endogenous *Msx2* expression was slightly higher at P0 than at P6. *In situ* hybridization analysis of *Msx2* expression in *Msx2*-overexpressing transgenic embryos was consistent with the *Msx2*-lacZ pattern and with the *in situ* hybridization results of Kim *et al.* (1998) (data not shown).

In human craniosynostosis syndromes that affect the sagittal suture, the development of craniosynostosis occurs in stages. The parietal bones first come into close apposition, then fuse at one or more sites over the length of the suture (Cohen, 1993). Fusion typically does not extend over the whole length of the suture, but occurs at a few sites. We asked whether *Msx2* transgenic mice exhibited a similar phenotype. An initial histological examination of the sutural morphology of *Msx2* transgenic mice at P6 revealed that the parietal bones had invaded the sutural space and overlapped (Fig. 3C), while in littermate controls, the parietal bones remained apart (Fig. 3B). This phenotype was observed in two independent transgenic lines (TG1 and TG2) in a total of 84% of transgenic pups (11/13). Overexpression of *Msx2* under the control of a segment of the *Msx2* promoter was therefore sufficient to cause the parietal bones to grow into the sagittal suture. The parietal bones did not fuse, however, indicating a difference between the human and the murine phenotypes (see Discussion).

Overexpression of Msx2 Causes an Increase in the Number of Proliferative Osteoblastic Cells in the Osteogenic Fronts of Calvarial Bones

To investigate the developmental mechanisms underlying the parietal bone growth phenotype in *Msx2* transgenic mice, we focused first on possible changes in populations of osteogenic cells within the suture. We used the molecular

markers alkaline phosphatase and bone sialoprotein to identify early-stage osteoblasts (preosteoblasts) and late-stage (mature) osteoblasts respectively (Aubin *et al.,* 1995). We also examined the expression of FGFR2 (bek isoform), both because it is expressed in the osteogenic front (Iseki *et al.,* 1997; Kim *et al.,* 1998) and because activating mutations in FGFR2 have a well-documented role in craniosynostosis syndromes (Wilkie, 1997). BrdU incorporation was used to assess the proliferation status of sutural cells. We carried out these studies in line TG1 (Fig. 3).

Grossly, the spatial distribution of alkaline phosphatase was similar in transgenic and nontransgenic controls (Figs. 4A–4C and 4F–4H). In both groups of animals, activity was prominent in the osteogenic front and was also detectable in osteoblastic cells above and below the bone. Fgfr2 staining was detectable in osteoblastic cells in the osteogenic front and was particularly intense in cells closest to the bone. It was also present at reduced intensity in sutural cells outside the osteogenic front (Figs. 4D and 4I). The spatial distribution of Fgfr2 did not change substantially in *Msx2* transgenics (Fig. 4I).

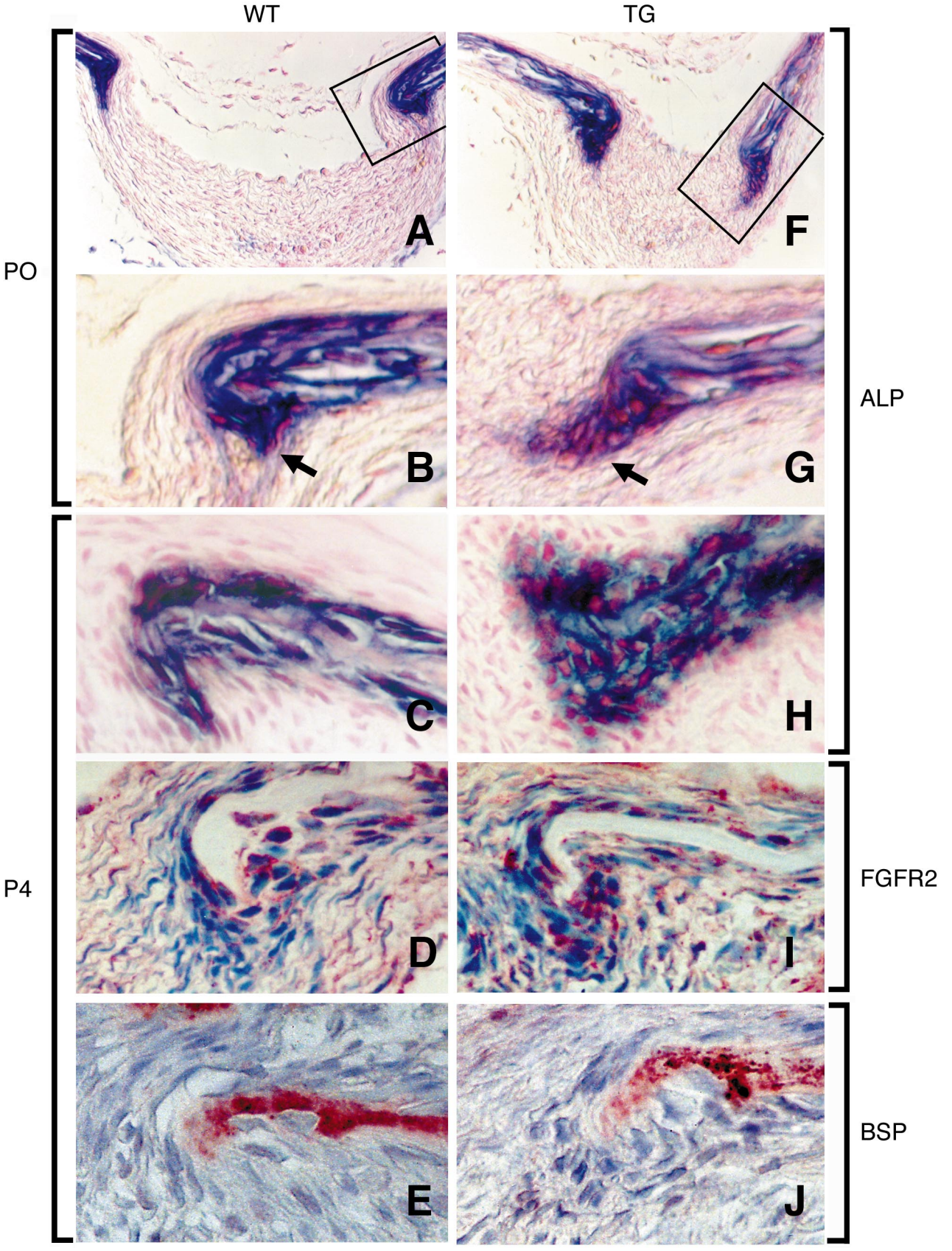
In both transgenic and control mice, bone sialoprotein was expressed in cells of the osteogenic front lateral to the tip, as well as in the region of osteoid deposition of the growing bone (Figs. 4E and 4J). Together with data on the expression of the *Msx2*–lacZ transgene (Fig. 2), these data show that within the osteogenic front, the *Msx2*–lacZ transgene was expressed predominantly in osteogenic cells that were not fully differentiated. The region of alkaline phosphatase stain medial to osteogenic front was larger in *Msx2*-transgenic mice than in control animals (compare Figs. 4B and 4G; Figs. 4C and 4H). BrdU incorporation studies on the sagittal suture showed that at both P0 and P4, BrdU was taken up predominantly by osteogenic cells located in the osteogenic front and in the periosteum (Figs. 5A–5H). There was little incorporation into cells of the central portion of the suture. At both P0 and P4, there was a significant enhancement in the number of BrdU-labeled cells of the parietal bone osteogenic front in *Msx2*-transgenic animals relative to littermate controls.

We quantitated differences between transgenic and nontransgenic mice in the numbers of alkaline phosphatase (ALP)-positive cells and BrdU-labeled cells in the osteogenic fronts at P0 and P4 (Fig. 6). Cells were counted under Nomarski optics in serial sections through the sagittal suture in three or more mice of line TG1. The characteristic appearance of osteoblastic cells in the osteogenic front made it straightforward to carry out such cell counts. At P0, control mice had an average of 16.6 ALP-positive cells per osteogenic front $\pm$ 3.4 (95% confidence interval), while *Msx2*-transgenic mice had an average of 28 $\pm$ 4.3 ALP-positive cells/osteogenic front. The difference between these mean cell numbers is statistically significant ($P < 0.05$). By P4, control mice had an average of 17.6 $\pm$ 3.7 ALP-positive cells/osteogenic front, while *Msx2*-transgenic mice had a mean of 26.6 $\pm$ 4.6 ALP-positive cells/osteogenic front ($P < 0.05$). Analysis of transgenic and control mice at prenatal stages E16.5 and E18.5 showed no significant differences in cell number per osteogenic front (not shown). These data show that overexpression of *Msx2* enhanced the number of ALP-positive osteogenic cells in the osteogenic front of the parietal bones at P0 and P4. A difference was also apparent in BrdU labeling. At P0, transgenic animals had an average of 3.1 $\pm$ 1.1 BrdU-labeled cells, while controls had an average of 0.6 $\pm$ 0.4 labeled cells ($P < 0.05$). By P4, there was an average of 11.5 $\pm$ 2.3 BrdU-labeled cells in the osteogenic front of transgenic mice compared to an average of 5.4 $\pm$ 1.7 in controls ($P < 0.05$). We note also that there was a significant increase in BrdU labeling in both transgenic and control groups between P0 and P4 ($P < 0.05$) Thus, the proportion of osteogenic front cells that took up BrdU increased during development in both groups, but was substantially higher at both stages in transgenic animals. We conclude that overexpression of *Msx2* caused an increase both in the number of alkaline phosphatase-positive cells and in the number of BrdU-labeled cells in the osteogenic front.

DISCUSSION

In an effort to understand the pathophysiology of craniosynostosis and the mechanisms that regulate skull morphogenesis, we used a segment of the *Msx2* promoter to overexpress *Msx2* in specific cell populations in the developing skull and sutures. We sought to mimic the effect of the apparently dominant-activating p148h mutation in the human *MSX2* gene in Boston-type craniosynostosis. The most significant new finding presented here is that *Msx2*-transgenic mice exhibit an increased number of proliferative osteoblastic cells in the osteogenic front in early postnatal stages. This effect on the osteogenic front is

FIG. 4. Influence of *Msx2* transgene expression on osteogenic lineage in the sagittal suture. Images of cross-sections of the sagittal suture of *Msx2* transgenic and control mice showing alkaline phosphatase activity (ALP, blue color, A–H) and immunostaining with antibodies against Fgfr2 (reddish color, D, I) and bone sialoprotein (BSP, E, J). (A–G) Images of P0 (newborn) animals; (C–J) images of P4 mice. The left (A–E) shows controls, the right (F–J) *Msx2* transgenics. B and G are enlargements of the boxed areas in A and F. Sections were visualized under differential interference contrast optics at 100× magnification. Note that at both P0 and P4 stages, there are more alkaline phosphatase-expressing cells in the osteogenic fronts (arrows) of transgenic mice than in littermate controls. Fgfr2 staining is evident in cells of the osteogenic front, as well as in osteoblastic cells adjacent to the bone (D, I). At P4, bone sialoprotein expression is present in cells of the matrix of the advancing bone (E, J).



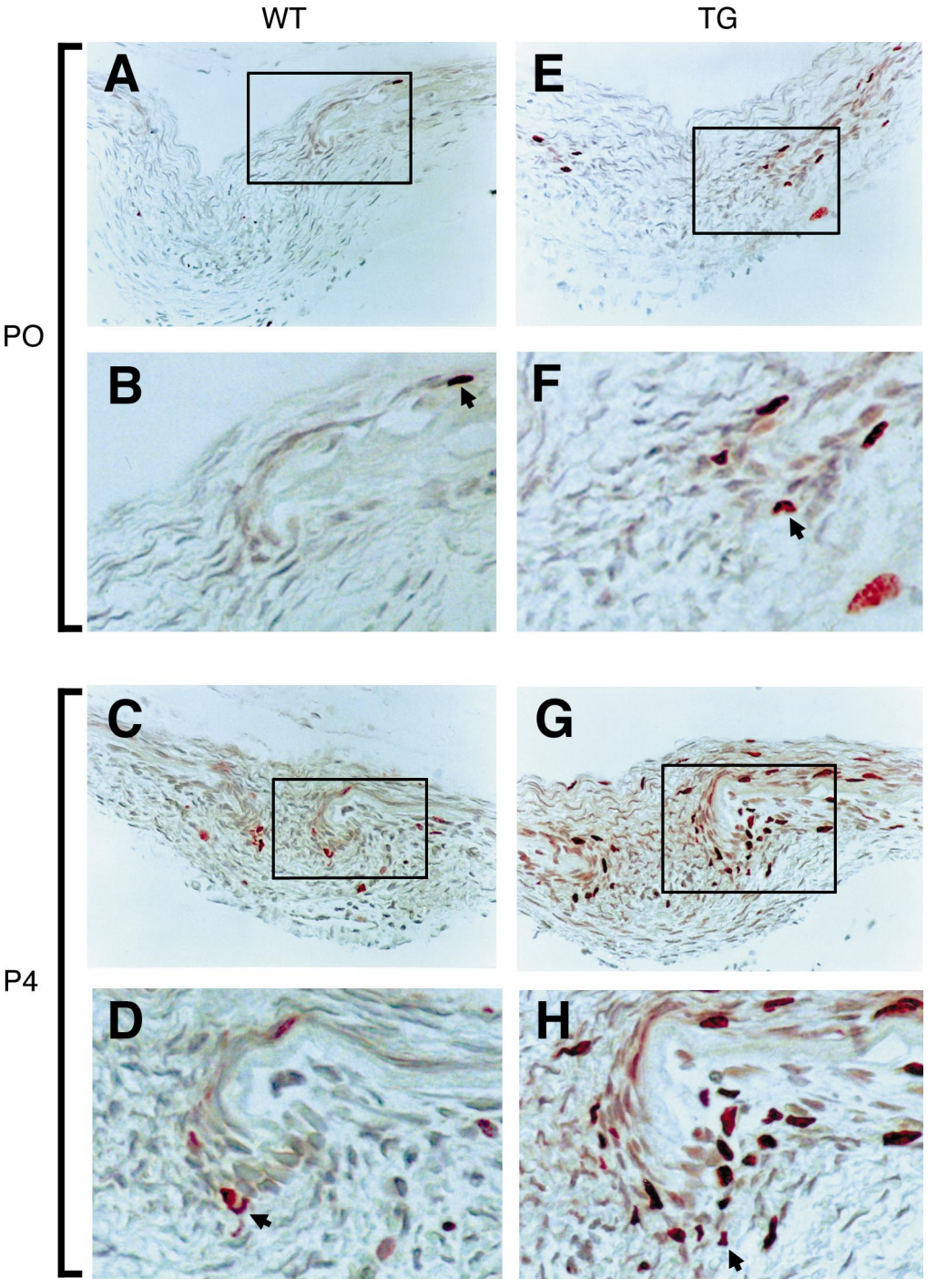


FIG. 5. Cell proliferation in sutural cell populations of *Msx2*-transgenic mice. BrdU was injected intraperitoneally into *Msx2*-transgenic and littermate control mice at P0 (newborn) and P4. Two hours after injection, calvarial tissue was harvested and processed as described under Materials and Methods. Sections through the midsagittal suture were stained with an antibody against BrdU and counterstained with hematoxylin. Stained sections were viewed under Nomarski optics. Images of control sutures are shown on the left, transgenic sutures on the right. B, F, D, and H are higher magnification images of the boxed regions in A, E, C, and G, respectively. Note that at both P0 and P4, the number of BrdU-labeled cells (arrow) in the osteogenic front is greater in *Msx2*-transgenic mice than in littermate controls.

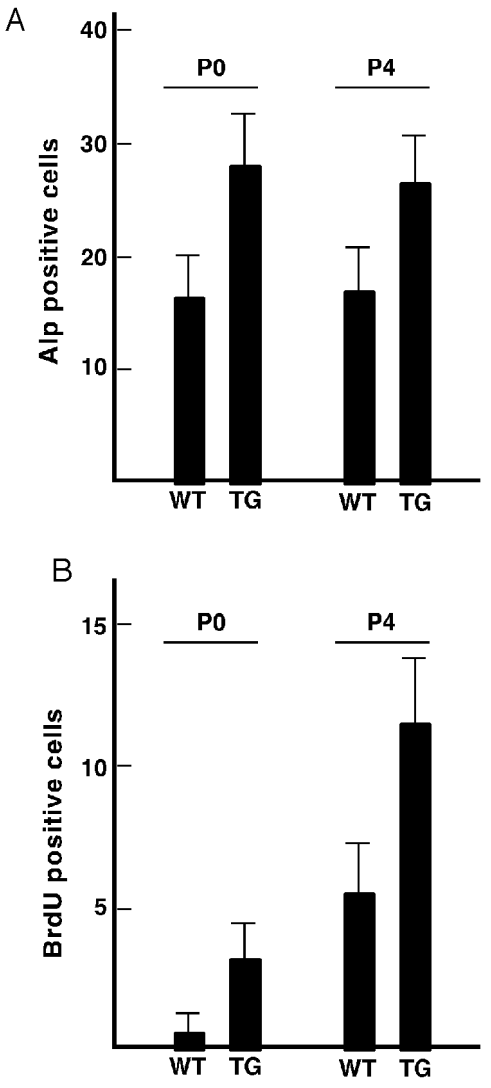


FIG. 6. Numbers of alkaline phosphatase-expressing and BrdU-labeled cells in calvarial osteogenic fronts of *Msx2* transgenic mice. Using light microscopy as illustrated in Figs. 4 and 5, we counted the number of alkaline phosphatase-expressing and BrdU-positive cells in osteogenic fronts of the midsagittal sutures of *Msx2*-transgenic and littermate control mice. A minimum of three mice were analyzed in each group. For each mouse, approximately seven serial sections were examined. Shown are the mean numbers of labeled cells per osteogenic front ± the 95% confidence intervals. The statistical analysis is described under Materials and Methods. WT, wild type (littermate control); TG, *Msx2*-overexpressing transgenic.

consistent with the view that *MSX2*-induced craniosynostosis in humans is a result not of premature differentiation of osteogenic cells but of a transient inhibition of differentiation. More generally, our findings underscore the point that a perturbation in the timing of proliferation and differentiation of a given cell population can have profound consequences on morphogenesis.

Spatiotemporal Pattern of Msx2 Transgene Expression

Marker analysis defined the pattern of *Msx2*–lacZ transgene expression relative to the state of differentiation of osteoblastic cells at the osteogenic front. The location of cells within the osteogenic front appears to be correlated with their state of differentiation: the closer the cell to the bone (i.e., the more lateral), the further along in differentiation. As expected, the terminal differentiation marker, bone sialoprotein, was detected in the lateralmost cells of the osteogenic front that were beginning to synthesize the osteoid matrix. Alkaline phosphatase, which is first expressed in osteoprogenitor cells as they make the transition to osteoblasts and continues to be expressed through much of osteoblast differentiation (Aubin *et al.*, 1995), was present in histologically osteoblastic cells throughout the osteogenic front. Fgfr2 was also expressed in the osteogenic front, consistent with the data of Iseki *et al.* (1997). A comparison of the patterns of these marker proteins with the expression of the −5.2 *Msx2*–lacZ transgene showed that the transgene is not detectably expressed in fully differentiated osteogenic cells, but in less differentiated (i.e., alkaline phosphatase-positive, bone sialoprotein-negative) cells of the osteogenic front, as well as in cells medial to the bone that do not express bone markers. In later stages (e.g., P4), *Msx2*–lacZ was expressed in a punctate pattern in cells of the medial osteogenic front. *In situ* hybridization analyses of *Msx2* expression in the sagittal suture are consistent with these results (Kim *et al.*, 1998; our unpublished results), suggesting that within the suture, as elsewhere in the embryo, the *Msx2*–lacZ transgene closely approximates the expression of the endogenous *Msx2* gene (Liu *et al.*, 1994).

Phenotypic Effects of Msx2 Overexpression and the Developmental Mechanisms Underlying Craniosynostosis

Since we used the 5.2-kb *Msx2* promoter segment to drive expression of the *Msx2* cDNA in transgenic mice, the lacZ staining pattern defines, to a first approximation, the expression of the *Msx2* cDNA transgene. Consistent with the expression of the *Msx2*–lacZ transgene in early-stage osteoblasts, we found that overexpression of *Msx2* enhanced the number of alkaline phosphatase-positive cells in osteogenic fronts of the sagittal suture. This enhancement was evident at both P0 and P4, but was not seen in embryos at E16.5 or E18.5 (not shown). TUNEL assays did not detect significant levels of apoptotic cells in the sutures between E12.5 and P4 in either transgenic or control mice (not shown), suggesting that a reduction in cell death does not account for the increase in osteogenic cell number in the osteogenic front.

BrdU labeling revealed that the total number of prolifer-

ating cells was higher at both P0 and P4 in *Msx2* transgenics than in controls. The effect of the transgene on BrdU labeling did not extend over the whole area of *Msx2* overexpression (defined by *Msx2*–lacZ expression), but was concentrated in the osteogenic front and periosteum, suggesting that *Msx2* overexpression was not sufficient to promote cell proliferation in nonosteoblastic sutural cells.

We showed previously that CMV-promoter-driven overexpression of *Msx2* in the developing skull and sutures caused the parietal bones to invade the sagittal suture and overlap (Liu *et al.,* 1995). This phenotype is generally similar to that produced by overexpression of *Msx2* under the control of its own promoter (Fig. 3). However, a significant difference is that CMV-driven overexpression of *Msx2* also induced ectopic bone above the parietal bone. This difference may be a consequence of the more generalized or higher-level expression of the CMV promoter compared to the *Msx2* promoter (Liu *et al.,* 1995). Taken together, our results with the CMV and *Msx2* transgenes suggest that overexpression/misexpression of *Msx2* is sufficient (i) to enhance parietal bone growth, (ii) to augment the number of osteoblastic cells in osteogenic fronts, and (iii) to induce cells that would not normally form bone to do so (in the case of the CMV–*Msx2* transgene). One interpretation of these findings is that overexpression/misexpression of *Msx2* has a positive effect on the development of osteoblastic cells from osteoprogenitors (Fig. 7).

An alternative possibility is suggested by the data of Dodig *et al.* (1998), who used *Msx2* sense- and antisense-expressing retroviruses to modulate *Msx2* expression in chick calvarial osteoblast cultures. They demonstrated that upregulated overexpression of *Msx2* mRNA throughout the culture period prevented the decline in Msx2 protein levels that normally occurs as the cells differentiate. This continuous, high-level expression of *Msx2* prevented osteoblastic differentiation and mineralization of the extracellular matrix. In contrast, expression of antisense *Msx2* RNA reduced proliferation and accelerated differentiation. These results indicate that abnormally high levels of *Msx2* expression can impede the final phases of osteoblastic differentiation and are consistent with findings that forced expression of *Msx2* in a myogenic cell line prevents terminal differentiation (Song *et al.,* 1992; Woloshin *et al.,* 1995).

These data provide an attractive hypothesis that explains our key observations and may also provide insight into the pathophysiology of Boston craniosynostosis (Fig. 7). Overexpression of *Msx2* in the sutures of transgenic mice may maintain osteoblastic cells in a proliferative, undifferentiated state longer than normal. This would explain the increased pool of early-stage osteoblastic cells found in the osteogenic fronts at both newborn and P4 stages, as well as our observation of increased BrdU labeling at P0 and P4. A change in the pattern of *Msx2* expression from the rather generalized pattern in the suture and osteogenic front, evident in newborn animals, to a more punctate pattern in the outer margins of the osteogenic front in P4 animals might then allow differentiation of this increased osteoprogenitor pool. This would result in enhanced growth of the parietal bones into the sutural space. In Boston craniosynostosis, the dominant-activating p148h mutation would, similarly, cause an increase in the size of the osteoprogenitor population in the osteogenic fronts. The ultimate differentiation of this population, consequent upon a decline in *Msx2* levels in the osteogenic front, would result in the overlap of calvarial bones. Fusion of the overlapped bones would occur subsequently through an as yet unknown mechanism.

While this hypothesis explains much of the data from *Msx2*-transgenic mice, it does not explain the presence of ectopic bone in transgenic mice in which *Msx2* expression is driven by the CMV promoter (Liu *et al.,* 1995). This phenotype raises the possibility that *Msx2* is capable of initiating a program of osteogenic differentiation when ectopically expressed in certain populations of calvarial cells. It is possible that *Msx2* has a positive role in the early stages of osteogenic differentiation, in addition to its apparent ability to inhibit the terminal differentiation of osteoblastic cells. It will be interesting to test this prediction by examining the effect of targeted inactivation of *Msx2* on calvarial cell populations.

To what extent do the phenotypes exhibited by the *Msx2* transgenic mice provide a model for Boston-type craniosynostosis or other craniosynostosis syndromes? Since Boston-type craniosynostosis is not described in detail histologically, we cannot compare our results in the mouse directly with the human pathology. However, other craniosynostosis disorders have been examined in sufficient detail to allow comparison. Synostosis of the sagittal suture typically involves localized fusion of the parietal bones at one or more sites (Cohen, 1993). Adjacent to the sites of bone fusion, both enhanced growth of bones into sutural space and enhanced osteoblast activity are usually seen (Cohen, 1993). Further evidence for enhanced osteoblast activity comes from studies showing that there is a significant increase in the number of alkaline phosphatase-positive cells isolable from sutural tissue of individuals affected with Apert's syndrome (Lomri *et al.,* 1998). These results suggest that enhancement of both the growth of calvarial bones and osteoblast activity accompanies craniosynostosis. That *Msx2*-transgenic mice show increased calvarial bone growth and osteoblast activity suggests that these mice model at least one aspect of the early phases of craniosynostosis. Why the calvarial bones do not fuse in such mice is unclear, though bone fusion apparently does not occur in older wild-type mice, in contrast to humans (Zimmermann *et al.,* 1998; our unpublished observations). The later stages of the program of suture development may therefore differ in mouse and human.

The p148h mutation in human *MSX2* behaves genetically as an autosomal dominant (Warman *et al.,* 1993; Muller *et al.,* 1993). We showed that this mutation enhances the binding affinity for DNA (Ma *et al.,* 1996) as well as the interaction with a putative partner protein, Miz1 (Wu *et al.,* 1997). Further, overexpression of both mutant and wild-

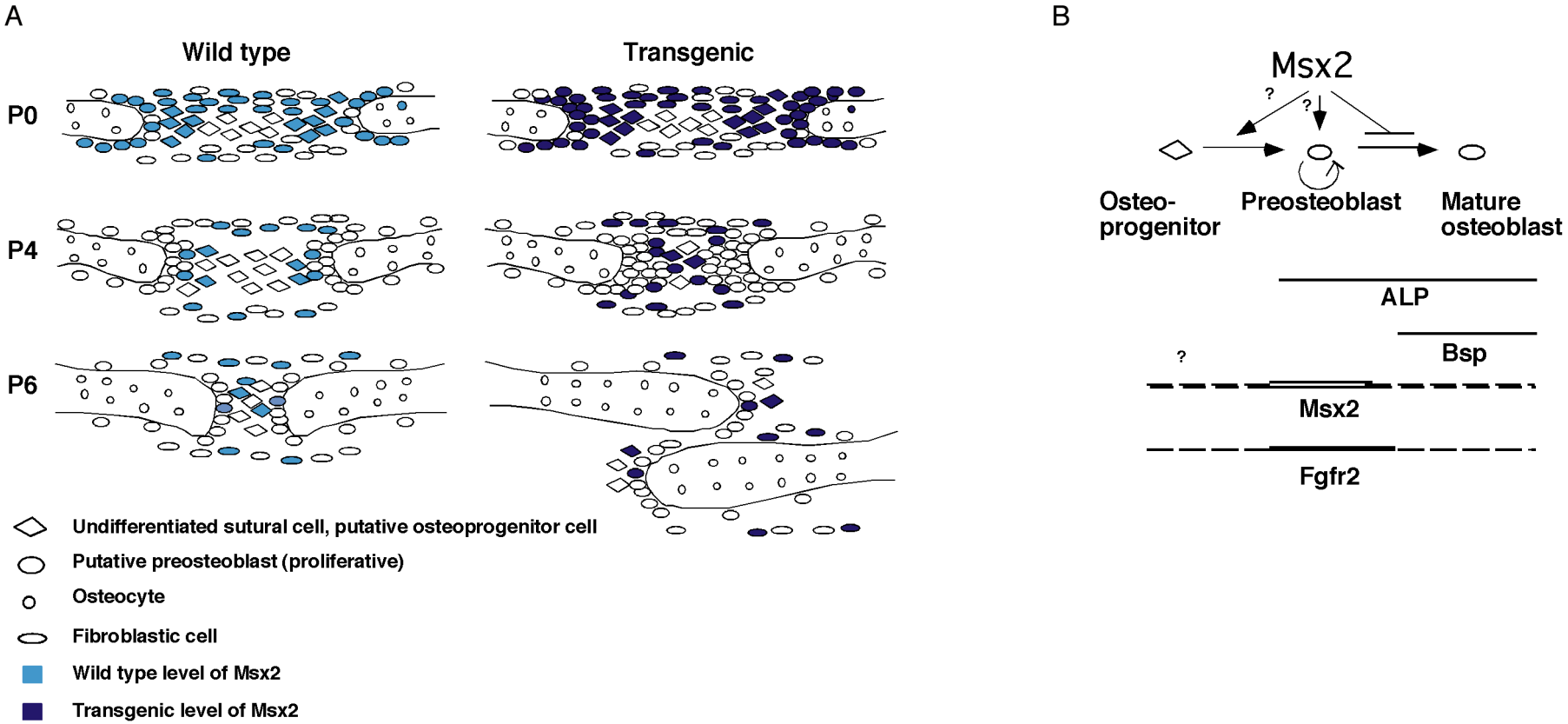


FIG. 7. Model of *Msx2* function in calvarial patterning and craniosynostosis. (A) Diagram showing influence of *Msx2* transgene on sutural cell populations. The *Msx2* transgene and the endogenous *Msx2* gene are expressed in fibroblastic cells, in sutural mesenchymal cells, and in osteoblastic cells (Kim *et al.*, 1998) (Fig. 2). Light blue represents endogenous *Msx2* expression, dark blue, overexpression of *Msx2* in transgenic mice. *Msx2*-expressing mesenchymal cells likely include osteoprogenitors (Aubin *et al.*, 1995). The left shows sutures of wild-type control mice, the right sutures of *Msx2*-transgenic mice. At P0, *Msx2* is expressed broadly in each of these sutural cell populations. *Msx2* transgenic mice exhibit increased numbers of proliferative osteoblastic cells (alkaline phosphatase positive, bone sialoprotein negative). We designate these cells putative preosteoblasts following Aubin's nomenclature (Aubin *et al.*, 1995), although we stress that these cells have not been shown functionally to be preosteoblasts. By P4, *Msx2* expression in osteoblastic cells has retreated to cells of the medial osteogenic front. *Msx2*-transgenic mice continue to show greater numbers of proliferative osteoblastic cells than controls. Calvarial bones of *Msx2*-transgenic mice are significantly closer together than controls. At P6, calvarial bones of *Msx2* transgenic mice overlap. (B) Diagram showing possible effects of *Msx2* on calvarial osteoblast lineage. The approximate stages of expression of alkaline phosphatase (ALP), bone sialoprotein (Bsp), Fgfr2, and *Msx2* are indicated by solid lines (Aubin *et al.*, 1995) (this work). Dotted lines signify reduced levels of expression. The model for *Msx2* effects is based on these observations: (i) CMV-driven misexpression/overexpression of *Msx2* results in ectopic cranial bone (Liu *et al.*, 1995); thus *Msx2* may stimulate osteoprogenitor development. (ii) BrdU-labeling of osteoblastic cells of the osteogenic front is increased in *Msx2*-transgenic mice, suggesting that *Msx2* may influence the proliferation of preosteoblasts. (iii) Overexpression of *Msx2* can inhibit the terminal differentiation of calvarial osteoblasts in culture (Dodig *et al.*, 1998).

type cDNA under the control of the CMV promoter caused the parietal bones to invade the sutural space (Liu *et al.*, 1995). These data led us to suggest that the mutation functions in a dominant-activating manner (Ma *et al.*, 1996). Our demonstration here that overexpression of the wild-type *Msx2* causes a cranial growth phenotype is consistent with this hypothesis. As expected, increased dosage of *Msx2* in the mouse mimics at least some of the effects of the apparently activating mutation in humans. We point out, however, that genetic proof that the p148h mutation can influence calvarial growth in the mouse will require the introduction of this mutation into the endogenous *Msx2* gene.

Winograd and co-workers used a human *MSX2* promoter segment to overexpress human *MSX2* (*huMSX2*) in transgenic mice (Winograd *et al.*, 1997). These mice died perinatally with exencephaly, as was the case with our homozygous *Msx2*-transgenic mice, though they did not exhibit a craniosynostosis phenotype. However, since we typically do not detect enhancement of calvarial bone growth until postnatal day 3 or later, the lack of a comparable phenotype in the *huMSX2* mice may be a result of the failure of these mice to survive long enough for the phenotype to become evident. It is not clear why the human *MSX2* mice exhibited a more severe neural tube phenotype than our mice. Neither the level nor the spatial expression pattern of the human *MSX2* transgene was characterized, leaving open the possibility that they differed significantly from the murine *Msx2* promoter that we used.

The Role of Msx2 in Signaling Pathways That Regulate Calvarial Morphogenesis

The osteogenic front expresses ligands and receptors of the Bmp, Fgf, and Shh pathways (Iseki *et al.*, 1997; Kim *et al.*, 1998). Might overexpression of *Msx2* affect one or more of these pathways? Implantation of FGF-containing beads in the murine suture promotes both proliferation and differentiation of osteogenic cells (Iseki *et al.*, 1997; Kim *et al.*, 1998). Fgf beads stimulate *Msx1* expression but apparently do not affect *Msx2* (Kim *et al.*, 1998). Implantation of beads containing Bmp4 increases the number of sutural cells and also activates *Msx2* expression (Kim *et al.*, 1998). Although the cell populations affected by Bmp4 were not identified, this increase in cell number is reminiscent of our finding of an increase in osteoblastic cell number in osteogenic fronts of *Msx2*-transgenic mice. *Msx2* may therefore act downstream of Bmp4 to promote proliferation of osteogenic cells within the suture.

One inconsistency in this view is that implantation of Bmp4 beads in the suture does not influence the proximity of calvarial bones (Kim *et al.*, 1998), while in *Msx2*-transgenic mice, the parietal bones ultimately invade the sutural space (Fig. 3). With the caveat that bead implantations may not accurately recapitulate endogenous developmental mechanisms, these data suggest that the activity of *Msx2* in the suture may extend outside the Bmp pathway. Epistasis studies in *Drosophila* have shown that the *Msx*-related gene, msh, is also influenced by EGF and hedgehog signaling (D'Alessio and Frasch, 1996). Enhanced *Msx2* activity in the mammalian suture may therefore affect more than one growth factor pathway.

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