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Concerted action of *Msx1* and *Msx2* in regulating cranial neural crest cell differentiation during frontal bone development

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Abstract

The homeobox genes *Msx1* and *Msx2* function as transcriptional regulators that control cellular proliferation and differentiation during embryonic development. Mutations in the *Msx1* and *Msx2* genes in mice disrupt tissue-tissue interactions and cause multiple craniofacial malformations. Although *Msx1* and *Msx2* are both expressed throughout the entire development of the frontal bone, the frontal bone defect in *Msx1* or *Msx2* null mutants is rather mild, suggesting the possibility of functional compensation between *Msx1* and *Msx2* during early frontal bone development. To investigate this hypothesis, we generated $Msx1^{-/-};Msx2^{-/-}$ mice. These double mutant embryos died at E17 to E18 with no formation of the frontal bone. There was no apparent defect in CNC migration into the presumptive frontal bone primordium, but differentiation of the frontal mesenchyme and establishment of the frontal primordium was defective, indicating that *Msx1* and *Msx2* genes are specifically required for osteogenesis in the cranial neural crest lineage within the frontal bone primordium. Mechanistically, our data suggest that *Msx* genes are critical for the expression of *Runx2* in the frontonasal subpopulation of cranial neural crest cells and for differentiation of the osteogenic lineage. This early function of the Msx genes is likely independent of the Bmp signaling pathway.

Keywords

cranial neural crest (CNC) cells; frontal bone; *Msx1*; *Msx2*; *Runx2*; proliferation; differentiation; and apoptosis

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Introduction

Frontal bones form the anterior aspect of the skull vault. During embryogenesis, cranial neural crest cells migrate into the presumptive frontal primordium, condense at the lateral aspect of the head and differentiate into osteoblasts, which constitute the frontal primordium. The primordium then proceeds to undergo apical extension and growth until adulthood. Meanwhile, the osteoblasts within the primordium synthesize bone matrix through intramembranous ossification (Couly et al., 1993; Jiang et al., 2002; Wilkie and Morriss-Kay, 2001). Extensive studies have been focused on the growth of frontal bones. Morriss-Kay and coworkers suggested that the development of the normal frontal bone must require mechanisms to ensure that both its morphology and its rate of growth are precisely matched to those of the developing brain (Iseki et al., 1999). This relationship is coordinated through tissue-tissue interactions between the brain, the developing meninges, the frontal bone primordium and the cranial sutures, the fibrous joint between two frontal bones (Antonopoulou et al., 2004; Holleville et al., 2003b; Ishii et al., 2003). However, the earliest aspect of frontal bone development, the initiation of osteogenesis in mesenchymal precursor cells, is largely unknown.

The CNC-derived ectomesenchyme is the sole source for populating the frontal bone primordium and its underlying meninges in mice (Jiang et al., 2002). The cranial neural crest (CNC) cells, a pluripotent cell population derived from the lateral ridges of the neural plate, migrate ventrolaterally to the frontonasal prominence and the branchial arches and give rise to the jaws, the anterior skull base and the anterior skull vault. The migration, proliferation and differentiation of CNC cells are regulated by growth factor signaling pathways and their downstream transcription factors before these cells become committed to an array of different phenotypes (Echelard et al., 1994; Graham and Lumsden, 1993; Lumsden, 1988; Noden, 1983; Trainor and Krumlauf, 2000). It is unknown, however, how the differentiation of osteoblasts is regulated in the CNC-derived cells. Understanding this event will shed light on the mechanism of lineage selection in the pluripotent precursors.

The homeobox genes *Msx1* and *Msx2* are highly conserved among various species and function as transcriptional regulators that control cellular proliferation and differentiation during normal embryonic development (Hill et al., 1989; Robert et al., 1989). *Msx1* is strongly expressed in CNC cells and plays a critical role in regulating epithelial-mesenchymal interactions during organogenesis (Hill et al., 1989; Lyons et al., 1992; Robert et al., 1989). Targeted null mutation of *Msx1* results in multiple craniofacial abnormalities including a frontal bone development defect (Satokata and Maas, 1994). *Msx2* null mutation causes pleiotropic defects in bone growth and ectodermal organ formation (Satokata et al., 2000). As the result of an osteoprogenitor proliferation defect, *Msx2* null mutant mice display defective skull ossification and persistent calvarial foramen, and the frontal primordium is reduced in size at the initiation stage of osteogenesis (Ishii et al., 2003), suggesting that *Msx2* plays a critical role in regulating calvarial morphogenesis. Significantly, the entire calvaria is missing in the $Msx1^{-/-};Msx2^{-/-}$ mutant, suggesting that *Msx1* and *Msx2* function together to regulate osteogenesis during calvaria development (Satokata et al., 2000). The complete absence of the calvaria including the frontal bones provides a unique opportunity of explore the initiation of the frontal primordium. We hypothesize that Msx1 and Msx2 act concertedly to regulate the establishment of the frontal bone primordium. Taking advantage of the profound frontal bone defect in the $Msx1^{-/-};Msx2^{-/-}$embryos, we investigated the cellular and molecular mechanisms by which *Msx1* and *Msx2* function together to regulate the fate of CNC cells during frontal bone development.

Our study demonstrates that *Msx* genes are critical for osteogenic lineage differentiation of CNC cells during frontal primordium formation. *Msx* genes control osteogenesis by regulating *Runx2* expression. This study represents a significant step towards the understanding of the

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molecular regulation of CNC fate determination and provides an animal model for the investigation of abnormal CNC development during calvarial morphogenesis.

Results

Msx1 and *Msx2* have critical and functionally redundant roles in regulating calvaria morphogenesis

To test the hypothesis that *Msx1* and *Msx2* function redundantly to regulate calvaria morphogenesis, we generated double knockout mice. In $Msx1^{-/-};Msx2^{+/-}$ and $Msx1^{+/-};Msx2^{-/-}$ mice, the calvarial ossification defect between the frontal bones was significantly enlarged as compared to the $Msx1^{-/-}$ mutant (Fig. 1A,D,G,J arrows). In contrast, the fontanel between the parietal bones was comparable in $Msx1^{-/-}$, $Msx1^{-/-};Msx2^{+/-}$, and $Msx1^{+/-};Msx2^{-/-}$ mice (Fig. 1A,D,G,J *). Thus, *Msx1* or *Msx2* null mutations in the background of haploinsufficiency of the complementary *Msx* gene significantly intensify the frontal ossification defects, whereas parietal bone development is largely unchanged. Next, we examined $Msx1^{-/-};Msx2^{-/-}$ double null mice and found that they were able to survive to E17 or E18 but showed no calvarial ossification with severely deformed craniobase and jaws (Fig. 1M–O). The only ossified bony elements in the skull were rudiments of mandible, maxilla and craniobase, although most of the cranial cartilage was present (Fig. 1M,N). The majority of the $Msx1^{-/-};Msx2^{-/-}$ embryos (43/47=91%) had exencephaly and a partially open neural tube. To rule out the possibility that the frontal bone defect was solely associated with embryos that had an open neural tube, we investigated the frontal bone development in the $Msx1^{+/-};Msx2^{-/-}$ mice with open neural tubes (see Fig. 5). Our data suggest that *Msx1* and *Msx2* have important and concerted roles in the regulation of frontal bone development.

Msx1 and *Msx2* are co-expressed in the presumptive frontal, but not parietal, primordium

Unlike the gradient of defects detectable in the frontal bone, there is no obvious parietal bone defect in $Msx1^{-/-}$ or $Msx2^{-/-}$ mice (Fig. 1D,G,J * versus arrow). To explore the possibility that Msx1 and Msx2 compensate for the function of each other completely during parietal bone development but only partially during frontal bone development, we documented the expression profiles of *Msx1* and *Msx2* in the presumptive primordium. Cranial skeletogenic mesenchyme derives from the mesoderm and neural crest. In the calvaria of mice, frontal bones are derived from cranial neural crest whereas parietal bones are derived from mesoderm (Jiang et al., 2002). These two embryonic origins can be mapped using the Cre/loxp system with LacZ as a reporter. At E10.5, all the CNC-derived mesenchyme in *Wnt1-Cre;R26R* mice are labeled with blue and condense in the frontonasal processes and the presumptive frontal primordium (Fig. 2A,B arrow), whereas the mesoderm-derived cells of *Mesp1-Cre/R26R* mice are stained blue and populate the future parietal primordium (Fig. 2A,B dotted line). Cells from these two origins are separated with a clear boundary. Interestingly, *Msx1* and *Msx2* were expressed only in the prospective frontal primordium (Fig. 1C–F arrow). There was no *Msx1* or *Msx2* expression in the prospective parietal primordium (Fig. 1C–F dotted line). At E12.5, both frontal and parietal primordia were well established, as indicated by alkaline phosphatase activity (Fig. 2G,I). *Msx1* is expressed only in the CNC-derived mesenchymal cells in the frontal bone primordium, not the parietal bone primordium (Fig. 2H,J,K,M,N arrows). *Msx2* was detectable in both the frontal and parietal primordia (Fig. 2L–P arrow). Thus, *Msx1* and *Msx2* were specifically co-expressed in the presumptive frontal bone primordium during calvaria development, suggesting possible concerted action in regulating the initiation of frontal bone development. In parietal bone development, however, there is no co-expression of *Msx1* and *Msx2* in the primordium and combined action is not likely to take place.

In addition to the divergent embryonic origins and differential expression of *Msx* genes, frontal and parietal primordia also vary in their rate of development. At E12.5, both primordia were

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positive for alkaline phosphatase activity (Fig. 2Q), however we detected osteoblast differentiation markers, such as *Dlx5* and *Osterix*, only in the frontal primordium (Fig. 2R,S arrow). Subsequently, the frontal bone matrix developed faster than the parietal bone matrix (Fig. 2T,U).

***Msx1* and *Msx2* are required for frontal bone primordium formation during the initiation of calvarial morphogenesis**

The failure of frontal bone development in *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice suggests that *Msx* genes may be specifically required for the initiation of frontal primordium morphogenesis. At E11.5, there is no clear mesenchymal condensation in wild type, *Msx1*$^{-/-}$ or *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ samples (Fig. 3A–C). At E12.5, the frontal bone primordium in wild type mice began to form as an aggregated cell mass above the developing orbit (Fig. 3D). This condensation of the CNC-derived mesenchyme prior to formation of the frontal bone is a critical step for normal development (Eames and Helms, 2004). In *Msx1* null mice, the frontal primordium was similar to that of wild type (Fig. 3E). There was no condensation of cells in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant (Fig. 3F). At E14.5, the frontal bone matrix began to form in both wild type and *Msx1* null mutant mice (Fig. 3G,H). There was some condensation of the CNC-derived cells in the frontal primordium of the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant, but no bone matrix formation (Fig. 3I). By E16.5, frontal bone formation had extended towards the top of the calvaria in wild type mice (Fig. 3J). In *Msx1* null mutant mice, frontal bone development had extended apically to a lesser extent than wild type (Fig. 3K). No bone matrix was ever detected in the frontal bone primordium of the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant with an open neural tube (Fig. 3L). This observation was later confirmed by bone differentiation marker analysis (see Fig. 5).

Loss of function of *Msx1* and *Msx2* does not affect the migration of CNC cells into the frontal primordium

The frontal primordium is composed exclusively of cranial neural crest-derived mesenchyme. In order to test whether the malformations of the frontal bone in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ animals resulted from a CNC migration defect, we generated *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice carrying a *Wnt1cre;R26R* transgene (*Msx1*$^{-/-}$*;Msx2*$^{-/-}$*;Wnt1cre;R26R*). The phenotype of these embryos was indistinguishable from that of unmarked *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice (data not shown). We observed comparable β-gal staining in whole embryo preparations of wild type and *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ samples at both E9.5 and E10.5, suggesting normal CNC migration into the craniofacial region in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice (Fig. 4A–D and data not shown). At the cellular level, there was no apparent difference in the contribution of the CNC-derived cells between wild type, *Msx1*$^{-/-}$ and *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice. Specifically, the frontonasal neural crest was primarily concentrated in the region above the eye where the frontal primordium was developing and within the nasal swellings in the wild type sample at E10.5 (Fig. 4A,B). There was normal migration of CNC cells into the region above the eye in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant (Fig. 4C,D). After the formation of the frontal primordium at E12.5, there was also no apparent difference in the distribution of CNC cells within the frontal primordium between wild type and *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice. CNC-derived cells condensed in a region dorsal to the developing eye where the frontal primordium was forming (Fig. 4E arrow). In the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant, there was no obvious deficiency of CNC-derived cells in the region of the frontal bone primordium (Fig. 4F). Taken together, our data indicate that a CNC migration defect was not responsible for the failure of frontal bone formation in *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice.

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CNC-derived mesenchymal precursors in the frontal bone region fail to differentiate into the osteogenic lineage

In mouse calvaria morphogenesis, a well-defined frontal primordium is visible at E12.5 with the expression of alkaline phosphatase and osteogenic lineage markers such as *Fgfr2*, *Dlx5*, *Runx2*, and *Osterix* in the CNC-derived mesenchymal cells (Fig. 5A–E). In *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ embryos, however, there is no expression of any of these osteogenic markers in the frontal primordium region (Fig. 5F–J). The CNC-derived frontal mesenchymal cells failed to express osteogenic lineage genes known to be critical for calvarial development at E14.5 (Fig. 5P–T) and E16.5 (Fig. 5Z) as well. Thus, our results strongly suggest that the CNC-derived mesenchymal precursors in the frontal bone region failed to commit into the osteogenic lineage.

Fgfr2 is a critical regulator for osteoprogenitor cell proliferation during frontal bone development (Iseki et al., 1999). Recent study shows that *Fgfr2*-deficient mice have bone development defects resulting from a decrease in osteoblast proliferation (Yu et al., 2003). To test whether *Fgfr2* acts downstream of Msx signaling, we compared the expression of *Fgfr2* in wild type and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant mice. In *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice, expression of *Fgfr2* in the presumptive frontal primordium was lost, whereas the expression of *Fgfr2* in the brain and surface epithelium remained comparable to the wild type littermate control (Fig. 5B,G). This specific absence of *Fgfr2* expression corresponds with the region of specific co-expression of *Msx1* and *Msx2* in the presumptive primordium. This loss of *Fgfr2* expression was also detected in the frontal primordium region of the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant at E14.5 (Fig. 5L,Q), again not in the brain and surface epithelium.

Next, we analyzed the expression of *Dlx5,* a critical mediator for calvarial osteoblast differentiation. Loss of *Dlx5* results in multiple craniofacial defects including malformed frontal bones (Acampora et al., 1999; Depew et al., 1999; Holleville et al., 2003a; Tadic et al., 2002). In vitro, Msx and Dlx5 are expressed at different stages of osteoblast differentiation (Ryoo et al., 1997) and Msx and Dlx5 can form heterodimers and regulate the promoter of the osteogenic lineage markers, *Osteocalcin* (Newberry et al., 1998; Zhang et al., 1997). To determine the effect of loss of both *Msx1* and *Msx2* on the expression of *Dlx5* during the differentiation of the CNC-derived frontal mesenchymal precursors, we examined the expression of *Dlx5* in wild type and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. At E12.5, we detected no *Dlx5* expression in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant presumptive frontal primordium, although well-defined *Dlx5* expression was present within the frontal primordium of the control sample (Fig. 5C,H). However, similar *Dlx5* expression in the brain was detected in both the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice and the control (data not shown). At E14.5, we also did not detect *Dlx5* expression in the frontal primordium of the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant, although expression in the brain was comparable in both the control and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice (Fig. 5M,R).

Finally, we examined the expression of osteogenic-lineage specific markers *Runx2* and *Osterix* in the frontal primordium to determine if an osteoprogenitor differentiation defect might be responsible for the failure of frontal primordium initiation in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant. *Runx2*, a *runt*-domain transcription factor, is expressed initially in the mesenchymal condensations of the developing skeleton, and the expression is strictly restricted to cells of the osteoblast lineage (Ducy et al., 1997). *Osterix*, a zinc finger-containing transcription factor, is expressed in the osteoblasts of all bones (Nakashima et al., 2002). Both *Runx2* and *Osterix* are required for osteoblast differentiation and osteogenesis in neural crest and mesoderm derived bones (Komori et al., 1997; Nakashima et al., 2002). By E12.5, both *Runx2* and *Osterix* were expressed in the frontal primordium of the wild type samples, whereas neither were detected in the presumptive frontal primordium of the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant (Fig. 5D,E,I,J). Neither *Runx2* or *Osterix* expression was detected in the frontal primordium region

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of *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice at E14.5 (Fig. 5S,T), suggesting a failure in osteogenesis. In contrast, the expression of both *Runx2* and *Osterix* was expanded in wild type mice at E14.5 (Fig. 5N,O).

The anterior neural pore failed to close in the majority of the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutants. The failure of osteoblast differentiation in the frontal primordium could be the result of excencephaly. However, our data suggest the contrary. A small number of *Msx1*$^{+/-}$*;Msx2*$^{-/-}$ mice (3 out of 240) were born with excencephaly. In these mice, apical extension of frontal bone is abolished but the CNC-derived frontal mesenchymal cells were able to differentiate into osteoblasts and form the orbital portion of the frontal bone at E16.5 (Fig. 5U,V). In contrast, the orbital portion of the frontal bone is missing in *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice at E16.5, along with the rest of the frontal bone (Fig. 5X,Y). In *Msx1*$^{+/-}$*;Msx2*$^{-/-}$ mice with excencephaly, the osteogenic lineage differentiation marker alkaline phosphatase can be detected at E12.5 (Fig. 5W). In *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice, we detected no alkaline phosphatase activity in the CNC-derived mesenchymal cells in the frontal primordium at E12.5 or E14.5 (Fig. 5F,P). The alkaline phosphatase staining of the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice was ambiguous in the frontal region at E16.5 (Fig. 5Z), but *in situ* failed to detect osteogenic lineage specific markers such as *Runx2* and *Osterix* (data not shown). Due to the scarcity of the excencephalic *Msx1*$^{+/-}$*;Msx2*$^{-/-}$ samples, we did not document the expression of osteogenic lineage markers in this group.

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Increased cell density fails to promote osteogenesis in the cultured CNC-derived frontal primordium of *Msx1*$^{-/-}$*/Msx2*$^{-/-}$ mice

In order to distinguish whether the differentiation defect of the CNC-derived frontal mesenchyme was the result of an intrinsic requirement for the *Msx* genes or a failure of CNC aggregation in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant with an open neural tube, we cultured these cells under an artificial high density (micromass) or monolayer condition. We dissected E11.5 presumptive frontal bone primordium from wild type and *Msx1*$^{-/-}$*/Msx2*$^{-/-}$ mutant embryos. Following twelve days of culture, an examination of the micromass cultures revealed nodule formation in wild type, *Msx1*$^{-/-}$, *Msx2*$^{-/-}$ and the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant samples (Fig. 6A–D). We were able to detect cartilage and bone formation in the wild type, *Msx1*$^{-/-}$, and *Msx2*$^{-/-}$ samples (Fig. 6E–G). Histological analysis confirmed the presence of alcian blue-positive cartilage structures with typical chondrocytes and compact, trabecular-like structures (Fig. 6I–K). We also detected deposits of calcium in wild type, *Msx1*$^{-/-}$, and *Msx2*$^{-/-}$ samples (Fig. 6M–O), but not in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ sample (Fig. 6P). In the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant, however, there was no indication of bone or cartilage development (Fig. 6H). Cross-section analysis showed that these cultured CNC cells failed to organize into any structure that resembles cartilage or bone (Fig. 6L). In addition, cultured frontal primordium CNC cells grown in a monolayer were able to express alkaline phosphatase in the wild type, *Msx1*$^{-/-}$, and *Msx2*$^{-/-}$ samples, confirming the successful differentiation of CNC into osteoblasts (Fig. 6Q–S). In contrast, there was no detectable alkaline phosphatase expression in cultured *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ CNC cells, indicating a failure of osteoblast differentiation (Fig. 6T). Taken together, our data suggest that there is an intrinsic requirement for *Msx1* and *Msx2* in the CNC-derived frontal bone primordium.

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Ectopic Runx2 restores osteogenic differentiation of the frontal CNC cells

The failure of CNC-derived frontal mesenchymal cells to differentiate into osteogenic lineage and to express osteogenic lineage specific transcription factor *Runx2* suggested that Runx2 may function downstream of Msx1 and Msx2 to promote the osteogenic differentiation of the CNC-derived frontal mesenchyme. To test this hypothesis, we transfected *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ frontal mesenchymal cells with a Runx2-expressing vector and examined osteogenic differentiation using alkaline phosphatase activity as marker. We detected the expression of GFP and Runx2 in transfected wild type and *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ cells (Fig. 7A–C, G). Upon the

ectopic expression of *Runx2*, *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ cells expressed alkaline phosphatase at a level comparable to that of the wild type control, whereas the negative control, *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ cells treated with empty vector, remained unstained. (Fig. 7D–F).

BMP signaling pathway during frontal primordium establishment

Msx genes have been reported to regulate the expression of *Bmp* genes (Bei et al., 2000; Brugger et al., 2004). To investigate whether an alteration in the BMP signaling pathway is responsible for the failure of differentiation of the CNC cells within the frontal primordium, we surveyed the expression of *Bmp2*, *Bmp4*, and *Bmp7* after CNC migration and before the establishment of the primordium in wild type samples (Fig. 8A–L). Of these, *Bmp4* is the only *Bmp* actively expressed in the presumptive primordium (Fig. 8E–H, arrow). *Bmp* expression in hair follicles (Fig. 8F,G) and in the midbrain served as positive controls (Fig. 8E–H). *Bmp2* and *Bmp7* were expressed in hair follicles (Fig. 8B,C,J,K), but were absent from the frontal primordium (Fig. 8D,L).

Surprisingly, the expression of *Bmp4* in the presumptive frontal primordium persisted in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ samples. The expression of Bmp4 in wild type, *Msx1*$^{-/-}$, *Msx2*$^{-/-}$, and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ samples was indistinguishable (Fig. 8M–Q). To support this finding, we analyzed the phosphorylation state of the pan-Bmp Smads. In wild type, *Msx1*$^{-/-}$, *Msx2*$^{-/-}$, and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ samples, we also detected the phosphorylation and nuclear translocation of BMP Smads, a result consistent with the maintenance of BMP4 signaling (Fig. 8R–V).

Discussion

Msx genes function in calvarial development

One intriguing feature of the calvarial phenotype associated with *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice is the absence of parietal as well as frontal bones. However, we conclude that the parietal bone defect is not a direct result of the loss of function of Msx1 and Msx2. First, neither *Msx1* nor *Msx2* is expressed in the mesoderm that gives rise to the parietal primordium. In the parietal primordium, *Msx2* is only expressed when osteogenic differentiation is visible, while *Msx1* is absent throughout the early parietal bone development. In addition, skeletal staining of newborn samples shows that there is no obvious parietal bone defect in single *Msx* mutants (Fig. 1), consistent with previous reports on *Msx1*$^{-/-}$ or *Msx2*$^{-/-}$ mice. Frontal bones are the only component of the skull vault that suffered developmental defects in both *Msx1* and *Msx2* null mutant mice. In *Msx1*$^{-/-}$ mice, a deficiency of the medial portions of the frontal bones results in an enlarged anterior fontanel. In the parietal bone, there are no osteogenic defects, other than that a small region of the parietal bones overlaps (Satokata and Maas, 1994). *Msx2*$^{-/-}$ mice have an enlarged anterior fontanel at E18.5, indicating that frontal bone ossification is delayed. In contrast, the parietal fontanel is similarly sized in wild type and *Msx2*$^{-/-}$ mice even though the interparietal and supraoccipital bones are small and abnormally shaped (Satokata et al., 2000). We also found that the parietal fontanel in *Msx1*$^{+/-}$;*Msx2*$^{-/-}$ and *Msx1*$^{-/-}$;*Msx2*$^{+/-}$ mice are comparable that of wild type, whereas the frontal fontanel were greatly enlarged.

Thus, the parietal bone defects in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice are likely secondary to those of the cranial neural crest cells, possibly as a result of compromised CNC-derived meninges, as this interaction is required for parietal ossification (Jiang et al., 2002). Conditional inactivation of *Tgfbr2* in the neural crest cells leads to compromised parietal development, as a result of defective meninges (Ito et al., 2003). In further support of this hypothesis, the expansion of CNC cells toward the apical midline is limited in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice (Fig. 4F), suggesting a meninges defect. Unfortunately, markers for the early development of meninges have not been identified and most *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutants die before E16.5, so the meninges defect

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and the function of meninges during parietal bone development still requires further investigation. Meanwhile, the function of meninges during the development of frontal bone also needs to be addressed using other conditional gene targeting approaches.

The function of *Msx* genes in CNC cell induction and migration

The frontonasal skeleton derives from rostral cranial neural crest cells, which arise from the diencephalon and midbrain (Santagati and Rijli, 2003). The failure of frontal bone early development could be a result of aberrant CNC cells induction or migration given the fact that the expression patterns of the *Msx* genes are closely related to the development of neural crest cells in several species. In mammals, the early expression of *Msx1* and *Msx2* demarcates the area from which neural crest cells emigrate (Hill et al., 1989; Mackenzie et al., 1991). Expression of *Msx1* and *Msx2* in the cranial neural crest continues during cell migration and colonization of the branchial arches (Catron et al., 1996; MacKenzie et al., 1992). Furthermore, forced expression of *Msx1* in *Xenopus* embryos induces neural crest cells, and inhibition of Msx1 activity by dominant negative Msx1 inhibits the production of neural crest cells (Tribulo et al., 2003). In *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice, there are defects in the specification or distribution of subpopulations of cranial neural crest cells. Reduced β-galactosidase staining was evident in the stream of neural crest from rhombomere 6 and 7 in *Wnt1-Cre;R26R;Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice (Ishii et al., 2005). Our data suggests that CNC migration into the frontal bone primordium is unaffected in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. Post-migratory CNC cells survived until the differentiation stages. One explanation for the lack of a neural crest induction defect may be functional redundancy of *Msx1* and *Msx2* with the remaining member of the *Msx* gene family, *Msx3*, which is expressed within the neural tube. However, the rostral boundary of *Msx3* expression is the rhombencephalon (Wang et al., 1996), whereas the frontal bone-contributing CNC cells derive from prosencephalon and mesencephalon. Therefore, the expression patterns of the three *Msx* genes are not consistent with an argument for functional overlap. In addition, *Msx1* and *Msx3* execute distinct functions in the neural tube when overexpressed (Liu et al., 2004). We consider it unlikely that *Msx3* compensates for the loss of *Msx1* and *Msx2* function. Another possibility is that the *Msx* gene family is not necessary for the formation and migration of cranial neural crest cells destined to populate the frontonasal process, but is instead involved in survival, proliferation and differentiation during and/or after migration. This model was also confirmed by previous work (Ishii et al., 2005).

Msx genes control CNC-derived osteoblast differentiation in the frontal primordium

The compromised frontal bone primordium and the failure of the frontal mesenchyme to express *Runx2* in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ embryos suggests that *Msx* genes are critical for the differentiation of post-migratory CNC cells into the osteogenic lineage. This differentiation defect is not secondary to the loss of CNC-derived cells within the presumptive primordium because our cell fate mapping data from *Wnt1-Cre;R26R;Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice reveals a comparable contribution from CNC-derived mesenchymal cells (see Fig. 4E,F). The differentiation defect is not due to the failure in neural tube closure either, because the orbital portion of the frontal bone is formed in *Msx1*$^{+/-}$;*Msx2*$^{+/-}$ embryos with excencephaly and the differentiation of post-migratory CNC cells followed the same temporal pattern as the wild type control (see Fig. 6). Reduced cell proliferation and condensation cannot explain the differentiation defect either, because culturing the E11.5 mesenchymal cells from the putative frontal primordium in an artificially high cell density failed to rescue the defect. Osteogenic culture condition failed to induce the differentiation in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ cells either, so the defect is more likely to be intrinsic.

The rudimentary maxilla and mandible present in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice appear to contradict a model positing the intrinsic requirement of Msx1 and Msx2 function for osteogenic lineage differentiation in all CNC cells. However, the maxilla and mandible are derived from

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neural crest cells originating in the hindbrain, whereas the frontal and nasal bone are derived from neural crest cells originating from forebrain and midbrain (Helms and Schneider, 2003). Thus, bones derived from the first branchial arch are partly formed in the absence of Msx1 and Msx2, but bones derived from the frontonasal process fail to form, suggesting that there may be an intrinsic requirement for Msx1 and Msx2 function during osteoblast differentiation in the frontonasal process-derived bones but not in the branchial-arch derived bones. It is also possible that Msx1 and Msx2 control some inductive step(s) in calvaria morphogenesis that is different from the mechanism involved in osteogenesis in jaws. More detailed studies on the tissue-tissue interaction during the establishment of the frontal bone primordium might shed light on this issue.

Multiple studies have addressed the function of *Msx* genes in osteogenesis (Cheng et al., 2003; Satokata et al., 2000; Shirakabe et al., 2001), but their conclusions have not been consistent. For example, stable, forced Msx2 expression in MC3T3E1 cells prevented osteoblast differentiation and matrix mineralization (Yoshizawa et al., 2004), but viral Msx2 induced osteogenic differentiation in the aortic myofibroblast (Cheng et al., 2003). The variation of cell types and developmental stages might contribute to the differences observed in the function of *Msx* genes in osteogenesis. Most reported functions of *Msx* genes have derived from studies in committed progenitor cells, although the expression of *Msx* genes occurs prior to the appearance of the osteoprogenitors. Limited study has been focused on the function of the *Msx* genes in the initiation of the frontal primordium. Null mutation of *Msx2* leads to a reduction in size of the frontal primordium (Ishii et al., 2003). Early differentiation markers, such as alkaline phosphatase and *Runx2,* were expressed in *Msx2*$^{-/-}$ mice with the same temporal pattern as wild type although the expression domain is reduced in size, suggesting that the CNC cells are competent to differentiate into the osteogenic lineage in the absence of Msx2 (Ishii et al., 2003). In contrast, *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice completely lack *Runx2* and *Osterix* expression in the frontal primordium, providing the first *in vivo* evidence for the requirement of *Msx* genes in the differentiation of the CNC-derived frontal mesenchymal cells into the osteogenic lineage. *Msx2* has been shown to induce alkaline phosphatase activity and promote calcification in *Runx2*-deficient mice (Cheng et al., 2003; Ichida et al., 2004). It is possible that *Msx* genes promote osteoblast differentiation in both *Runx2*-dependent and *Runx2*-independent manners.

Msx genes are critical for the expression of *Runx2* in the frontal primordium

The failure of CNC-derived mesenchymal cells to express *Runx2* and *Osterix* in the absence of *Msx1* and *Msx2* may prevent osteogenic differentiation. Runx2 is an essential transcription factor controlling osteoblast differentiation. Null mutation of *Runx2* leads to a complete lack of ossification in both neural crest and mesoderm derived bones (Komori et al., 1997). In primary bone marrow stromal cells, exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization (Byers and Garcia, 2004). We have shown above that the ectopic expression of *Runx2* in *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ frontal mesenchyme partially restored the differentiation of CNC-derived cells into osteogenic lineage, based on alkaline phosphatase activity. The partial rescue of differentiation may be due to incomplete transfection efficiency or to a requirement for additional factors. Previously, Msx2 has been reported to promote osteoblast differentiation independent of Runx2 (Ichida et al., 2004). Expression of Runx2 with a stably-expressing cell line or virus transfection with efficiency over 90% might help resolve this issue in the future. Nevertheless, our data suggests that Runx2 mediates the effect of Msx1 and Msx2 on the differentiation of CNC-derived frontal mesenchyme into the osteogenic lineage. However, to date, there is no evidence for a direct interaction of Msx with the Runx2 promoter (Otto et al., 2003). It is also possible that Msx genes regulate *Runx2* expression indirectly through a network of other factors.

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Osterix is expressed in all developing bones and the mesenchyme of membranous bones which failed to differentiate into osteoblasts in *Osterix* null mutant (Nakashima et al., 2002). However, the stable expression of *Osterix* in fibroblasts is not sufficient for osteogenic lineage commitment. Forced expression of *Osterix* stimulates osteopontin expression but not the activity of alkaline phosphatase or the expression of *Collagen type I* and *Osteonectin* (Kim et al., 2006). *Osterix* is not expressed in *Runx2* null mutant mice, but *Runx2* is expressed in *Osterix* null mutant mice (Nakashima et al., 2002). Also, the *Osterix* promoter contains putative binding sites for *Msx2* and *Runx2*. Both Msx2 and Runx2 mildly increase *Osterix* promoter activity (Lu et al., 2006). Osterix likely functions downstream of Runx2.

Msx genes control the differentiation of frontal CNC cells through signaling pathways other than BMP

BMPs function as one of the important regulators of *Msx* genes (Brugger et al., 2004) (Tribulo et al., 2003) (Zhang et al., 2003b). Both *Msx1* and *Msx2* can be induced by BMP4 during early tooth and suture development (Bei and Maas, 1998; Kim et al., 1998). Interestingly, *Msx* can also function to regulate Bmp expression in tooth development and digit regeneration (Bei et al., 2000; Bei and Maas, 1998; Han et al., 2003). BMPs have been also associated with the formation, migration, and differentiation of neural crest cells and skeletal morphogenesis (Barembaum and Bronner-Fraser, 2005; Goldstein et al., 2005; Kanzler et al., 2000; Kleber et al., 2005; Mbalaviele et al., 2005). However, it has been difficult to determine the function of BMP signaling in osteogenesis using individual *Bmp* deficient mice. Most Bmp knockout mice exhibit mortality too early to detect skull defects (Chen et al., 2004; Winnier et al., 1995; Zhang and Bradley, 1996). Others show no obvious skull defects (Kingsley et al., 1992; Solloway et al., 1998; Yan et al., 2001) or produce an increase in bone density (Daluiski et al., 2001). However, *Bmp1* deficient mice suffer reduced ossification of frontal, parietal, and interparietal bones (Suzuki et al., 1996) and *Bmp7* knockout mice have a skull patterning defect (Luo et al., 1995).

BMP4 has been reported to be involved in intramembranous bone growth. Exogenous BMP4 can increase tissue volume in calvarial culture, but does not accelerate osteogenic differentiation (Kim et al., 1998). In hematopoietic stem cells, conditional inactivation of *Bmpr1a* leads to an increase in the number of osteoblastic cells (Zhang et al., 2003a). Based on the comparable BMP signaling activity in the presumptive frontal primordium of $Msx1^{-/-};Msx2^{-/-}$ mice (see Figure 8) and the persistance of *Bmp4* expression in the first branchial arch (Ishii et al., 2005), we conclude that Bmp signaling is not likely required for osteogenic lineage differentiation in the frontal primordium. Due to the functional redundancy among BMPs and the early lethality of *Bmp2;Bmp4* or *Bmp5;Bmp7* double knockout mice, we have not been able to compare the calvarial phenotypes of compound *Bmp* and *Msx* mutants.

The reduced *Fgfr2* expression within the frontal primordium in the $Msx1^{-/-};Msx2^{-/-}$ samples suggests that FGF signaling might be compromised. In humans, gain-of-function mutations in *FGFR1*, *FGFR2*, *FGFR3*, and *MSX2* are linked to craniosynostoses (Cohen, 1997; Jabs et al., 1994; Jabs et al., 1993; Lomri et al., 1998; Muenke et al., 1997). In mice, however, inactivation of Fgf genes leads either to lethality too early to allow analysis of frontal primordium development (Arman et al., 1998; Deng et al., 1994; Feldman et al., 1995; Meyers et al., 1998) or to no obvious skull defects (Colvin et al., 1996; Hebert et al., 1994; Min et al., 1998; Ortega et al., 1998). However, there is evidence that FGF signaling is involved in calvarial development. In calvarial culture, FGF4 accelerates the ossification of the suture and induces its early closure (Kim et al., 1998). FGF2 can rescue the compromised osteogenitor proliferation of *Tgfr2* conditional knockout mice (Sasaki et al., 2006).

Other signaling pathways such as *TGF-β*, *Wnt* and *Hh* have also been shown to be important in regulating bone formation (Gaur et al., 2005; Ito et al., 2003; Mansukhani et al., 2000).

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However Shh and its receptor, Ptc, are not expressed during the initial establishment of the frontal primordium (Kim et al., 1998). Previous studies have suggested that Wnt signaling may be involved in osteogenesis in long bone development or cell lines, but the role of Wnt in skull development remains unclear (Hu et al., 2005; Monkley et al., 1996; Spencer et al., 2006).

In conclusion, we have shown that Msx1 and Msx2 act in concert to regulate the establishment of the CNC-derived frontal bone primordium. Moreover, *Msx* genes act upstream of *Runx2* to control osteogenesis during the development of the frontal bone primordium.

Experimental Procedures

Animals and tissue preparation

We generated $Msx1^{-/-};Msx2^{-/-}$ mice carrying the *Wnt1cre;R26R* transgene by crossing $Wnt1cre^{Tg/+};Msx1^{+/-};Msx2^{+/-}$ with $R26R^{Tg/+};Msx1^{+/-};Msx2^{+/-}$ mice. Embryonic age was determined as previous described (Theiler, 1989). Genotyping was performed as described previously (Chai et al., 2000; Saga et al., 1999; Satokata et al., 2000; Satokata and Maas, 1994; Soriano, 1999).

Detection of β-galactosidase (lacZ) activities

Whole embryos (E10.5) or frozen sectioned tissues were stained for β–galactosidase activity according to standard procedures (Chai et al., 2000)

Histology, skeleton staining and in situ hybridization

Histological analysis and whole mount skeleton staining of wild type and $Msx1^{-/-};Msx2^{-/-}$ samples were carried out as previously described (Ito et al., 2003). In situ hybridization was performed according to standard procedures (Xu et al., 2005).

Micromass and monolayer cell culture

The frontal bone primordium tissue block above the eye was dissected and removed from wild type and $Msx1^{-/-};Msx2^{-/-}$ embryos at E11.5. Cells were dissociated in dissociation buffer (0.1% collagenase, 0.1% trypsin EDTA in DPBS) for 15 minutes at 37ºC. Dissociated cells were pipetted into single cell suspension and plated at a concentration of 3.5×10^6 cells/ml in a 96-well plate in micromass culture medium (36% DMEM high glucose with pyruvate, 54% F12, 10% FCS, 50 μg/ml ascorbic acid) for 6 days. From day 7 to day 12, cultures were maintained in mineralization medium (50 μg/ml ascorbic acid, 10mM β-glycerophosphate in α–MEM) and terminated on day 12. For monolayer cell culture, cells were plated at 5×10^3 cells/ml in mineralization medium (10% FBS, 50 μg/ml ascorbic acid, 10mM β-glycerophosphate in α–MEM) for 7 days.

Constructs and transfection

pGFP-C1 vector, Runx2 cDNA were kindly given by Dr. Baruch Frenkel. The sequences of the constructs were confirmed by DNA sequence analysis. The empty pCMV5 vector was used as control. Transfection of primary frontal primordium mesenchymal cells was carried out using Lipofectmin 2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected 3 days after plating and were harvested at day 8.

Quantitative PCR analysis

Total RNA from the cultured cells were extracted using RNeasy mini kit and treated with RNase-free DNase I (Qiagen) following the manufacturer's protocol. The Superscript III with an oligo$(dT)_{20}$ primer (Invitrogen) was used for the first-strand synthesis. We carried out real-time RT-PCR on the iCycler (Bio-rad) with gene-specific primers and using SYBR Green. All

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reactions were under the following cycling protocol: 3 min heat start at 95°C and 40 cycles of denaturation at 95°C for 1 min, annealing and extension at 60°C for 1 min. We normalized relative expression ratios to β-actin. Primer sequences are available on request.

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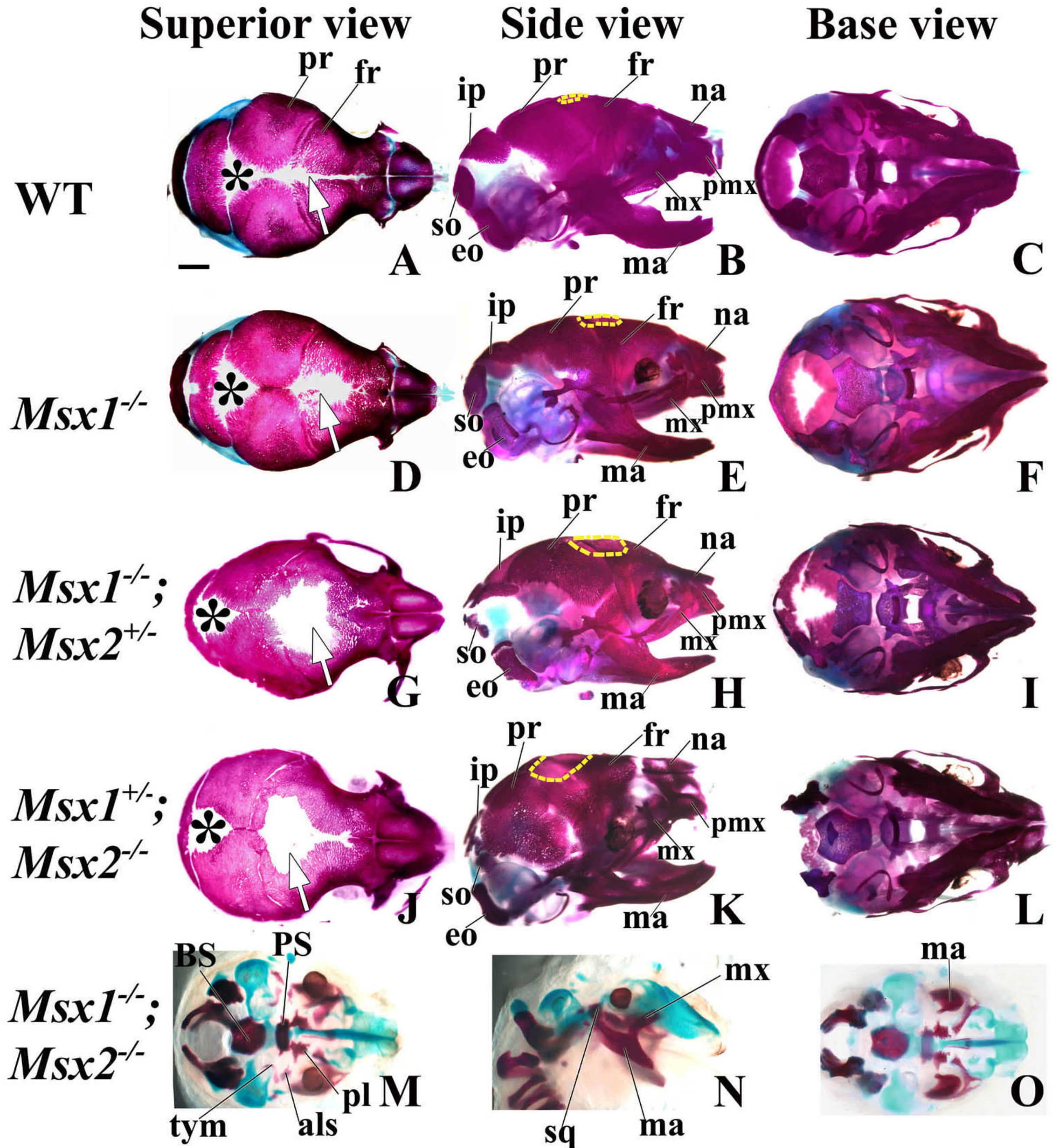

Fig. 1.

Msx1 and Msx2 are critical regulators for calvarial morphogenesis. Superior, side and base views of newborn skull preparations stained with Alcian blue and Alizarin Red (red= bone; blue= cartilage). (A–F) In the *Msx1*$^{-/-}$mice, there is a large gap between the two frontal bones in the superior view (arrow) and side view (yellow dotted line) of the skull. (G–L) In *Msx1*$^{-/-}$;*Msx2*$^{+/-}$ and *Msx1*$^{+/-}$;*Msx2*$^{-/-}$ mice, the defect between the two frontal bones is significantly enlarged. (M–O) *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice show complete agenesis of the calvaria and most of the bony elements of the skull. (als) alisphenoid (BS) basisphenoid (eo) exoccipital (fr) frontal (ip) interparietal (ma) mandible (mx) maxilla (na) nasal (pmx) premaxilla (pr) parietal (PS) presphenoid (so) supraoccipital (sq) squamosal (tym) tympanic.

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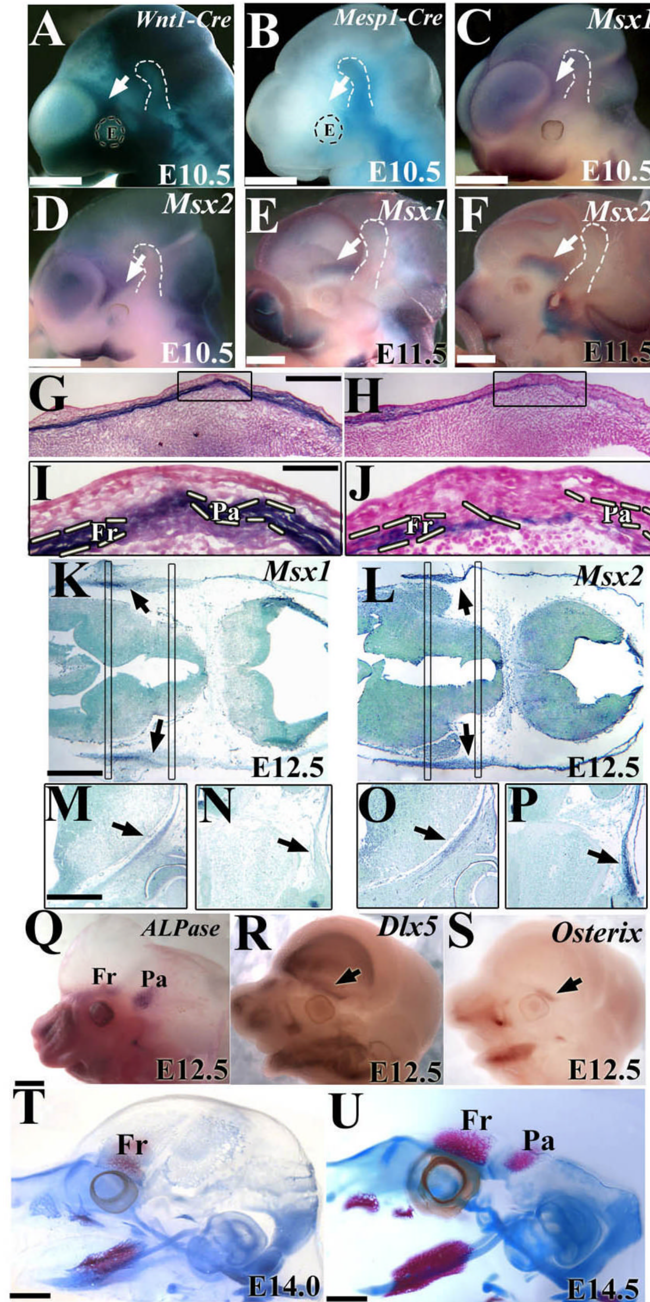

Fig. 2.

Msx1 and Msx2 are co-expressed in the prospective frontal primordium but not the prospective parietal primordium during their initiation. (A,B) After X-gal staining of *Wnt1-Cre;R26R* and *Mesp1-Cre;R26R* mice at E10.5, neural crest cells (arrows) and mesoderm mesenchyme (broken line) are clearly labeled separately with a distinct boundary. (C–F) Whole mount *in situ* hybridization of *Msx1* and *Msx2* at E10.5 and E11.5. The expression of *Msx1* and *Msx2* (arrows) are restricted to the CNC cell domain, with no expression in the mesoderm mesenchyme domain (broken lines). (G, I) Alkaline phosphatase staining of the horizontal section of the calvaria. Frontal and parietal primordia are stained blue. Open box is enlarged in I. (H, J) X-gal staining of the horizontal section of the *Wnt1-Cre;R26R* embryos. CNC-

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derived mesenchyme is stained blue. Open box is enlarged in J. (K–P) *In situ* hybridization of *Msx1* (K) and *Msx2* (L) in the horizontal section of the calvarial primordium at E12.5. Open bars indicate the section planes in M (*Msx1* in the frontal primordium), N (*Msx1* in the parietal primordium), O (*Msx2* in the parietal primordium), and P (*Msx2* in the parietal primordium). Arrows indicate the expression of *Msx1* and *Msx2*. (Q) Whole mount alkaline phosphatase staining at E12.5. Both frontal (Fr) and parietal primordia (Pa) are positive. (R,S) Whole mount in situ hybridization of *Dlx5* and *Osterix* at E12.5. The frontal primordium expresses both *Dlx5* and *Osterix* (arrows) but the parietal primordium does not. (T) Skeletal staining at E14.0 reveals the formation of the frontal bone (Fr) and the lack of any parietal bone staining. (U) Skeletal staining at E14.5 shows that the frontal bone (Fr) has developed further than the parietal bone (Pa).

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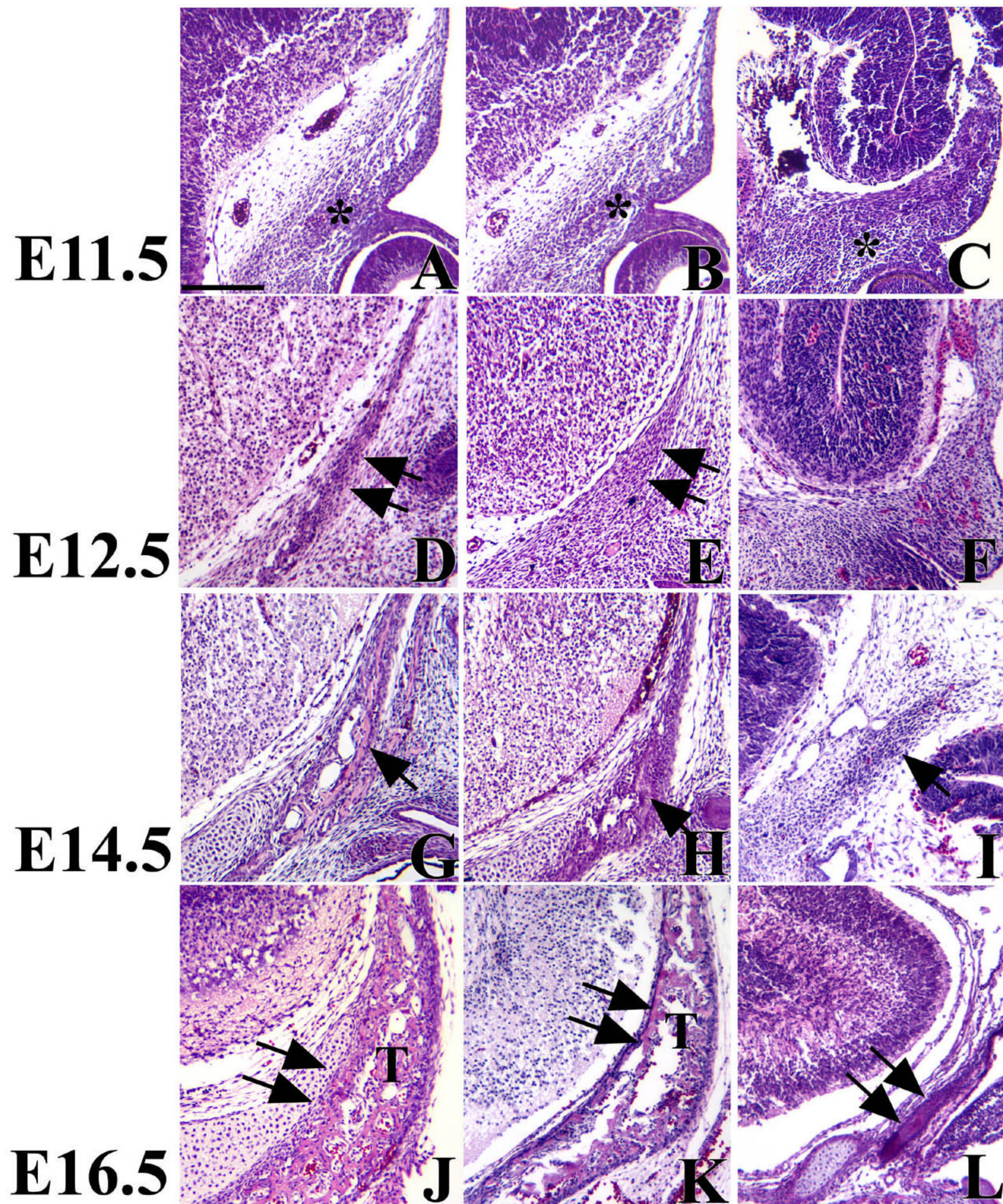

Fig. 3.
Compromised frontal primordium in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ embryos. Time course showing the development of the frontal primordium in wild type, *Msx1*$^{-/-}$, and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. (A–C) At E11.5, there is no obvious difference in cell density in the presumptive frontal primordium (*) between wild type and mutant littermates. (D–F) At E12.5, the mesenchyme forms a condensation (double arrow) in both wild type and the *Msx1*$^{-/-}$ mutant. The *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ embryos do not have a frontal bone primordium. (G–I) At E14.5, bone matrix is visible (arrow) within the frontal bone primordium of the wild type and *Msx1*$^{-/-}$samples. In the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutants, bone matrix fails to form (arrow). (J–L) At E16.5, frontal bone formation is evident in the wild type and the *Msx1*$^{-/-}$sample (double arrow) with the trabecular

structure (T). There is no bone matrix in the *Msx1*$^{-/-}$*; Msx2*$^{-/-}$ mutant, but there is an ectopic structure at the roof of the orbital cavity comprised of condensed mesenchymal cells (double arrows).

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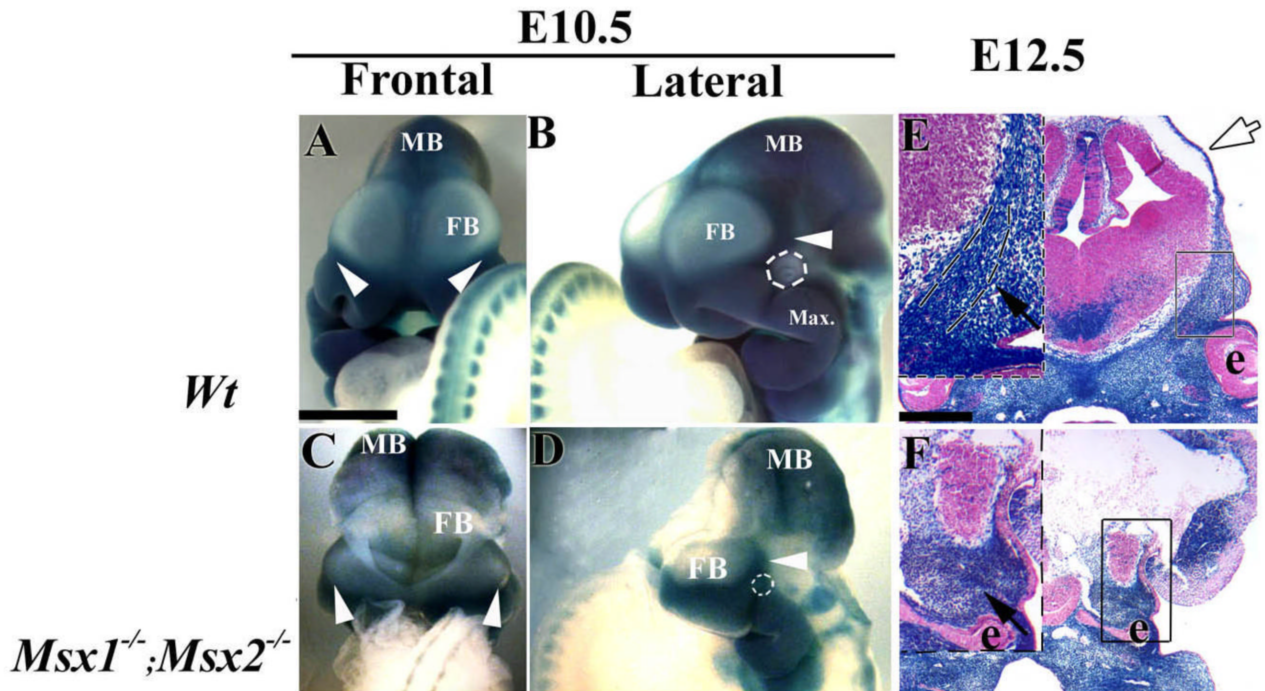

Fig. 4.

CNC cell migration is unaffected in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. LacZ staining of Wnt1-Cre;R26R (A,B,E) and Wnt1-Cre;R26R;Msx1$^{-/-}$;Msx2$^{-/-}$ (C,D,F) mice. (A–D) At E10.5, CNC cells have populated the first branchial arch and the frontonasal process, as indicated by positive β-gal staining (blue color). The CNC that contributes to the development of frontal bone primordium has migrated above the developing eye (arrowhead). The eye (e) is highlighted by a dotted white line. (FB) forebrain (MB) midbrain (Max) maxillary prominence (E–F) Cross-sections of E12.5 heads from wild type and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. CNC-derived mesenchyme in the putative frontal primordium is outlined with broken lines. Inserts show the enlarged CNC-derived frontal primordium (arrow). White open arrow points to the apical expansion of CNC cells (E).

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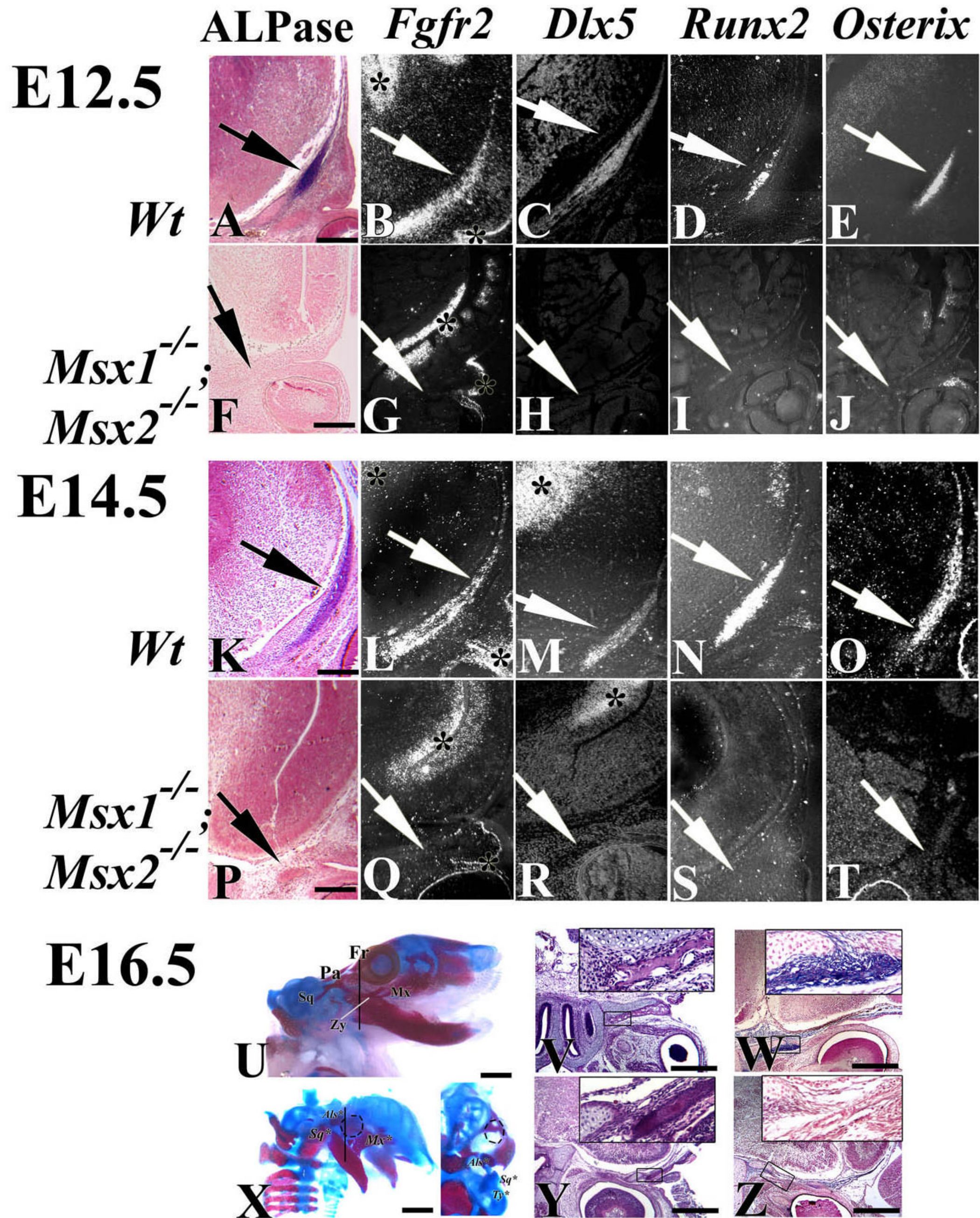

Fig. 5.

Failure of osteogenic progenitor cell differentiation in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. Alkaline phosphatase staining and in situ hybridizations of *Fgfr2*, *Dlx5*, *Runx2*, and *Osterix* in wild type and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice at E12.5, E14.5 and E16.5. (A–E) Well-defined frontal primordium with alkaline phosphatase (ALPase) activity and expression of *Fgfr2*, *Dlx5*, *Runx2*, and *Osterix* in wild type mice at E12.5. (F–J) Osteogenic markers were not detected in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant at E12.5. (K–O) At E14.5, frontal primordium with osteogenic differentiation markers expression expanded in the wild type mice. (P–T) None of the osteogenic markers expressed in the wild type frontal primordium were detected in the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mutant. (U,X) Side view (and top views) of the skeletal staining of E16.5

head shows that the orbital portion of the frontal bone is formed in the *Msx1*$^{+/-}$*;Msx2*$^{+/-}$ mutant with excencephaly but not in the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mutant. The black line indicates the section plane shown in V and Y. (V, Y) Coronal section of the frontal bone. The box in V and Y is shown enlarged in the insert. (W,Z) Alkaline phosphatase staining of the coronal section of the frontal primordium (box, enlarged in insert). The alkaline phosphatase activity is visualized with blue stain in the *Msx1*$^{+/-}$*;Msx2*$^{+/-}$ mice at E12.5 (W). Notice the lack of alkaline phosphatase staining (blue) in the presumptive frontal primordium region of the *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice (Z). Fr: frontal bone, Mx: Maxillary, Pa: parietal bone, Sq: squamous bone, Zy: zygomatic arch.

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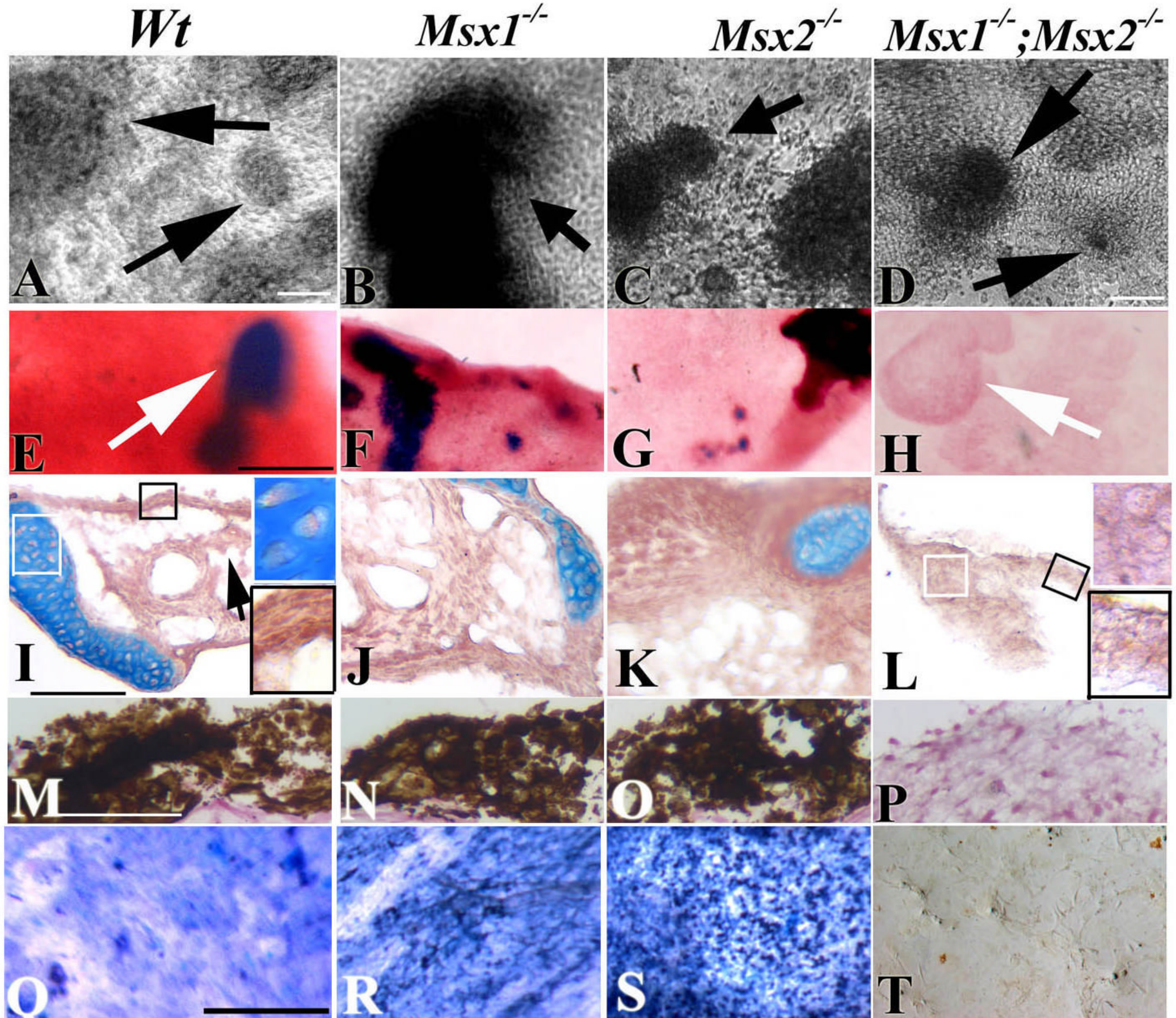

Fig. 6.

Artificially-enhanced cell density fails to rescue the differentiation defect of the CNC-derived mesenchymal cells in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. (A–D) Phase contrast light microscopy of micromass cultures of frontal primordium mesenchymal cells from wild type, *Msx1*$^{-/-}$, *Msx2*$^{-/-}$ and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mice. Arrows indicate nodule-like masses. (E–H) Whole-mount Alcian blue and Alizarin Red staining of the micromass cultures (cartilage in blue and bone in red). White arrows indicate the nodule-like masses. (I–L) Cross-sections of the samples in E–H. Staining of the wild type, *Msx1*$^{-/-}$, and *Msx2*$^{-/-}$ samples reveal trabecular-like structures (arrow) and condensed multiple layer structure (black box, enlarged image at the lower right insert). Cartilage development (in blue) and chondrocytes (white box, enlarged at the upper right inset). In the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ sample, there is no detectable bone or cartilage development. The black and white-boxed area (enlarged images are shown as inserts) does not contain any osteocytes or chondrocytes. (M–P) Von Kossa staining of micromass cultures reveals calcium deposition. (Q–T) Alkaline phosphatase activity (blue staining) in monolayer cultures of CNC-derived mesenchymal cells.

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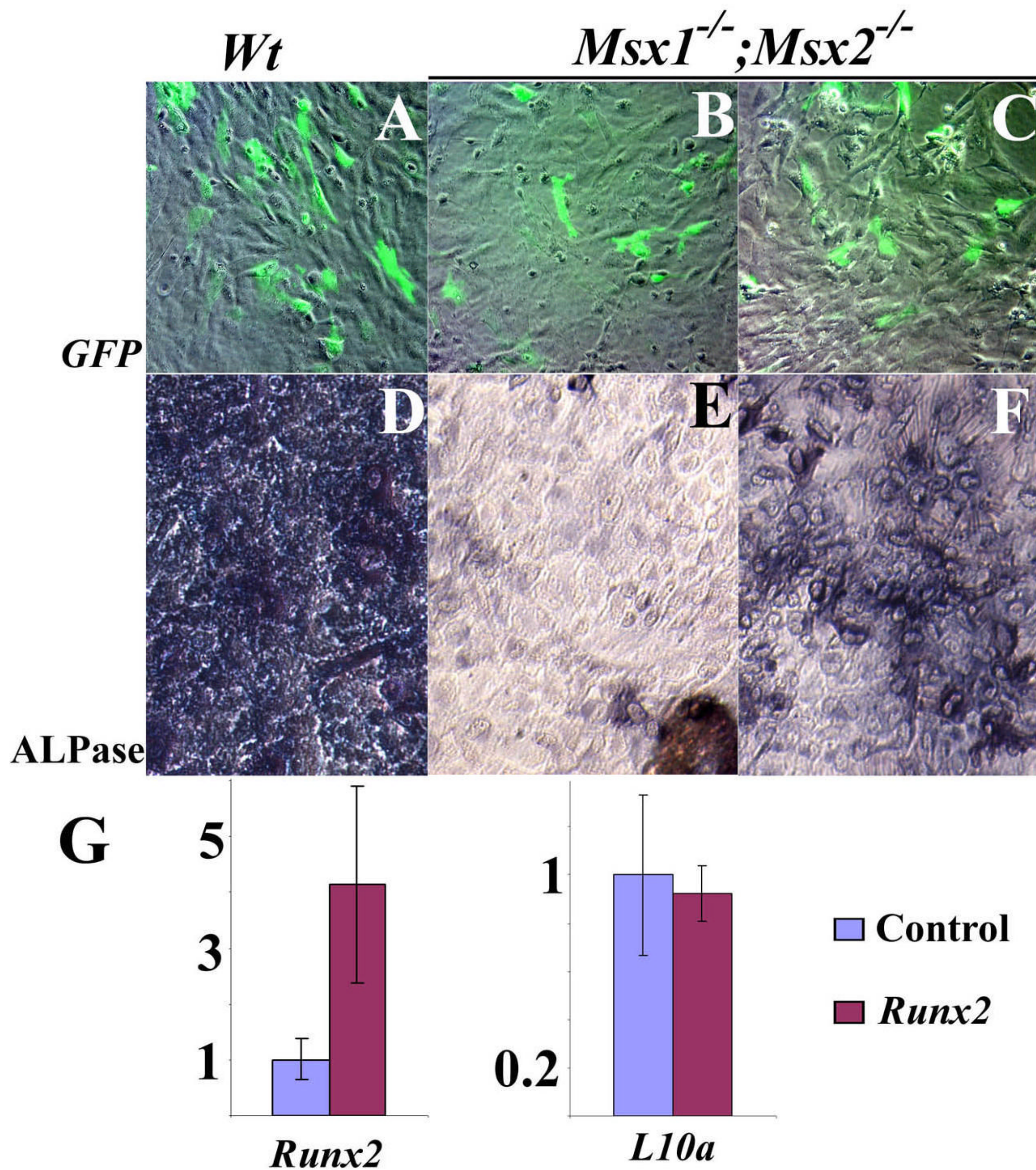

Fig. 7.

Ectopic expression of Runx2 rescued the differentiation defect of the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ frontal mesenchyme. (A–C) Merged images of phase contrast light microscopy and GFP fluorescence. (D–F) Alkaline phosphatase staining of wild type and *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ frontal mesenchyme culture after transfection. (G) Real-time PCR confirmed the ectopic expression of Runx2. Gene expression was normalized using β-actin. Ribosomal protein L10a was used as negative control.

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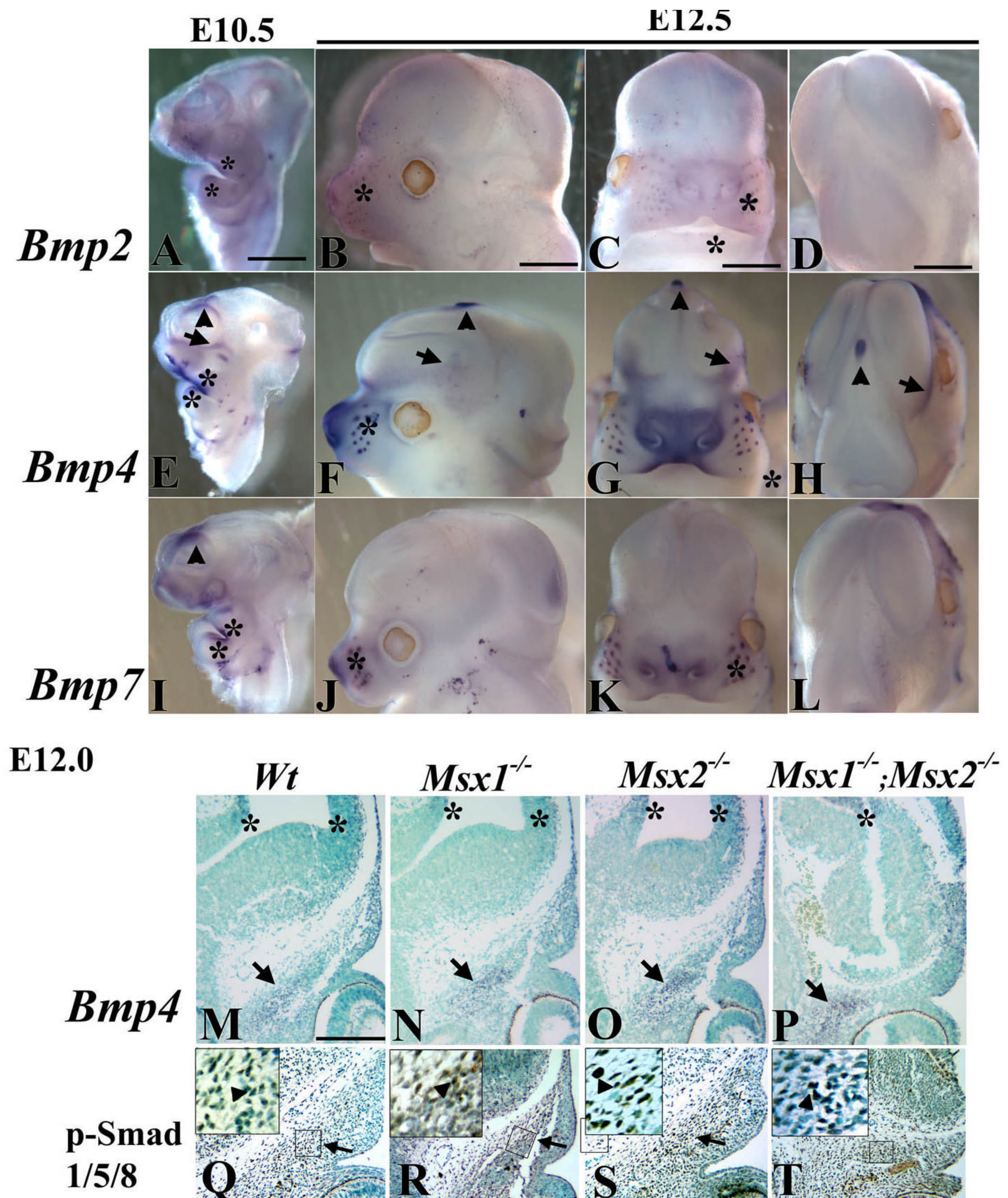

Fig. 8.

Bmp signaling pathway is largely unaltered. (A–L) Whole mount *in situ* hybridization of *Bmp2*, *Bmp4*, and *Bmp7* of E10.5 and E12.5 wild type mice. The expression in the whiskers and branchial arches (*) served as control. *Bmp4* is expressed in the frontal and midbrain region at E10.5 (arrow and arrowhead) and the frontal primordium and midbrain at E12.5 (arrow and arrowhead). *Bmp2* and *Bmp7* were not detected in the frontal primordium at E12.5. (M–Q) *In situ* hybridization of Bmp4 in sections from the presumptive frontal primordium at E11.5. Bmp4 is expressed in the future frontal primordium in wild type, *Msx1*$^{-/-}$, *Msx2*$^{-/-}$, and *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ samples (arrow). The expression of Bmp4 in the brain (*) served as control. (R–V) Immunostaining of phosphor-Smad1/Smad5/Smad8 in the presumptive frontal

primordium at E11.5. Phosphorylation of Smad1/5/8 is detected in all samples (arrow). Insert: enlarged box area.

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