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Msx1 and Dlx5 function synergistically to regulate frontal bone development

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Abstract

The *Msx* and *Dlx* families of homeobox proteins are important regulators for embryogenesis. Loss of *Msx1* in mice results in multiple developmental defects including craniofacial malformations. Although *Dlx5* is widely expressed during embryonic development, targeted null mutation of *Dlx5* mainly affects the development of craniofacial bones. *Msx1* and *Dlx5* show overlapping expression patterns during frontal bone development. To investigate the functional significance of *Msx1/Dlx5* interaction in regulating frontal bone development, we generated *Msx1* and *Dlx5* double null mutant mice. In *Msx1*^{-/-};*Dlx5*^{-/-} mice, the frontal bones defect was more severe than that of either *Msx1*^{-/-} or *Dlx5*^{-/-} mice. This aggravated frontal bone defect suggests that *Msx1* and *Dlx5* function synergistically to regulate osteogenesis. This synergistic effect of *Msx1* and *Dlx5* on the frontal bone represents a tissue specific mode of interaction of the *Msx* and *Dlx* genes. Furthermore, *Dlx5* requires *Msx1* for its expression in the context of frontal bone development. Our study shows that *Msx1/Dlx5* interaction is crucial for osteogenic induction during frontal bone development.

Keywords

Msx1; *Dlx5*; frontal bone; cranial neural crest cell

Introduction

Cranial neural crest (CNC) cells are a population of multipotent embryonic progenitor cells that migrate ventrolaterally from the neural tube, populate the frontonasal prominence and the branchial arches, and contribute extensively to the formation of mesenchymal structures in the head and neck (Chai et al., 2000). Frontal bones form the anterior aspect of the skull vault. The CNC-derived ectomesenchyme is the sole source for populating the frontal bone primordium and its underlying meninges in mice (Jiang et al., 2002). During the development of the frontal bone, CNC 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 frontal bone primordium synthesize bone matrix through intramembranous ossification (Couly et al., 1993; Jiang et al., 2002; Wilkie and Morriss-Kay, 2001; Han et al 2007).

The migration, proliferation and differentiation of CNC cells are regulated by growth factor signaling pathways and their downstream transcription factors. (Echelard et al., 1994;

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Graham and Lumsden 1993; Lumsden, 1988; Noden, 1983; Trainor and Krumlauf, 2000). The *Msx* and *Dlx* families of homeobox genes are expressed in the CNC cells to specify differential fates (Bendall and Abate-Shen, 2000). Expression of *Msx* and *Dlx* genes is primarily restricted to the sites of epithelial-mesenchymal interaction during organogenesis (Morasso et al., 1995; Ferrari et al., 1995; Sumoy et al., 1995). Several lines of in vivo evidence suggest that the *Msx* and *Dlx* families of homeobox proteins may represent regulatory genes that preferentially support skeletal tissue development (Jabs et al., 1993; Satokata and Maas., 1994; Mina et al., 1995). A notable feature of *Msx* and *Dlx* expression patterns is their relationship to the pattern of cellular proliferation and differentiation in multiple cell lineages that contribute to embryonic and adult structures (Bendall and Abate-Shen, 2000). In general, *Msx* gene expression is restricted to cells that are proliferating, whereas *Dlx* gene expression is primarily found in regions undergoing differentiation, or capable of doing so (Ryoo et al., 1997; Bendall and Abate-Shen, 2000).

Selective protein-protein interactions play an essential role in specifying the actions of homeoproteins (Zhang et al., 1997; Bendall and Abate-Shen, 2000). *Msx* and *Dlx* recombinant proteins form heterodimers via their homeodomains and reciprocally inhibit each other's activities (Zhang et al., 1997). In osteoblast differentiation, *Msx* activity counteracts the osteogenic-inducing property of *Dlx* during osteoblast differentiation (Kim et al., 2004). Thus, functional antagonism through heterodimer formation may provide a mechanism for regulating the transcriptional actions of *Msx* and *Dlx* in vivo (Zhang et al., 1997; Alappat et al., 2003). On the other hand, it has also been suggested that *Msx* and *Dlx* genes act independently in regulating the development of craniofacial skeleton (Levi et al., 2006). We have recently demonstrated that *Msx1* and *Dlx5* operate in parallel in regulating downstream target gene expression during palatogenesis (Han et al., 2009). These multiple modes of action of *Msx1* and *Dlx5* allow for diverse functional outcomes during organogenesis.

In both *Msx1*^{-/-} and *Dlx5*^{-/-} mice, frontal bone development was adversely affected. To investigate the functional significance of *Msx1/Dlx5* interaction in regulating frontal bone development, we generated *Msx1*^{-/-};*Dlx5*^{-/-} mice. The frontal bones of *Msx1*^{-/-};*Dlx5*^{-/-} mice showed even more severe defects than either *Msx1*^{-/-} or *Dlx5*^{-/-} mice. This aggravated frontal bone defect suggests that *Msx1* and *Dlx5* function synergistically to regulate osteogenesis during frontal bone development. In addition, *Msx1* appears to be required for *Dlx5* expression within the developing frontal bone primordium. Our study demonstrates that *Msx1* and *Dlx5* are involved in a tissue specific interaction in regulating frontal bone morphogenesis.

Materials and Methods

Generation of *Msx1*^{-/-};*Dlx5*^{-/-} mice

We generated *Msx1*^{-/-};*Dlx5*^{-/-} mice by crossing *Msx1*^{+/-};*Dlx5*^{+/-} mice with *Msx1*^{+/-};*Dlx5*^{+/-} mice. Embryonic age was determined as previously described (Theiler 1989). Genotyping was performed as described previously (Satokata and Maas, 1994; Depew et al., 1999; Han et al., 2007). All mice are maintained in a C57BL/6 background.

Histology, skeleton staining and in situ hybridization

Histological analysis and whole mount skeleton staining of wild type and mutant samples (n=3 per genotype per stage) were carried out as previously described (Ito et al., 2003). For in situ hybridization, embryos were fixed in 4% paraformaldehyde overnight at 4°C and processed into serial paraffin sections. Digoxigenin (Roche)-labeled anti-sense RNA probes were applied according to standard procedures (Xu et al., 2005; Han et al., 2007). Five

samples were analyzed from each genotype (wild type control, *Msx1*^{-/-}, *Dlx5*^{-/-}, and *Msx1*^{-/-};*Dlx5*^{-/-}) for each osteogenic differentiation marker per developmental stage.

Analysis of cell proliferation and apoptosis

Cell proliferation was assayed using BrdU incorporation. BrdU (5-bromo-2'-deoxyuridine, Sigma) solution was injected intraperitoneally with 100 µg body weight in E13.5 pregnant mice. One hour after injection, mice were sacrificed and embryos were fixed in 4% paraformaldehyde in PBS and processed. Detection of BrdU labeled cells was performed using a BrdU Labeling and Detection kit following the manufacturer's protocol (Zymed). Five randomly selected sections were analyzed for the number of BrdU positive cells over the total number of cells per unit area. Five samples were used in each genotype for the cell proliferation analysis. TUNEL assays to detect apoptosis were performed using the In Situ Cell Death Detection (fluorescent) kit (Roche Molecular Biochemicals) following the manufacturer's protocol. Five samples were used from each genotype for this analysis. ANOVA was used for statistical study and a p value of <0.05 was considered statistically significant.

Culture of frontal mesenchymal cells

The frontal bone primordium tissue block above the eye was dissected and removed from control and *Msx1*^{-/-} embryos at E12.5. Cells were dissociated in dissociation buffer (0.1% collagenase, 0.1% trypsin EDTA in DPBS, Gibco) for 15 min at 37°C. Dissociated cells were pipetted into single cell suspension and plated. Normal culture medium consisted of MEM-alpha (Invitrogen) containing 20% fetal bovine serum, 2 mM L-glutamine (Invitrogen), 55 nM 2-mercaptoethanol (Gibco) and 25 U/ml penicillin and 25 µg/ml streptomycin sulfate (Invitrogen). The cell culture medium was replaced every 48 hours until the end of experiment.

Transfection of *Msx1*-DNA construct into frontal bone primordium mesenchymal cells

Using the pAdTrack-CMV vector, *Msx1*-DNA construct containing GFP was generated. The sequences of the constructs were confirmed by DNA sequence analysis. The empty pAdTrack-CMV vector containing GFP was used as control. Transfection was carried out 3 days after plating of E12.5 frontal bone primordium cells as previously described (Han et al., 2007), and the transfected cells were harvested at day 6.

Quantitative PCR analysis

The mRNA levels of cultured cells were analyzed by real-time quantitative RT-PCR (Bio-Rad iCycler system). The mRNA was extracted and reverse-transcribed into cDNAs by using SuperScript™ First-Strand (Invitrogen life technologies). Real-time PCR was performed using a SYBR super mix kit (Bio-Rad) as previously described (Oka et al., 2007; Han et al. 2007).

Results

Msx1 and *Dlx5* have a synergistic effect on frontal bone morphogenesis

Msx1 and *Dlx5* are crucial regulators for cranial neural crest cell fate determination (Han et al., 2003; 2009). The frontal bone is entirely derived from cranial neural crest cells. In order to evaluate the functional significance of *Msx1* and *Dlx5* interaction in regulating CNC-derived osteogenesis, we generated *Msx1* and *Dlx5* double knockout mice and focused our study on frontal bone development. These *Msx1/Dlx5* mutant mice were able to survive until newborn stage, and we examined their calvaria at this stage. We confirmed the previous findings of calvarial ossification defects in frontal bones of *Msx1*^{-/-} and *Dlx5*^{-/-} mice (Fig.

1A–C). In *Dlx5*^{-/-} mice, this ossification defect was more enlarged than that of *Msx1*^{-/-} mice (Fig. 1C). The calvarial ossification defect of the frontal bones in *Msx1*^{-/-};*Dlx5*^{-/-} embryos was significantly enlarged compared to either *Msx1* or *Dlx5* single mutant mice (Fig. 1D). In addition to the defects in the frontal bones, we detected significantly enlarged bone defects in the parietal bones (Fig. 1A–D), occipital and supraoccipital bones, and smaller mandible (Fig. 1E–H), and sphenoid bones (Fig. 1I–L) of *Msx1*^{-/-};*Dlx5*^{-/-} mice. We decided to investigate the genetic interaction of *Msx1* and *Dlx5* in regulating frontal bone development because of our previous investigation on the concerted action of *Msx1* and *Msx2* in regulating frontal bone development (Han et al., 2007). Significantly, the more severe phenotype in the frontal bone of *Msx1*^{-/-};*Dlx5*^{-/-} embryos suggests that *Msx1* and *Dlx5* have a synergistic effect on frontal bone morphogenesis.

Compromised frontal bone primordium development in *Msx1*^{-/-};*Dlx5*^{-/-} embryos

To evaluate frontal bone morphogenesis, we examined the initiation and progressive development of the frontal bone primordium. In wild type mice, the frontal bone primordium begins to form as an aggregated cell mass above the developing orbit at E12.5 (Han et al., 2007). This condensation of the CNC-derived mesenchyme prior to formation of the frontal bone is a crucial step for normal frontal bone development (Eames and Helms, 2004; Han et al., 2007). In *Msx1* or *Dlx5* single null mice the frontal bone primordium was similar to that of wild type (Fig. 2A–C). However, we detected reduced condensation of mesenchymal cells in *Msx1*^{-/-};*Dlx5*^{-/-} mice (Fig. 2D). At E13.5, *Msx1*^{-/-};*Dlx5*^{-/-} mutant mice showed delayed development of the frontal bone primordium (Fig. 2E–H and inserts). At E14.5, bone matrix began to form within the frontal bone primordium in wild type, *Msx1*^{-/-}, and *Dlx5*^{-/-} mutant mice (Fig. 2I–J). In *Msx1*^{-/-};*Dlx5*^{-/-} mutant mice, bone matrix formation was detectable at E14.5, but the extent of bone matrix formation was reduced compared with *Msx1* or *Dlx5* single null mice (Fig. 2L). By E16.5, frontal bone formation had extended toward the top of the calvaria in wild type mice (Fig. 2M). In *Msx1* and *Dlx5* null mutant mice, frontal bone development had extended apically to a lesser extent than that of wild type (Fig. 2N, O). We detected only limited formation of frontal bone in *Msx1*^{-/-};*Dlx5*^{-/-} embryos (Fig. 2P). These findings suggest that there is a delay in formation of the frontal bone primordium and defect in the ossification process of the frontal bone primordium in *Msx1*^{-/-};*Dlx5*^{-/-} mice. This underdevelopment of frontal bone in *Msx1*^{-/-};*Dlx5*^{-/-} embryos, compared with *Msx1*^{-/-} or *Dlx5*^{-/-} mice, provides supportive evidence suggesting a synergistic mode of interaction of *Msx1* and *Dlx5* in regulating osteogenesis during frontal bone morphogenesis.

Reduced cell proliferation within the frontal bone primordium in *Msx1*^{-/-};*Dlx5*^{-/-} embryos

To explore the cellular mechanism for the underdevelopment of the frontal bone in *Msx1*^{-/-};*Dlx5*^{-/-} mutant embryos, we investigated whether there was a decrease in cell proliferation, an increase in apoptosis or a combination of both in the frontal bone primordium. At E13.5, cell proliferation activity within the frontal bone primordium, as measured by BrdU incorporation, was significantly reduced in *Msx1*^{-/-};*Dlx5*^{-/-} mutant embryos (n=5, *P*<0.001, see methods for details) as compared with wild type control, *Msx1*^{-/-}, or *Dlx5*^{-/-} embryos. In addition, *Msx1*^{-/-} and *Dlx5*^{-/-} embryos showed decreased cell proliferation activity (n=5, *P*<0.05) compared to wild type control. The decrease in cell proliferation activity was greater in *Dlx5*^{-/-} embryos than *Msx1*^{-/-} embryos (Fig. 3A–E). Thus, our data suggest that *Msx1* and *Dlx5* function synergistically to promote cell proliferation within the frontal bone primordium during calvarial morphogenesis.

To investigate whether an increase in apoptosis might have contributed to the failure of frontal bone development in the *Msx1*^{-/-};*Dlx5*^{-/-} mice, we performed TUNEL assay to detect apoptosis in the frontal bone primordium. At 14.5, no apoptotic activity was

detectable in the frontal bone primordium (Fig. 3F–I). At E16.5, we detected some TUNEL positive signals in the developing frontal bone. However, apoptotic activity is indistinguishable in wild type, *Msx1*^{-/-}, *Dlx5*^{-/-} and *Msx1*^{-/-};*Dlx5*^{-/-} embryos (Fig. 3J–M), suggesting that loss of *Msx1* and *Dlx5* does not affect cell survival during frontal bone development. Taken together, these findings are consistent with a model in which reduced cell proliferation is responsible for the underdevelopment of frontal bone primordium and the reduction in size of frontal bone matrix in *Msx1/Dlx5* mutant mice.

Compromised osteogenic lineage differentiation within the frontal bone primordium in *Msx1*^{-/-};*Dlx5*^{-/-} mice

To investigate the possibility that a defect in osteoprogenitor differentiation might be responsible for the underdevelopment of frontal bone in *Msx1*^{-/-};*Dlx5*^{-/-} mice, we analyzed the expression of osteogenic lineage differentiation markers including alkaline phosphatase, *Runx2*, *Osterix*, *Osteopontin*, and *Osteocalcin* (n=4 per genotype per differentiation marker). At E14.5, the frontal bone matrix begins to form in the developing frontal bone primordium (Han et al., 2007). Thus, we investigated the differentiation status of osteoprogenitor cells within the frontal bone primordium at E12.5 and E14.5.

First, we examined the expression of alkaline phosphatase as an early osteogenic differentiation marker. In *Msx1*^{-/-};*Dlx5*^{-/-} mice, we failed to detect alkaline phosphatase expression at E12.5 (Fig. 4D), although it was detectable in wild type control, *Msx1*^{-/-}, and *Dlx5*^{-/-} samples (Fig. 4A–C). At E14.5, we detected alkaline phosphatase expression in the developing frontal bone in wild type, *Msx1*^{-/-}, *Dlx5*^{-/-}, and *Msx1*^{-/-};*Dlx5*^{-/-} embryos, but the region of alkaline phosphatase expression was reduced in *Msx1*^{-/-};*Dlx5*^{-/-} embryos compared to the control (Fig. 4E–H). Next, we examined the expression of osteogenic lineage specific markers *Runx2* and *Osterix*. *Runx2*, a runt-domain transcription factor, is expressed initially in the mesenchymal condensations of the developing skeleton, and its 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, and *Osterix* is likely to function downstream of *Runx2* (Komori et al., 1997; Nakashima et al., 2002; Han et al., 2007). We found that the expression of *Runx2* was reduced in *Msx1*^{-/-} and *Dlx5*^{-/-} mutant mice. The expression of *Runx2* was further reduced and limited to the lower part of the frontal bone primordium in *Msx1*^{-/-};*Dlx5*^{-/-} mice (Fig. 4I–L). The expression of *Osterix* was also reduced in *Msx1*^{-/-};*Dlx5*^{-/-} mice as compared to the control (Fig. 4M, P). Quantitative PCR analysis revealed that *Osterix* expression was significantly reduced in *Msx1*^{-/-};*Dlx5*^{-/-} mice relative to the control (Fig. 4P').

Finally, we investigated mature stage osteogenic lineage differentiation makers, *Osteopontin* and *Osteocalcin*. *Osteopontin* is secreted by osteoblastic cells and deposited into the bone matrix (Butler, 1989; Kasugai et al., 1991). *Osteocalcin* is known as bone gamma-carboxyglutamate (gla) protein. It is localized intracellularly and in all the extracellular compartments, and is concentrated at the mineralization front during bone matrix formation (Nefussi et al., 1997). In wild type, *Msx1*^{-/-}, and *Dlx5*^{-/-} mice, we detected expression of *Osteopontin* and *Osteocalcin* in the developing frontal bone (Fig. 4Q–T and data not shown). *Osteopontin* expression showed gradual reduction in *Msx1*^{-/-}, and *Dlx5*^{-/-} mice, respectively. In *Msx1*^{-/-};*Dlx5*^{-/-} embryos, we failed to detect the expression of *Osteopontin* at E14.5 (Fig. 4T). We also detected a delay in *Osteocalcin* expression in *Msx1*^{-/-};*Dlx5*^{-/-} embryos (data not shown). These findings suggest that there was a defect in the bone matrix maturation process in *Msx1*^{-/-};*Dlx5*^{-/-} mutant mice. Overall, our osteogenic lineage differentiation marker analysis and phenotypic analysis indicate that osteoprogenitor cells in

Msx1^{-/-}; *Dlx5*^{-/-} embryos have limited osteogenic potentials and a delay in bone matrix formation and maturation as compared with wild type control, *Msx1*^{-/-} or *Dlx5*^{-/-} mice.

Interaction of *Dlx5* and *Msx1* within the frontal bone primordium

Msx1 and *Dlx5* show overlapping expression patterns in the frontal bone primordium (Han et al., 2007). In order to test whether *Msx1* and *Dlx5* interaction plays a crucial role in regulating frontal bone development, we first tested if *Msx1* and *Dlx5* regulate each other's expression.

At E11.5, we detected *Msx1* but not *Dlx5* expression in the presumptive frontal bone primordium in wild type embryos. In *Dlx5*^{-/-} embryos, expression of *Msx1* was detectable, but *Dlx5* expression was not detectable in *Msx1*^{-/-} mice (Fig. 5A–D). Thus, *Msx1* appears to be expressed earlier than *Dlx5* in the presumptive frontal bone primordium. At E12.5, *Msx1* was expressed in the frontal bone primordium in wild type and *Dlx5*^{-/-} mice (Fig. 5E, F). *Dlx5* expression was also detectable in wild type mice at this stage (Fig. 5G). However, we failed to detect the expression of *Dlx5* in *Msx1*^{-/-} embryos (Fig. 5H). These findings suggest that the expression of *Msx1* within the frontal bone primordium begins earlier than *Dlx5*, and its expression requires *Msx1* in the context of frontal bone development.

Adenoviral mediated exogenous *Msx1* expression restores *Dlx5* expression in *Msx1*^{-/-} frontal mesenchymal cells

To test the hypothesis that *Msx1*-mediated *Dlx5* expression plays an important role during frontal bone development, we transfected *Msx1*^{-/-} frontal mesenchymal cells with *Msx1*-expressing adenoviral vectors containing GFP and examined the expression of *Dlx5* (Fig. 6A–C). Following three days of culture, cells from the frontal bone primordium were transfected with either control-GFP vector or *Msx1*-GFP vector. Cultured cells from the frontal bone primordium were successfully transfected with the adenoviral vector as demonstrated by GFP expression three days after the adenoviral transfection (Fig. 6C). We detected the expression of *Dlx5* in wild type control cells, whereas the expression of *Dlx5* in *Msx1*^{-/-} frontal mesenchymal cells was down-regulated. The level of *Msx2* expression in *Msx1*^{-/-} frontal mesenchymal cells was comparable to that of wild type control (Fig. 6D). After adenoviral-mediated expression of *Msx1*, frontal bone mesenchymal cells expressed *Dlx5* in *Msx1*^{-/-} mice at a level comparable to that of the wild-type control (Fig. 6E). In treatment with empty vectors, the reduced expression level of *Dlx5* in *Msx1*^{-/-} cells persisted (Fig. 6E). The expression of *Msx2* was comparable in wild-type control cells and vector transfected *Msx1*^{-/-} cells (Fig. 6E). Furthermore, transfection of *Msx1* did not alter the expression of *Msx2*, suggesting that the regulation of *Msx2* expression is independent of *Msx1*. Thus, ectopic expression of *Msx1* restored *Dlx5* expression in frontal mesenchymal cells and cell proliferation activity in the *Msx1*^{-/-} sample (Fig. 6E). Collectively, this experiment shows that restored *Msx1* expression in cultured frontal bone primordium cells can rescue *Dlx5* expression and cell proliferation activity in *Msx1*^{-/-} samples.

Discussion

Msx1 and *Dlx5* have a synergistic effect on frontal bone morphogenesis

During frontal bone development, *Msx1* and *Dlx5* show overlapping expression patterns (Han et al., 2007). Our study demonstrates that *Msx1* and *Dlx5* genes have a synergistic effect on frontal bone morphogenesis. Loss of *Msx1* and *Dlx5* results in a more severe frontal bone defect compared with either *Msx1* or *Dlx5* single null mutant. This synergistic interaction of *Msx1* and *Dlx5* during frontal bone development controls the proliferation and differentiation of osteoprogenitor cells as well as expression of osteogenic lineage differentiation markers including alkaline phosphatase, *Runx2*, *Osterix*, *Osteopontin*, and

Osteocalcin. We conclude that the reduced cell proliferation and limited osteogenic potentials that result from the defective interaction between *Msx1* and *Dlx5* are responsible for the underdevelopment of the frontal bone primordium and the reduction in size of the frontal bone matrix in *Msx1*^{-/-};*Dlx5*^{-/-} embryos.

This synergistic interaction of *Msx* and *Dlx* genes differs from previously published in vitro studies that show functional antagonism of the *Msx* and *Dlx* proteins based on the overlapping expression patterns of *Msx* and *Dlx* genes during murine embryogenesis and reciprocal inhibitory transcriptional properties of *Msx* and *Dlx* proteins (Zhang et al., 1997, Alappat et al., 2003). More recently, we have shown that *Msx1* and *Dlx5* operate in parallel in regulating downstream target gene expression during palatogenesis (Han et al., 2009). Clearly, *Msx1* and *Dlx5* can work either antagonistically, in parallel, or synergistically to regulate downstream target gene expression, depending on the cellular context. With this dynamic operating mechanism, the spatial and temporal specific interaction between *Msx1* and *Dlx5* produces a diverse array of functional outcomes that control organogenesis during embryonic development.

***Dlx5* requires *Msx1* for its expression in the context of frontal bone development**

During frontal bone morphogenesis, the expression of *Msx1* begins earlier than that of *Dlx5*. This sequential expression is related to the role of *Msx1* and *Dlx5* in the development of the CNC-derived frontal bone. The *Msx* gene family is involved in survival and proliferation during and/or after migration of CNC cells to the frontonasal skeleton (Ishii et al., 2005; Han et al., 2007). *Dlx* genes are all expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon, and in distal regions of extending appendages (Acampora et al., 1999). Among *Dlx* genes, *Dlx5* has the unique property of being expressed in condensing, differentiating and developing cartilages, bones and teeth (Acampora et al., 1999; Depew et al., 1999; Jeong et al., 2008). In osteogenic lineage differentiation of precursor cells, *Msx* and *Dlx5* are expressed at different stages (Ryoo et al., 1997, Newberry et al., 1998). The level of *Msx* gene expression is high in proliferating cells, and decreases after osteoprogenitor cells reach confluence. However, *Dlx5* expression is not detectable in the non-differentiated osteoblasts, but it is up-regulated during osteoblast differentiation (Ryoo et al., 1997). *Dlx5* is crucial for regulating the expression of the osteogenic lineage marker, *Osteocalcin* and promoting the maturation of bone cell phenotypes (Ryoo et al., 1997, Newberry et al., 1998, Depew et al., 1999).

This sequential expression pattern of *Msx1* and *Dlx5* during proliferation and differentiation of osteoprogenitor cells and the restoration of *Dlx5* gene expression in *Msx1*^{-/-} frontal bone primordial cells following exogenous *Msx1* expression suggest that *Dlx5* requires *Msx1* for its expression in the context of frontal bone development. However, we have yet to identify the regulatory mechanism by which *Msx1* directly or indirectly promotes *Dlx5* gene expression during calvarial morphogenesis. Nevertheless, the interaction between *Msx1* and *Dlx5* must play an essential role in promoting osteogenic differentiation. This genetic interaction affects both CNC-derived and mesoderm-derived cells in the calvaria, implying the synergistic interaction between *Msx1* and *Dlx5* might be a widely used mechanism in regulating calvaria morphogenesis.

Functional redundancy of *Msx* signals in frontal bone development

As frontal bone development proceeds to the later stages (E14.5), expression of *Dlx5* was detectable in *Msx1*^{-/-} mice. There are several possibilities for the delayed expression of *Dlx5* in the developing frontal bone of *Msx1*^{-/-} mice. One explanation may be functional redundancy of *Msx1* with the remaining member of the *Msx* gene family, *Msx2*. Consistent with this hypothesis, the overall frontal bone defect as well as the cell proliferation and

osteoprogenitor differentiation activity phenotypes in the frontal bone are less severe in *Msx1* null mutant mice compared with *Dlx5*^{-/-} mice. Moreover, our previous study demonstrated the concerted action of *Msx1* and *Msx2* in regulating CNC cell proliferation and differentiation as well as the functional redundancy of *Msx* genes during frontal bone development (Han et al., 2007). In *Msx1*^{-/-};*Msx2*^{-/-} mice, frontal bone development was completely absent, and the expression of *Dlx5* was not detectable in the frontal bone primordium (Han et al., 2007). In this study, the expression of *Msx2* was maintained in frontal bone primordium of *Msx1*^{-/-} embryos. Thus, *Msx2* may be responsible for inducing and maintaining *Dlx5* expression during later stages of the frontal bone primordium development in *Msx1*^{-/-} embryos.

Another possible explanation for the expression of *Dlx5* in *Msx1*^{-/-} embryos is BMP4 signaling. During osteogenic lineage differentiation of the dental mesenchyme, *Dlx5* may be a component of the BMP4 signaling pathway (Zhang et al., 2003). Ectopic expression of *Bmp4* in the dental mesenchyme in the absence of *Msx1* is able to initiate the induction of the osteogenic lineage, possibly by activating the expression of *Dlx5* (Zhang et al., 2003). In the developing avian skull, BMP signaling was shown to regulate *Dlx5* expression specifically in proliferating preosteoblasts, implying that *Dlx5* functions downstream of BMP signaling in regulating osteoblast differentiation (Holleville et al., 2003, 2007). In our previous study, we detected *Bmp4* expression in the frontal bone primordium of *Msx1*^{-/-} mice (Han et al., 2007). However, we consider it unlikely that BMP4 signaling is responsible for the expression of *Dlx5* in *Msx1*^{-/-} embryos. Our previous work showed persistent expression of *Bmp4* and pan-Bmp Smads in the presumptive frontal bone primordium in the *Msx1*^{-/-};*Msx2*^{-/-} embryos, but *Dlx5* expression was not detectable (Han et al., 2007). In addition, we detected *Bmp4* gene expression in the developing frontal bone of *Dlx5*^{-/-} and *Msx1*^{-/-};*Dlx5*^{-/-} embryos (data not shown). These findings imply that the *Bmp4* pathway is not directly related to *Msx1* and *Dlx5* mediated osteogenic differentiation during frontal bone development.

Msx1 and *Dlx5* are two crucial transcription factors that regulate an array of biological activities during organogenesis. Future studies that aim to elucidate the molecular mechanism of *Msx1* and *Dlx5* interaction must consider their relationship in a temporally and spatially specific manner. Multiple studies have shown that the dynamic mechanisms of *Msx1* and *Dlx5* interaction provide versatility and allow for divergent outcomes in regulating organogenesis.

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Fig. 1. *Msx1* and *Dlx5* have a synergistic effect on frontal bone morphogenesis

Superior, side and base views of newborn skull preparations from wild type (WT), *Msx1*^{-/-}, *Dlx5*^{-/-} and *Msx1*^{-/-};*Dlx5*^{-/-} mice stained with Alcian Blue and Alizarin Red (red, bone; blue, cartilage). (A–D) In the superior view, the space between the frontal and parietal bones (arrow heads) is visible. The frontal bone defect of *Msx1*^{-/-};*Dlx5*^{-/-} mice is significantly enlarged. (E–L) In addition to frontal bone defects, the *Msx1*^{-/-};*Dlx5*^{-/-} embryos showed significantly enlarged bone defects between the occipital bones (E–H), and sphenoid bones (I–L). (bs) basisphenoid, (eo) exoccipital, (fr) frontal, (ip) interparietal, (ma) mandible, (mx) maxilla, (na) nasal, (pmx) premaxilla, (pr) parietal, (ps) presphenoid, (so) supraoccipital

Fig. 2. Compromised frontal bone primordium in *Msx1*^{-/-};*Dlx5*^{-/-} mice

Time course from E12.5 to E16.5 showing the development of the frontal bone primordium using H&E staining in wild type (WT), *Msx1*^{-/-}, *Dlx5*^{-/-}, and *Msx1*^{-/-};*Dlx5*^{-/-} mice. (A–D) At E12.5, the mesenchyme forms a condensation (arrowheads) in wild type, *Msx1*^{-/-}, and *Dlx5*^{-/-} mice. The condensation of the mesenchymal cells is reduced in *Msx1*^{-/-};*Dlx5*^{-/-} embryos (D). (E–H) At E13.5, development of frontal bone primordium is delayed in *Msx1*^{-/-};*Dlx5*^{-/-} mice. Arrowheads indicate frontal bone primordium and the area has been enlarged in inserts. (I–L) At E14.5, bone matrix is visible (arrowhead) within the frontal bone primordium of the wild type, *Msx1*^{-/-}, and *Dlx5*^{-/-} samples. Bone matrix formation is reduced in *Msx1*^{-/-};*Dlx5*^{-/-} mice (L). Inserts highlight the difference in bone matrix formation between control and *Msx1*^{-/-};*Dlx5*^{-/-} mice. (M–P) At E16.5, frontal bone formation is evident in the wild-type, *Msx1*^{-/-}, and *Dlx5*^{-/-} samples (arrowheads). In *Msx1*^{-/-};*Dlx5*^{-/-} mice, bone matrix formation is compromised. Scale Bars: 100 μ m.

Fig. 3. Reduced cell proliferation within the frontal primordium in *Msx1*^{-/-}; *Dlx5*^{-/-} embryos (A–E) BrdU staining of wild type (WT), *Msx1*^{-/-}, *Dlx5*^{-/-} and *Msx1*^{-/-}; *Dlx5*^{-/-} mice at E13.5. Positive BrdU signals (brown dots in enlarged inserts) indicate active cell proliferation in the frontal bone mesenchyme of wild type control samples (A). In *Msx1*^{-/-} mice (B), cell proliferation activity is reduced, and there is an even greater decrease in cell proliferation activity in the frontal bone primordium of *Dlx5*^{-/-} mice (C). The reduction of cell proliferation is most severe in *Msx1*^{-/-}; *Dlx5*^{-/-} embryos (D). (E) Statistical analysis of cell proliferation activity within the frontal bone primordium. **P*<0.05, ****P*<0.001 (F–M) TUNEL assays of wild type (WT), *Msx1*^{-/-}, *Dlx5*^{-/-}, and *Msx1*^{-/-}; *Dlx5*^{-/-} mice at E14.5 and E16.5. Dotted lines indicate the frontal bone primordium. Scale Bars: 100 μm.

Fig. 4. Compromised osteogenic progenitor cell differentiation in *Msx1*^{-/-}; *Dlx5*^{-/-} mice
 Alkaline phosphatase staining at E12.5 (A–D) and E14.5 (E–H) and in situ hybridization analysis (positive signal in dark blue) of Runx2 (I–L), Osterix (M–P), and Osteopontin (Q–T) at E14.5 of wild type (WT) *Msx1*^{-/-}, *Dlx5*^{-/-}, and *Msx1*^{-/-}; *Dlx5*^{-/-} mice. (P') Quantitative PCR analysis shows a significant reduction in *osterix* expression in *Msx1*^{-/-}; *Dlx5*^{-/-} mice as compared to the control samples. Arrowheads indicate positive signal in the frontal bone mesenchyme. Open arrow indicates negative osteopontin expression in the frontal bone primordium (T). Scale Bars: 100 μm.

Fig. 5. Interaction of *Dlx5* and *Msx1* within the frontal bone primordium

(A–D) Whole mount in situ hybridization analysis of *Msx1* (A, B) and *Dlx5* (C, D) expression in E11.5 wild type (WT), *Msx1*^{-/-}, and *Dlx5*^{-/-} mice. Arrowheads indicate positive signal. White dotted area indicates eye. Open arrowheads indicate negative *Dlx5* expression in C and D. (E–H) In situ hybridization analysis of *Msx1* (E, F) and *Dlx5* (G, H) in E12.5 wild type (WT), *Msx1*^{-/-}, and *Dlx5*^{-/-} mice. Arrowheads indicate positive signal. Open arrowhead shows negative *Dlx5* expression in H. Scale Bar: 100 μ m.

Fig. 6. Adenovirus-mediated expression of *Msx1* restores *Dlx5* expression in *Msx1*^{-/-} frontal bone primordium mesenchymal cells
 (A, B) Phase contrast images of cultures of E12.5 wild type (WT) and *Msx1*^{-/-} (B) frontal bone primordium mesenchymal cells. (C) Immunofluorescence of cultured *Msx1*^{-/-} frontal bone primordium mesenchymal cells transfected with *Msx1*-expressing adenoviral vectors containing GFP. (D) RT-PCR of *Msx1*, *Dlx5*, *Msx2* and *Gapdh* in cultured wild type (WT) and *Msx1*^{-/-} frontal bone primordium mesenchymal cells. Graphs show quantified data with error bars. **P*<0.05. (E) RT-PCR of *Msx1*, *Dlx5*, *Msx2* and *Gapdh* in cultured wild type frontal bone primordium mesenchymal cells transfected with empty vector (WT + Vc) and *Msx1*^{-/-} cultured frontal bone primordium mesenchymal cells transfected with *Msx1*-expressing adenoviral vectors (*Msx1*^{-/-} + Ad-*Msx1*). Graphs show quantification of data with error bars. **P*<0.05. Vc: pAdTrack-CMV vector, Ad-*Msx1*: pAdTrack-CMV vector containing *Msx1*-DNA construct. Scale Bars: 100 μ m.