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REVIEW

Msx homeobox gene family and craniofacial development

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ABSTRACT

Vertebrate *Msx* genes are unlinked, homeobox-containing genes that bear homology to the *Drosophila muscle segment homeobox* gene. These genes are expressed at multiple sites of tissue-tissue interactions during vertebrate embryonic development. Inductive interactions mediated by the *Msx* genes are essential for normal craniofacial, limb and ectodermal organ morphogenesis, and are also essential to survival in mice, as manifested by the phenotypic abnormalities shown in knockout mice and in humans. This review summarizes studies on the expression, regulation, and functional analysis of *Msx* genes that bear relevance to craniofacial development in humans and mice.

Key words: *Msx genes, craniofacial, tooth, cleft palate, suture, development, transcription factor, signaling molecule.*

INTRODUCTION

Vertebrate craniofacial organs form from multiple embryonic tissues including the cranial neural crest derived cells, prechordal mesoderm, and the embryonic craniofacial ectoderm. Normal craniofacial morphology develops as a consequence of complex interactions between these embryonic tissues, and requires precise regulation of cell movement, growth, patterning, and differentiation of craniofacial tissues. Genetic studies have revealed the involvement of numerous genes in these processes, including genes encoding a variety of transcription factors, growth factors and receptors[1]. Mutations in genes that influence any of these processes would cause craniofacial abnormalities, such as facial clefting and craniosynostosis, which are among the most frequent congenital birth defects in humans[2]. Among the critical factors involved in craniofacial development are members of the *Msx* homeobox gene family. The vertebrate *Msx* genes were initially cloned from mice and identified as homologous to the *Drosophila muscle segment homeobox* gene (*msh*)[3, 4]. Subsequently, *Msx* genes have been isolated from a variety of organisms, including ascidians[5, 6], sea urchin[7], zebrafish [5, 8], frogs[9], birds[10-12], and humans[13]. The mammalian *Msx* gene family consists of 3 physically unlinked members, named *Msx1*, *Msx2*, and *Msx3*[14, 15]. *Msx3* is only expressed in the dorsal neural tube, in a pattern resembling that of the prototypical *Drosophila msh* gene[16, 17]. However, in developing vertebrate embryos, *Msx1* and *Msx2* are widely expressed in many organs; particularly at the sites where epithelial-mesenchymal interactions take place[15]. Most notably, *Msx1* and *Msx2* are strongly expressed in the developing craniofacial regions in an overlapping manner to some extent, indicating a role for *Msx* genes in craniofacial development[18-21].

Craniofacial morphogenesis

During embryonic development, the face and neck are derived from swellings or buds of embryonic tissue, the branchial arches that originate bilaterally on the head. The neural crest cells generate most of the skeletal and connective tissue structures of the craniofacial region, while the mesoderm forms the musculature and endothelial lining of arteries of the future face and neck. The establishment of pattern in the craniofacial region is partly determined by the axial origin of the neural crest

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cells within each arch and partly by regional epithelial-mesenchymal interactions[22, 23]. In the mouse embryo, cranial neural crest cells originate from the posterior midbrain-hindbrain regions and migrate ventrolaterally into the branchial arches[24-28]. Within the branchial arches, the different populations of crest cells do not intermingle, but instead maintain the positional cues acquired by their rostral-caudal origins in the brain. This segregation of crest cell populations is established early in organogenesis by the apoptotic elimination of crest cells from specific levels of the hindbrain, giving rise to three distinct streams of migratory crest cells. Although this patterning of crest cells depends upon their rostral-caudal origin, this pattern does show some level of plasticity[29-31]. For example, the knockout of *Hoxa-2* in mice caused the second arch to produce skeletal elements normally found in the first arch. This result suggests that the Hox genes can specify pattern in arches caudal to the first arch, which does not express this class of genes[32]. Further patterning of the crest cells within the arches involves a reciprocal series of epithelial-mesenchymal interactions mediated by several growth factor signaling pathways[33-38].

The mammalian face develops from the coordinated growth and differentiation of five facial primordia, the single medial frontonasal prominence, the paired maxillary prominences, and paired mandibular prominences, which are located around the primitive mouth or stomodeum, as illustrated in Fig 1a and 1b. As development proceeds in the frontonasal prominence, localized thickenings of the surface ectoderm called nasal placodes develop. These placodes invaginate, while their margins thicken, to form the nasal pits and the lateral and medial nasal prominences. The maxillary prominences of the first branchial arch grow toward the future midline of the face. They fuse with the lateral nasal prominence on each side, then fuse with the medial nasal prominences, and finally with the intermaxillary segment of the frontonasal process to form the upper jaw and lip. In a similar way, the paired mandibular primordia fuse along their medial edge to form the lower jaw and lip. The frontonasal prominence forms the forehead and nose. Fusion of these approaching primordia results in the formation of a bilateral epithelial seam, which is later replaced by connective tissue[39-41] giving rise to a confluent lip. Clefts of the upper lip occur as a result of the failure of the maxillary prominence to merge with the medial nasal prominences on one side (producing a unilateral cleft) or on both sides (producing bilateral clefts). Failure of fusion of the paired mandibular prominences occurs far less frequently and results in clefts of the lower lip and jaw[42].

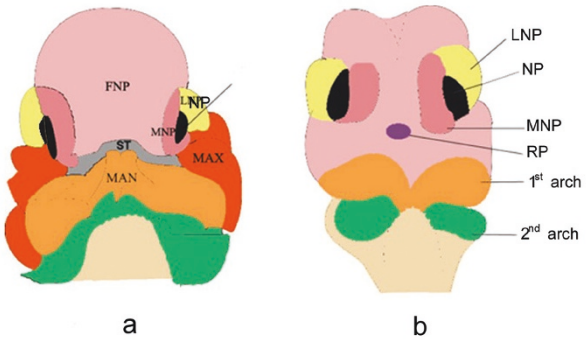

Fig 1. A schematic of the developing face in human and mouse embryos. (**a**) Frontal view of a head from a 37-day-old human embryo. (**b**) Frontal view of a head from a E10.5 mouse embryo. The medial frontonasal prominence (FNP, shown in light pink), the paired maxillary prominences (MAX, shown in red) and the paired mandibular prominences (MAN, shown in orange) constitute the five facial primordia that surround the primitive oral cavity, the stomodeum (ST, shown in grey). The nasal pits (NP, shown in black) are flanked by the lateral and medial nasal prominences (LNP in yellow and MNP in dark pink) which originate as placodes in the frontonasal prominence. The second branchial arch is indicated in green. RP, entrance to Rathke's pouch shown in magenta.

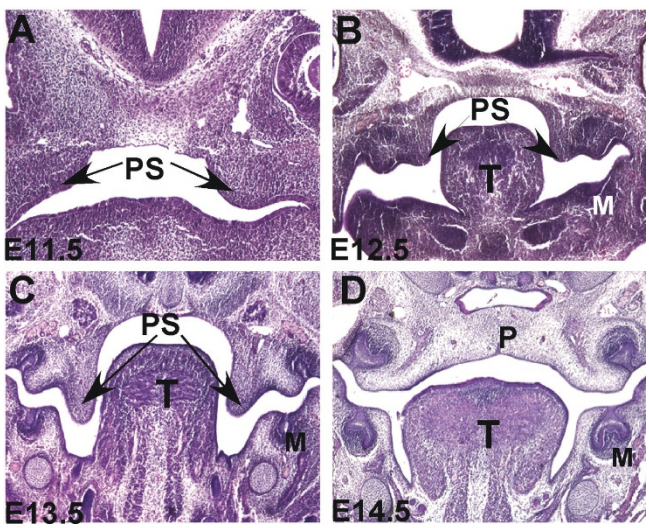

Fig 2. Histological sections of the mouse embryonic head showing representative stages of the developing secondary palate. (**A**) Frontal view of an E11.5 head showing bilateral palatal shelves projecting internally from the maxillary primordial. (**B** and **C**) the paired palatal shelves at E12.5 (**B**) and E13.5 (**C**) are vertically oriented on either side of the tongue. (**D**) At E14.5 the shelves are horizontally oriented above the dorsum of the tongue and are fused medially to form a closed palate. Abbreviations: M, molar tooth bud; P, palate; PS, palatal shelf; T, tongue.

Craniofacial morphogenesis continues with the outgrowth and fusion of tissues that form the palate or the roof of the mouth. The palate forms from two primordia, the primary palate and the secondary palate. A single, median, wedge-shaped mass of mesenchyme extending internally from the frontonasal prominence forms the primary palate. The secondary palate develops bilaterally as two vertical projections, the palatal shelves, from the internal surfaces of the maxillary prominences (Fig 2). As morphogenesis proceeds, the shelves become oriented horizontally allowing them to approach each other and fuse medially. Failure of the palatal shelves to fuse leads to a cleft palate. A number of human congenital syndromes such as Treacher Collins Syndrome and Pierre Robin Syndrome have accompanying craniofacial abnormalities, which include a cleft palate[43]. Misregulation of the timing, rate, or extent of outgrowth of the palatal shelves results in clefts of the palate[44, 45]. Failure of fusion of the palatal shelves often, though not always, occurs in conjunction with cleft lip[46].

Another important morphogenetic event in the facial tissues is odontogenesis and this phase of craniofacial morphogenesis has been extensively studied. Tooth formation is regulated by inductive tissue interactions between the oral epithelium and the subjacent mesenchyme of the first arch. The four histologically distinct stages of tooth development are: 1) the dental lamina, 2) the bud, 3) the cap, and 4) the bell stage (Fig 3)[47]. In the mouse, tooth initiation becomes morphologically distinguishable at E11.5 by the thickening of the dental epithelium to form the dental lamina at the prospective sites of tooth formation. The cells of the dental lamina proliferate and on E12.5, start to invaginate into the underlying mesenchyme. At the bud stage, the mesenchyme proliferates and condenses around the invaginating epithelial bud. As a result of differential proliferation, the dental epithelium next convolutes around the condensed mesenchyme (now referred to as the dental papilla) in the cap (E14.5) and bell stages (E16.5). E14.5 marks the onset of the definitive stages of tooth morphogenesis. In the cap and bell stages, transient signaling centers called primary and secondary enamel knots develop in the epithelium. They serve as organizing centers of tooth morphogenesis and cusp formation. In the final steps of odontogenesis, enamel-secreting ameloblasts and dentin-secreting odontoblasts differentiate from the dental epithelium and mesenchyme, respectively. Thus, an intricate set of epithelialmesenchymal interactions generates the species-specific pattern of odontogenesis.

Another craniofacial structure pertinent to our discussion on *Msx* genes is the skull. The skull (Fig 4) is a composite of multiple bones that are organized primarily into the neurocranium which includes the cranial vault and the viscerocranium that comprise the facial bones as well as the palatal, pharyngeal, temporal and auditory bones. The neurocranium whose function is to protect the brain and the sense organs derives from mesenchyme of both neural crest and mesodermal origin. The viscer-

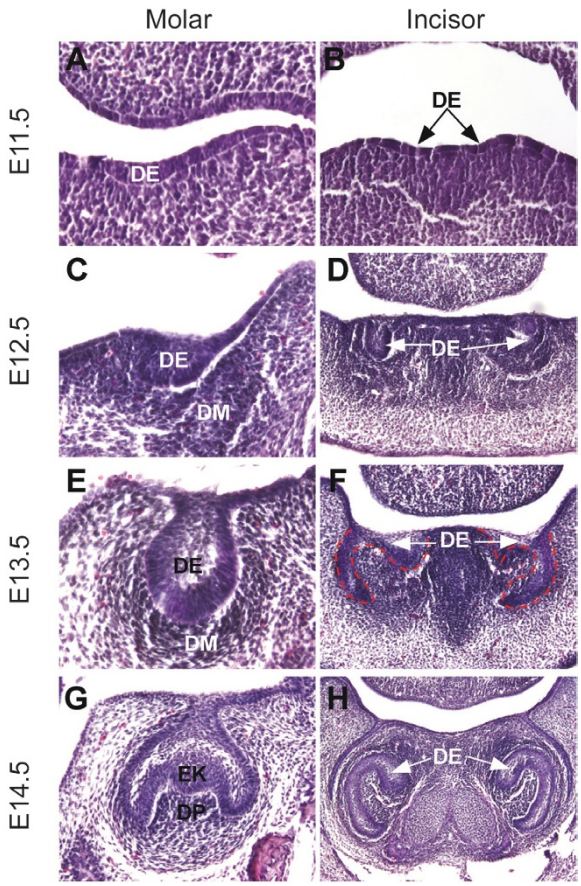

Fig 3. The representative stages of early tooth development in the mouse embryo. (**A**, **C**, **E**, **G**) molar tooth germ stages; (**B**, **D**, **F**, **H**) incisor tooth germ stages. (**A** and **B**) Dental lamina stage (E11.5): the oral epithelium thickens locally to form the molar and incisor tooth germs. (**C** and **D**) Early bud stage (E12.5): the epithelial thickening invaginates into the subjacent mesenchyme which condenses around the epithelial bud. (**E** and **F**) Late bud stage (E13.5): increased proliferation of the dental epithelium causes it to invaginate further into the dental mesenchyme; (**G** and **H**) Cap stage (E14.5): differential proliferation within the dental epithelium causes a population of the dental mesenchyme, the dental papilla, to be surrounded by the convoluting dental epithelium. Abbreviations: DE, dental epithelium; DM, dental mesenchyme; DP, dental papilla; EK, enamel knot.

ocranium by contrast is formed solely from neural crest mesenchyme. The calvaria or skull vault is formed by intramembraneous ossification of radially growing, discrete, mesenchymal condensates over the expanding brain. Fibrous, non-osteogenic membranes called sutures or fontanelles (wider sutures occurring at the juncture of several bones) separate the resulting calvarial bones formed by accretion growth. Further growth of the skull occurs by apposition at the lateral edges of the sutures, which are populated by highly proliferative preosteoblasts. Such a mechanism accommodates the constantly expanding brain. Unlike the skull vault, the base of the skull develops by endochondral ossification of the cartilaginous chondrocranium. Failures of inception, nonsynchronized growth or untimely ossification are some of the mechanisms contributing to dysmorphic development of the skull. The contribution of *Msx* genes in maintaining the delicate balance between proliferation and differentiation during pre- and post-natal skull morphogenesis will be elaborated upon later in this review.

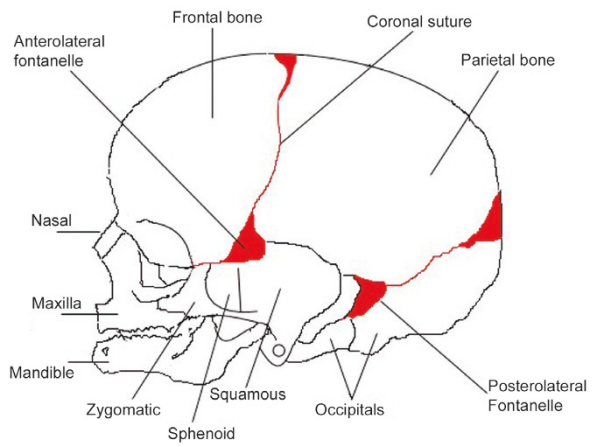

Fig 4. A schematic of the human skull showing the calvarial bones, fontanelles and sutures.

Msx genes encode transcription repressors

The Msx proteins are important modulators of craniofacial, limb, and nervous system development[16, 48, 49]. They are regulatory proteins that function as transcriptional repressors *in vitro* and *in vivo*[48, 50-55]. Protein-protein interactions, which engage residues within their homeodomain guide target gene selection and transcription regulation[53, 54]. The Msx homeodomain interacts directly with the TATA binding protein (TBP), the core component of the general transcription complex to execute transcription repression. Importantly, this ability to interact with members of the basal transcription machinery and affect transcription is not contingent upon their DNA-binding function[51]. Msx proteins also interact with other homeodomain proteins to regulate transcription. Heterodimers formed between *Msx1* and other homeodomain proteins such as *Dlx2*, *Dlx5*, *Lhx2* and *Pax3* result in mutual functional antagonism *in vitro*[55-57]. It is believed that tissues in which expression of *Msx1* overlaps these other proteins there may be such a regulatory mechanism in place. Although, both Msx1 and Msx2 show similar DNA-binding site preference as well as the ability to repress transcription they display different biochemical properties by virtue of unique N-terminal domains, which confer Msx2 with a greater affinity for DNA while rendering Msx1 a more potent repressor. A study of the three dimensional structure of Msx1 homeodomain/DNA complex reveals two major deviations from that of other homeodomain/DNA complexes[58]. Firstly, the presence of two non-canonical proline residues confers great stability and order to the N-terminal arm of the homeodomain, which tracks the minor groove of the DNA. Secondly, the DNA bound by the Msx1 homeodomain shows a 28° bend compared to the normal 21° observed with other homeodomain proteins.

Expression of Msx genes during craniofacial development

Overlapping expression of *Msx1* and *Msx2* are seen at multiple sites of tissue-tissue interaction including the craniofacial regions[20, 48, 59]. Through the course of murine craniofacial development, both *Msx1* and *Msx2* are detected in the forming skull and meninges, the distal aspects of the facial primordia, the associated sense organs, and teeth[18-20]. In the developing skull, *Msx1* and *Msx2* are expressed in the suture mesenchyme and dura mater. While *Msx1* expression extends into the postnatal stages of skull morphogenesis, *Msx2* registers a sharp decline in expression after birth.

The earliest restricted distribution of *Msx1* during tooth development is evident around E11.0 in the dental mesenchyme at the lamina stage, and the expression increases in the condensing dental mesenchyme at the bud stage (Fig 5). At the morphogenetic cap stages both the dental papilla and follicle express *Msx1* maximally. The expression begins to level off prior to the differentiation of the odontoblasts and ameloblasts. In the late stages of tooth morphogenesis, *Msx1* expression

is clearly absent from the root sheath epithelium and is rather weak in the dental pulp[60]. By extrapolation, it appears that *Msx1* does not support root morphogenesis in the developing tooth. Apart from the tooth, *Msx1* expression has been examined in the developing palate. Reports of a weak, diffuse expression of *Msx1* in the palatal mesenchyme provided the first evidence that *Msx1* may have a direct role in palate development[18, 61]. A more detailed analysis by Zhang et al[62] has reported that *Msx1* expression in the palatal mesenchyme is confined to the anterior portion of the developing palatal shelves.

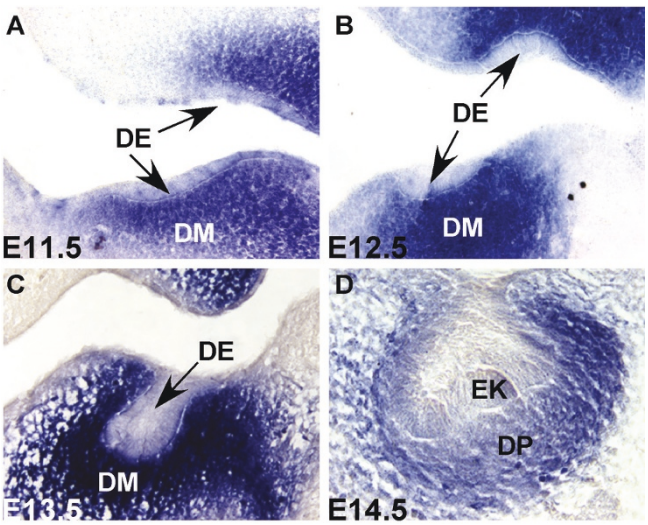

Fig 5. Murine *Msx1* expression in the early molar germs. *Msx1* expression is confined to the dental mesenchyme at E11.5 (**A**), E12.5 (**B**), E13.5 (**C**) and E14.5 (**D**). Maximal expression is seen at the bud stages E12.5 and E13.5. Abbreviations: DE, dental epithelium; DM, dental mesenchyme, DP, dental papilla; EK, enamel knot.

MSX2 expression is detectable by 7.5 weeks of human embryonic development[63]. In humans, the precursors of the orofacial skeleton such as the mandibular and maxillary bones, Meckle's cartilage, and tooth germs all express *MSX2*[63]. At the bud stage of developing tooth germ, *MSX2* is detectable in the vestibular lamina, and both the dental epithelium and mesenchyme. Later in development, *MSX2* expression is lost from the dental mesenchyme but is seen in the enamel knot and vestibular epithelium of the cap stage tooth. In mice, *Msx2* expression is continuous in the molar and incisor tooth germs [20, 61]. Unlike the developing incisors, the molar tooth germs show asymmetric distribution of *Msx2* at all developmental stages. Contrary to *Msx1*, whose expression is confined to the mesenchyme throughout tooth development, *Msx2* expression can be detected in both the epithelial and mesenchymal compartments of the developing tooth germs. The earliest indication of asymmetric expression is its buccal distribution seen within the invaginating dental lamina of molar tooth germs. At the cap stage, *Msx2* expression is prominently seen in the components of the enamel organ (the enamel navel, septum and knot) as well as the inner enamel epithelium. With the onset of the bell stage, *Msx2* expression is lost from the inner enamel epithelium as they differentiate into the ameloblasts. Instead, strong expression of *Msx2* is detected in the odontoblasts and the subondontoblastic regions of the dental papilla. Thus, the spatial and temporal expression of *Msx1* and *Msx2* genes appear to correlate with crucial aspects of craniofacial morphogenesis. In the next section we will consider the molecular hierarchy controlling their expression as well as their role in craniofacial patterning.

Regulation of Msx1 and Msx2 expression and facial patterning

The regulation of *Msx* gene expression is accomplished by diverse mechanisms involving retinoids, antisense 'quenching', growth factor regulation, and complementary/antagonistic interaction with other transcription factors. Retinoid regulation of *Msx* genes was speculated following the identification of a retinoic acid-responsive enhancer element in the 5′ flanking region of human *MSX1*[64]. Functional *in vivo* evidence was later provided by Chen *et al*[65] who showed that endogenous retinoids control the spatial expression of *Msx1* by delimiting its expression to the posterior regions of quail embryos at the gastrulation and neurulation stages. Thus retinoids are important regulators of normal *Msx1* expression in avians. In contrast, in murine embryonic palate mesenchymal cells, retinoic acid appeared to inhibit *Msx1* expression[66]. In addition, the 5' upstream region of murine *Msx1* was characterized as having multiple enhancer elements including three potential NFkB-binding sites and an Msx1 consensus binding site[67-71]. These studies indicate multiple regulations of *Msx1* expression.

Blin-Wakkach *et al*[72] reported the presence of endogenous *Msx1* antisense RNA in mice, rats and humans. It was suggested that the proportion of the sense and antisense transcripts determines the amount of functional protein available. Upregulation of the antisense RNA appears permissive for the differentiation of craniofacial structures, specially those associated with

mineralized matrices, while the sense form maintains the cells in a proliferating state[72].

Membraneous bone and cranial suture development rely upon growth factor signals transduced by *Msx* genes [11, 73, 74]. *In vitro* assays indicate that suture patency is controlled by the differential regulation of *Msx* genes by growth factors[74]. It was demonstrated that exogenous addition of FGF4 to the suture mesenchyme or the osteogenic front stimulates mesenchymal *Msx1* expression and cell proliferation, which promotes suture closure. In similar assays, application of BMP4 could induce both *Msx1* and *Msx2* in the suture mesenchyme resulting in a concomitant increase in tissue thickness. It is proposed that the BMP4-Msx signaling pathway regulates the balance between committed and uncommitted osteogenic cells in the suture. In addition to the regulation by BMPs via direct effect of Smad4 on the *Msx2* promoter, *Msx2* expression is also activated by a bi-functional zinc finger protein YY1 independent of BMP signaling pathway[75, 76].

The branchial arches are largely populated by cranial neural crest cells that migrate from the midbrain-hindbrain regions. The streams of neural crest cells exiting the dorsal aspects of the hindbrain are sculpted through apoptotic elimination of crest cells originating in rhombomeres 3 and 5[22, 78-80]. This apoptotic removal of the neural crest cells is mediated by BMP4-induced *Msx2* expression only in odd-numbered rhombomeres [81].

In the developing face, complimentary expression of *Msx1* and *Barx1* in the mandibular mesenchyme specifies patterning events including tooth formation[82, 83]. Also, the overlapping expression of *Msx* and *Dlx* genes together with the evidence that members of the two families form heterodimers *in vitro* reveals a putative mechanism for controlling facial patterning events *in vivo* [55, 84]. In the mandibular arch of chick embryos *Msx1* expression correlates with areas of cell proliferation while *Msx2* shows localization to regions either marked for programmed cell death or specified towards the formation of nonchondrogenic tissues[21]. The mesial localization of *Msx1* in the chick mandibular arch can be reconciled with a role in promoting mandibular arch outgrowth by extrapolation from the truncated mandibular arch phenotype reported in *Msx1* mutant mice. Further studies in the chick demonstrate that the expanded induction of *Msx* genes by ectopic application of BMP4 or BMP2 results in bifurcation of the facial skeleton and increased proliferation in the mandibular primordia[85].

In mice, *Msx1* is expressed downstream of *dHAND*, a mesenchymal transcription factor in the Endothelin-1 signaling pathway[86]. *Msx1*, *Msx2* and the bHLH transcription factor *dHAND* are expressed in an overlapping fashion in the distal mesenchyme of the branchial arches. In *dHAND*-null embryos *Msx1* expression is lost and the branchial arches become hypoplastic. *Msx2* expression remains unaltered in these mutants. Upon detailed examination of this phenotype it was concluded that *Msx1* is essential for the development of neural crest-derived mesenchyme of the branchial arches[86].

Msx1 is common to multiple growth factor signaling pathways and serves in the orchestration of inductive events essential to organogenesis. Therefore, we find its repeated use in the BMP, FGF, Endothelin and SHH signaling pathways. BMP2, BMP4, FGF2, FGF4, FGF8, and FGF9 represent growth factors from the oral and/or the dental epithelia that are capable of inducing *Msx1* expression in the subjacent mesenchyme of the mandible and maxilla[87, 88]. The mesenchymal expression of several growth and transcription factors, namely, *Bmp4*, *Fgf3*, *Dlx2*, *syndecan-1,* and *Ptc* in turn show dependence on *Msx1* expression[87, 89-91].

Between E9.5 and E13.5 *Msx1* shows a broad distribution distally, overlapping the presumptive incisor regions, in the mandible and maxilla. Subsequently *Msx1* expression becomes localized to the condensing mesenchyme of both the molar and incisor tooth germs. Curiously this shift in *Msx1* expression is preceded by a shift in *Bmp4* expression pattern[92]. Studies show that *Msx1* and *Bmp4* are induced in the dental mesenchyme by epithelial *Bmp4*[87, 89]. Once induced a positive feedback loop comes into play between *Msx1* and *Bmp4* in the dental mesenchyme maintaining the levels of both genes throughout tooth morphogenesis[47, 89, 92]. This same mechanism accounts for the spatial restriction of *Msx1* expression around late bud stage to the ondontogenic mesenchyme[92]. Thus, *Msx1* acts epistatic to mesenchymal *Bmp4*, a candidate factor that signals back to the dental epithelium allowing tooth morphogenesis to proceed to the cap stage[89, 93]. *Msx1* is also required in the *Fgf8* signaling pathway for the induction of *Fgf3* in the dental mesenchyme[90]. The two pathways appear to be independent of each other and occur in parallel during early odontogenesis. While BMPs can induce both *Msx1* and *Msx2* in dental mesenchyme, FGFs can only induce *Msx1*[87, 88, 90]. *In vitro* experiments sug-

gest that *Msx1* and *Msx2* mediate the inductive effects of BMP7 on mandibular morphogenesis as well as the initiation phase of odontogenesis[94]. The induction of *Ptc*, a downstream target of *Shh* signaling, in the dental mesenchyme is contingent upon *Msx1*, which is co-expressed with *Ptc*[91]. Conditional ablation of *Shh* specifically in the dental epithelium does not alter *Msx1* or *Msx2* expression. This proves that *Msx1* and *Msx2* are not targets of *Shh* signaling.

In conclusion, there appears to be multiple levels of regulation of *Msx* expression at the level of transcription, translation, and protein function, which contribute to the normal patterning and morphogenesis of the face.

Mutations in Msx1 cause tooth agenesis and cleft palate

In human, mutations in the *MSX1* gene cause orofacial clefting and tooth agenesis[95-101]. The homeodomain of *MSX1* is pivotal in mediating its multiple functions such as DNA-binding, protein-protein interactions, protein stability, and transcription repression. A missense mutation resulting in an arginine to proline substitution within the homeodomain of MSX1 causes selective tooth agenesis in humans, an autosomal dominant phenotype affecting the second premolars and third molars of the secondary dentition[95]. Biochemical and functional analyses of the mutant protein established haploinsufficiency of *MSX1* as the molecular basis underlying this phenotype[102]. The mutant protein exhibited reduced stability as a result of structural perturbations and failed to interact with DNA or its cognate protein factors. Accordingly its ability to function as a transcriptional repressor was greatly impaired. The increased sensitivity to *MSX1* gene dosage appears to be specific to humans. Curiously, in mice although a null mutation in the *Msx1* gene resulted in a complete failure of tooth development, mice heterozygous for *Msx1* did not present with any tooth abnormalities[103, 104] (see below). Despite the different phenotypes exhibited by different dosages of *Msx1* in humans and mice, its importance in tooth morphogenesis remains undisputed.

Wolf-Hirschhorn syndrome (WHS) is a congenital human syndrome results from a deletion of the *MSX1* locus on chromosome 4[105]. Phenotypic manifestations of this syndrome featuring midline fusion defects, ear defects, supernumerary teeth, and microcephaly involve regions that express *MSX1* in the mouse embryo[18, 19, 106, 107]. Furthermore, a nonsense mutation in *MSX1* accounts for the genetic etiology of Witkop syndrome characterized by tooth agenesis and nail dysgenesis[100].

In contrast to the haploinsufficiency of *MSX1* in human [102], genetically engineered *Msx1* heterozygous mutation in mice does not result in any visible phenotypes [103, 104]. However, mice carrying *Msx1* null mutation die right after birth, and exhibit severe craniofacial abnormalities[103, 104]. These phenotypes include cleft palate, an absence of alveolar processes, and an arrest of tooth development at the bud stage, thus mimicking the phenotype observed in humans carrying *MSX1* mutations.

In the mouse *Msx1* mutants the dental mesenchyme fails to condense around the dental epithelial buds causing a bud stage arrest of molar tooth germs[103]. *Msx1*-deficiency leads to significantly reduced expression of *Bmp4* in the dental mesenchyme of arrested tooth germs [89]. Gene expression studies also indicate that *Msx1*$^{-/-}$ embryos show ablation of *Fgf3* expression and downregulation of *Lef1*, *Ptc*, *Dlx2*, and *syndecan-1* expression in the dental mesenchyme[89, 90, 91]. On the other hand, tenascin expression is unchanged in these mutants. A non-cell autonomous secondary effect of *Msx1* deficiency is the loss of *Shh* and *Bmp2* expression in the dental epithelium[93]. The bud stage arrest is lifted upon the addition of exogenous BMP4 or by trangenic expression of *Bmp4* thereby bypassing the need for *Msx1* function[89, 93, 108]. As a result, tooth development proceeded past cap stage *in vitro* engendering a near complete rescue in kidney capsule cultures and a restoration of alveolar bone formation in transgenic mice [89, 108, 109]. Analysis of gene expression showed that *Lef1*, *Dlx2*, *Shh* and *Bmp2* expression is restored following ectopic or exogenous BMP-4 expression. Thus, *Msx1*-dependent mesenchymal expression of *Bmp4* is critical for tooth morphogenesis and alveolar bone formation. An accompanying defect in proliferation observed in *Msx1*-deficient embryos is attributed to a separate pathway involving FGFs where *Msx1* mediates the induction of *Fgf3* and *syndecan-1*, a low-affinity FGF receptor[89, 90]. Bei *et al*[109] using tissue recombination established that *Msx1* imparts early and late functions to tooth development. In the early phase it acts epistatic to *Bmp4* in the bud stage dental mesenchyme. The *Msx1*-induced *Bmp4* from the dental mesenchyme instructs the overlying dental epithelium to form the enamel knot, which guides tooth morphogenesis [93, 110]. After the cap stage, tooth development be-

comes independent of *Msx1* function. During the later cyto-differentiation stage *Msx1* maintains the survival of odontoblasts and the dental pulp[109].

In humans and mice loss of Msx1 function results in non-syndromic clefts of the secondary palate and tooth agenesis[95, 97, 103, 104]. Until recently, there has been much speculation surrounding the role of *Msx1* in palate development. It was previously believed that the cleft secondary palate observed in *Msx1*-deficient mice occurs as a consequence of a primary defect in tooth development[18, 19, 103]. This contention was disproved recently by functional assays using a transgenic mouse model where the *Msx1* promoter directs the expression of human *Bmp4* in developing tooth and palate in the *Msx1*$^{-/-}$ background leading to rescue of neonatal lethality in some mice[62, 93, 108]. Notably, all of the surviving *Msx1*$^{-/-}$*/Tg* mice exhibited a closed palate although they still lacked teeth. Thus, the transgene could specifically rescue the cleft palate phenotype independent of the tooth phenotype.

Msx1 is expressed in the anterior mesenchyme of the developing palate from E11.5 to E13.5. The posterior regions of the developing palate do not express *Msx1*. The expression of *Msx1* in the palatal mesenchyme is weak relative to its expression in the dental mesenchyme [62]. In the *Msx1* null mutant embryos the paired palatal shelves elevate normally but fail to make contact and fuse[103]. This failed fusion between the palatal shelves of *Msx1*$^{-/-}$ embryos is the result of significantly lower levels of cell proliferation in their anterior region leading to growth impairment[62]. Examination of gene expression levels revealed significant downregulation of *Bmp4* levels in the palatal mesenchyme, *Shh* in the medial edge epithelium and *Bmp2* in both the epithelium and mesenchyme of E13.5 *Msx1* mutant embryos. Following transgenic rescue of the cleft palate the gene expression and proliferation levels in the anterior palate are restored to normalcy. Bead implantation experiments indicate that *Msx1* directly regulates *Bmp4* expression while its effects on *Shh*, *Bmp2* and mitogenesis are indirect. Thus, through its regulation of specific growth factor expression *Msx1* maintains growth of the anterior palate during mammalian palatogenesis.

Middle ear defects in Msx1-deficient mice

Msx1-deficient mice show anomalous development of the malleus, one of the three middle ear ossicles[103]. The malleus is shorter than in wild types and it constituent part, the processus brevis is absent in these null mutants. The gross morphology of the remaining middle ear ossicles, the incus and the stapes appears normal. Similar to the tooth germs, the malleal primordia in the mutant embryo show reduced expression of *Bmp4*[111]. The transgenic expression of *Bmp4* in the *Msx1* mutant background failed to affect a rescue and the surviving mice still lacked the processus brevis, which allows the measurement of auditory evoked potentials to assess the functional significance of the malleal processus brevis. It was demonstrated that the malleal processus brevis is dispensable for sound transmission and balance in mice [111].

Craniofacial defects associated with Msx2 mutations

Role of *Msx2* in craniofacial development was initially revealed by a mutation in the *MSX2* gene causing Boston-type craniosynostosis in humans[112]. Boston-type craniosynostosis is characterized by the premature fusion of skull bones together with certain orofacial bone abnormalities. This mutation in the homeodomain of the MSX2 protein increased its DNA binding affinity and was believed to represent a gain-of-function mutation[6, 112]. Conversely, haploinsufficiency of *MSX2* causes midline cranial defects reflected in the occurrence of wide-open fontanels in the skull vault[113]. Thus MSX2 function is required for normal skull and suture morphogenesis.

A species-specific dependence on *MSX2* dosage was established variously through knock-out strategies, transgene expression and mutation studies. While haploinsufficiency in human *MSX2* results in parietal foramina the *Msx2* heterozygous mice manifest no abnormal phenotype[113, 114]. In contrast, *Msx2* homozygous null mice have a calvarial foramen similar to humans with *MSX2* haploinsufficiency[114]. Liu *et al* [115] showed that mice carrying a missence mutation within the *Msx2* homoeodomain developed craniosynostosis similar to mice that overexpress the wild type allele. Again trangenic mice expressing human *MSX2* exhibit multiple craniofacial defects including exencephaly[116].

Several studies have been undertaken to divulge the molecular mechanism and pathophysiology of Boston-type craniosynostosis. Essentially a proline to histidine substitution at position 7 of the homeodomain of Msx2

missense mutation increases the binding affinity of Msx2 for its target DNA without altering the binding site specificity while increasing the Msx2-DNA complex stability[6, 117]. The outcome is an augmentation of the normal function of Msx2. Overexpression and misexpression of *Msx2* transgene in mice gave a similar phenotype registering pronounced growth of the calvarial bones and an increased number of proliferating osteoblasts at the osteogenic front[115, 118].

In a normal developing cranium, *Msx2* is required to maintain a proliferating population of osteoblast progenitors at the osteogenic front[118]. Functional studies show that overexpression of *Msx2* impedes osteoblast differentiation while antisense inhibition promotes differentiation[119]. The reduction in the length of the axial and appendicular skeleton in *Msx2* null mice lends credence to its role in regulating the proliferation of bone-forming cells[120].

The temporal and spatial expression of *Msx2* in the suture mesenchyme and dura matter prior to birth appears to correlate with its role in the regulation of suture patency during prenatal development. The widespread occurrence of BMP4 in the developing suture as well as its ability to induce both *Msx1* and *Msx2* in the suture *in vitro* has been demonstrated[74]. Furthermore, genes typically expressed in terminally differentiated osteoblasts such as *collagen-I* and *osteocalcin* were shown to be regulated by Msx2[54, 121, 122, 123]. These data suggest that the modulation of *Msx* genes by BMPs and other factors in the developing suture regulates the rate of differentiation of the osteoblasts at the advancing osteogenic fronts and thereby calvarial osteogenesis. Collectively, the evidence that both gain- and loss-of-function mutations in *Msx2* cause craniofacial defects indicates that the precise regulation of *Msx2* expression and optimal level of Msx2 protein are crucial for normal development of craniofacial organs.

Msx1 and Msx2 function redundantly in craniofacial development

Several biochemical, expression, and knockout studies suggest that *Msx1* and *Msx2* are functionally redundant. Investigations at the biochemical level reveal that MSX1 and MSX2 have common DNA-binding and transcriptional properties[48]. They both recognize the same DNA consensus site and function as transcriptional repressors. Further, structural comparisons reveal that *Msx1* and *Msx2* only differ in one amino acid in their homeodomains. Spatial and temporal expressions of *Msx1* and *Msx2* in mice show a certain degree of co-localization. Consistent with this co-localization pattern, *Msx1/Msx2* double mutant mice exhibit synergistic defects in calvarial, tooth, ear, limb, hair follicle, and mammary gland development[90, 111, 114]. Therefore, while the *Msx2* single mutant mice show either incomplete or delayed ossification of the calvarial bones resulting in calvarial patency, the double mutant mice are deficient in calvarial ossification. With respect to tooth, hair follicle and mammary gland development *Msx1* and *Msx2* show functional redundancy through the early stages of organogenesis, coinciding with their overlapping expression pattern. However, the participation of *Msx2* in the early stages of tooth development appears non-essential since mice heterozygous for *Msx1* but lacking functional *Msx2* do not exhibit early tooth defects[90]. Tooth development proceeds normally through the lamina, bud, and cap stages in the *Msx2*-deficient mice. A requirement for *Msx2* in tooth development is seen in the late stages of organogenesis following the downregulation of *Msx1* expression. Anomalous tooth development in the *Msx2*-deficient mice becomes evident at E16.5 when the stellate reticulum and stratum intermedium fail to develop normally resulting in the degeneration of the ameloblast and ultimately the enamel organ. It is postulated that *Msx2* participates in the regulation of the spatiotemporal expression of the amelogenin gene during tooth development [124]. In the absence of *Msx1*, the phenotype of *Msx2* mutant mice is greatly amplified. Molar tooth development arrests at the dental lamina stage in *Msx1*/*Msx2* compound mutants unlike $Msx1^{-/-}$ mice that exhibit a bud-stage arrest[90, 103]. Thus, at the tooth initiation stages *Msx1* and transiently expressed *Msx2* function redundantly. Similarly additive hypomorphism is observed in the developing middle ear of compound *Msx* mutants. While $Msx1^{-/-}$ mice lack the processus brevis, mice double null for *Msx1* and *Msx2* genes are lacking the manubrium as well as the processus brevis. In the *Msx1*/*Msx2* double mutant mice there is a failure in hair follicle induction unlike $Msx2^{-/-}$ mice where the pelage hairs form but are lost prematurely owing to defects in hair maintenance[114, 125]. Similarly, in the absence of *Msx1* and *Msx2* the mammary gland epithelium does not invaginate while a loss of *Msx2* alone results in a sprout stage arrest of the mammary gland. Therefore in the tooth, hair and mammary gland, a loss of *Msx1* and *Msx2* results in a more severe phenotype

than those resulting from the loss of any one. Functional redundancy between *Msx1* and *Msx2* is also evident from limb deformities that are manifested by double knockout mice[126]. Accordingly, in a single mutant background the limbs develop normally, however, mice that are null for both *Msx1* and *Msx2* develop limbs with lack of antero-posterior polarity, absence of radius, marked inhibition of apoptosis in the interdigital regions, and polydactyly[127]. Taken together, these expression and functional analysis data support the hypothesis that functional redundancy exists between the *Msx* genes.

CONCLUSION

Members of the *Msx* homeobox gene family are among the critical factors involved in craniofacial development. These regulatory proteins function as transcriptional repressors and are involved in the modulation of craniofacial, limb and nervous system development. Of the three members, *Msx1* and *Msx2* have been given special consideration in this discussion because of their incontrovertible relevance to craniofacial morphogenesis. Loss-of-function and gain-of-function studies show that in mice as in humans *Msx1* and *Msx2* are required for normal craniofacial morphogenesis. Although there is a species-specific difference in the dosage requirements of Msx genes in humans and mice, both show similar phenotypes. Lastly we have considered the functional redundancy between *Msx1* and *Msx2* by comparisons of single, double, and compound mutant phenotypes which gives greater hypormorphism in organs where there is overlapping expression of the *Msx* genes. In summary, recent advances have revealed important roles for *Msx* genes in embryogenesis as well as the functional properties of Msx proteins. It would be of great interest to identify the direct downstream target genes of Msx proteins in *vivo* and their associated cellular processes including proliferation, apoptosis, cell adhesion and migration during organogenesis.

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