Y. Wang¹, H. Kong¹,
G. Mues¹, and R. D'Souza^{1*}

¹Department of Biomedical Sciences, Texas A&M University Health Science Center Baylor College of Dentistry, 3302 Gaston Ave., Dallas, TX 75246, USA; *corresponding author, rdsouza@bcd.tamhsc.edu.

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ABSTRACT

Mutations in the transcription factors *PAX9* and *MSX1* cause selective tooth agenesis in humans. In tooth bud mesenchyme of mice, both proteins are required for the expression of *Bmp4*, which is the key signaling factor for progression to the next step of tooth development. We have previously shown that Pax9 can transactivate a 2.4-kb *Bmp4* promoter construct, and that most tooth-agenesis-causing *PAX9* mutations impair DNA binding and *Bmp4* promoter activation. We also found that Msx1 by itself represses transcription from this proximal *Bmp4* promoter, and that, in combination with Pax9, it acts as a potentiator of Pax9-induced *Bmp4* transactivation. This synergism of Msx1 with Pax9 is significant, because it is currently the only documented mechanism for Msx1-mediated activation of *Bmp4*. In this study, we investigated whether the 5 known tooth-agenesis-causing *MSX1* missense mutations disrupt this Pax9-potential effect, or if they lead to deficiencies in protein stability, protein-protein interactions, nuclear translocation, and DNA-binding. We found that none of the studied molecular mechanisms yielded a satisfactory explanation for the pathogenic effects of the Msx1 mutations, calling for an entirely different approach to the investigation of this step of odontogenesis on the molecular level.

KEY WORDS: tooth agenesis, *Msx1*, *Pax9*, *Bmp4*, missense mutation.

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Msx1 Mutations: How Do They Cause Tooth Agenesis?

INTRODUCTION

To date, several mutations in the homeodomain transcription factor *MSX1* and the paired domain transcription factor *PAX9* have been identified in tooth agenesis patients (Vastardis *et al.*, 1996; Stockton *et al.*, 2000; Lidral and Reising, 2002; Das *et al.*, 2003; Lammi *et al.*, 2003; Mostowska *et al.*, 2003, 2006; Jumlongras *et al.*, 2004; Chishti *et al.*, 2006; Kapadia *et al.*, 2006; Xuan *et al.*, 2008; Wang *et al.*, 2009a). These findings had been inspired and supported by animal studies showing that Msx1 and Pax9 are co-expressed in dental mesenchyme during the early stages of tooth development, and that homozygous deletion of Msx1 or Pax9 results in an arrest of tooth development at the bud stage (Chen *et al.*, 1996; Peters *et al.*, 1998; Bei *et al.*, 2000). Mesenchymal expression of bone morphogenetic protein 4 (*Bmp4*), a key signaling factor for tooth development, is down-regulated in the *Msx1* or *Pax9* knockout animal model. Furthermore, single heterozygous *Pax9*^{+/-} or *Msx1*^{+/-} mutant mice have a normal dentition, whereas the compound *Pax9*^{+/-}*Msx1*^{+/-} double-heterozygous mice lack lower incisors, and with incomplete penetrance, third molars (Chen *et al.*, 1996; Nakatomi *et al.*, 2010). This phenotype can be partially rescued by transgenic expression of *BMP4* in dental mesenchyme, indicating a genetic interaction between Pax9 and Msx1 and confirming *Bmp4* as a downstream target of these two transcription factors (Nakatomi *et al.*, 2010).

Our previous *in vitro* studies supported this hypothesis: Using a proximal 2.4-kb *Bmp4* promoter fragment, we have shown that Pax9 but not Msx1 can directly activate the expression of *Bmp4*. Msx1, however, can physically interact with Pax9 and potentiate Pax9's activation of *Bmp4* expression (Ogawa *et al.*, 2006; Wang *et al.*, 2009a), thus providing a tentative explanation for the activation of *Bmp4* by Msx1. We have recently tested our *in vitro* molecular model using tooth-agenesis-causing *PAX9* paired domain mutations (Wang *et al.*, 2009a). *In vitro* testing showed that most of the Pax9 mutants had lost or reduced DNA binding ability and decreased transcriptional activities with *Bmp4* promoter constructs. The extent of these functional defects correlated well with the severity of the dental phenotype seen in the families carrying the mutations. One of the Pax9 mutations had lost synergism with Msx1.

The aim of the present study was to investigate if *MSX1* mutations result in tooth agenesis because the mutant proteins lose their ability to cooperate with *PAX9*, or if inadequacies in other functional properties of the mutant Msx1 proteins can be discovered by *in vitro* testing. Here, we analyzed all 5 tooth-agenesis-causing missense mutations in the *MSX1* gene with our *in vitro* system to test our hypothesis about the molecular mechanism of *Bmp4* activation in tooth bud mesenchyme. We conclude that the original hypothesis is untenable with regard to Msx1's molecular mechanism of action.

Table. Synopsis of the Characteristic Phenotypical and Functional Features of Wild-type *Msx1* and Tooth-agenesis-causing *Msx1* Mutants

	Wild-type	M61K	A194V	R196P	A219T	A221E
Inheritance		AD ^a	AD/I.P. ^a	AD	AR ^a	AD
Tooth phenotype ^b		12 (8)	14 (10)	11 (7)	18 (15.5)	12 (8)
Localization	Nuclear	Nuclear	Nuclear	Nucl clump	Nuclear	Nuclear
Co-IP	+	+	+	+	+	+
Half-life	+++	++	+++	+	+	+++
Synerg, <i>Bmp4</i> ^c		Less	Slightly up	Like WT	Less	More
Synerg, <i>Msx1</i> ^c		Like WT	Slightly up	Slightly up	Repressed	Slightly up
DNA-binding		More	Like WT	None	None	Like WT
Reference		Lidral and Reising, 2002	Mostowska <i>et al.</i> , 2006	Vastardis <i>et al.</i> , 1996	Chishti <i>et al.</i> , 2006	Xuan <i>et al.</i> , 2008

^aAD, autosomal-dominant; AR, autosomal-recessive; I.P., incomplete penetrance.

^bTooth phenotype: showed average number of missing teeth, and the number in parentheses excludes third molars.

^c"Synerg, *Bmp4*" represents synergism with Pax9 on the *Bmp4* promoter, and "Synerg, *Msx1*" represents synergism with Pax9 on the *Msx1* promoter.

MATERIALS & METHODS

Construction of Expression Plasmids and Site-directed Mutagenesis

The mammalian expression vector pCMV-Pax9 with a c-Myc epitope tag and pCMV-*Msx1* with a FLAG epitope tag were used as previously described (Ogawa *et al.*, 2006). *In vitro* site-directed mutagenesis was performed with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) to construct pCMV-*Msx1* with the M61K, A194V, R196P, A219T, and A221E mutations. Similar vectors were constructed with *Msx2* and *Dlx1*, 2, and 5 coding sequences.

We used the pEYFP-C1 vector (Clontech Laboratories, Palo Alto, CA, USA) to construct YFP-*Msx1* fusion proteins to determine subcellular localization. All constructs were sequenced entirely to confirm the correct reading frame and point mutation.

Protein Half-life Assay

We over-expressed FLAG-*Msx1* wild-type or mutant proteins in COS7 cells. After overnight culture, cells were treated with 90 mM cycloheximide (CHX) for 1 to 6 hrs to inhibit cellular protein synthesis. The abundance of *Msx1* proteins was subsequently examined by Western blot with samples taken at 0, 1, 2, 4, and 6 hrs after the start of CHX treatment. Actin was used as quantification control.

Western Blot Analysis and Co-immunoprecipitation

These were performed as described previously (Wang *et al.*, 2009a). Primary antibodies included anti-FLAG M2 (Stratagene), anti-actin (Abcam, Cambridge, MA, USA), anti-Pax9 M-18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO, USA). Visualization was achieved with mouse or goat horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). All blots were done in triplicate.

Gel Shift and Super Shift Assay

The oligonucleotide probe used for gel shift assays was the 26mer tgaccctatgtaattgcattcctgaa, which contains the previously established home domain-binding site 12B (Catron *et al.*, 1993). Electrophoretic mobility shift assays (EMSA) were performed with the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions and in triplicate.

Subcellular Localization

To demonstrate *in vivo* expression of wild-type and mutant *Msx1*, we transfected COS7 cells (in triplicate) with wild-type *Msx1* or mutant *Msx1* in the pEYFP-C1 vector and examined them 24 hrs later with a Nikon Eclipse TE2000-U fluorescence microscope. Vectors containing only the *Msx1* homeodomain or *Msx1* sequences without homeodomain were used as controls.

Reporter Assay

Msx1 expression vectors were co-transfected with a *Bmp4* promoter-reporter or a *Msx1* promoter-reporter construct [p2.4*Bmp4*-Luc and p3.5*Msx1*-Luc, described previously (Ogawa *et al.*, 2006)] into COS7 cells in a 12-well format. The pCMV-SPORT β-Gal plasmid (Invitrogen, Carlsbad, CA, USA) was used as an internal control. For each well, 500 ng of pCMV-Pax9, 250 ng of pCMV-*Msx1*, 150 ng of promoter-reporter construct, and 100 ng of pCMV-SPORT β-Gal were co-transfected. Transfection was performed in duplicate and repeated 3 times. Twenty-four hours after transfection, cell extracts were assayed by the luciferase assay system (Promega, Madison, WI, USA) and a β-gal assay kit (Invitrogen).

RESULTS

Review of Phenotypes and Mutations

All 5 *Msx1* missense mutations studied cause quite severe phenotypes, with great intra- and inter-familial variation (Table), in

particular, the A219T substitution, which is inherited in an autosomal-recessive mode in two consanguineous families. The A194V mutation presented with incomplete penetrance, suggesting that other gene variants may be involved. Premolars are affected most often, followed by molars and incisors. The average number of missing teeth is comparable with the observations made in patients with *PAX9* mutations.

Sequence Alignment and Structure-based Predictions

Alignment of the *Msx1*, *Msx2*, and *Msx3* amino acid sequences of human and mouse, as well as *Msx1* sequences from diverse species, revealed that the 5 affected residues are conserved. Based on the crystal structure of the *Msx1* homeodomain complex with DNA (Hovde *et al.*, 2001), we compared the mutant and wild-type residues in the 3D model (Fig. 1). A194V and R196P are located in helix 2, A219T and A221E in helix 3, and M61K is positioned N-terminal of the homeodomain in a conserved stretch of amino acids known as one of the two groucho/TLE-binding domains in *Msx1*. The R196P mutation has the most severe effect; it will diminish DNA binding and destabilize the α -helix at the same time. The A219T and A221E mutations introduce a hydrophilic amino acid in place of the hydrophobic alanine; the A194V substitution has only a minor effect on structural stability.

Effects of Mutations on the Half-life of *Msx1* Proteins

The half-life of *Msx1* was decreased by all mutations except A194V (Fig. 2A). The R196P and A219T mutants had the shortest half-life, dropping more than three-fold. The protein half-life of the M61K and A221E was reduced to less than half compared with that of the wild-type protein.

DNA Binding to an *Msx1* Consensus Site

Gel-shift and supershift assays show that the DNA-binding ability of the *Msx1* protein is abolished by the mutations R196P and A219T (Fig. 2B). Interestingly, R196P affects the same amino acid that is mutated to histidine in the *MSX2* homeodomain (R172H) in a family diagnosed with parietal foramina (Wilkie *et al.*, 2000) and has been reported to cause 85% reduction in DNA-binding. The loss of DNA-binding of the R196P mutation can be easily deduced from the structural prediction (Fig. 1B). The M61K, A194V, and A221E mutant proteins show the same patterns of shifted bands as the wild-type sequence. This finding is plausible, since M61K is located outside of the DNA-binding

Figure 1. Sequence alignments and 3D structure prediction. (A) Protein sequence alignment of human *MSX1/2* and mouse *Msx1/2/3*. The positions of mutated amino acids are indicated with grey shading. The secondary structure of the *Msx1* homeodomain is shown above the sequence. Two of the mutations are located within helix 2 and the other two in helix 3. (B) Protein-DNA interaction models of the *Msx1* homeodomain with and without the missense mutations. The homeodomain protein chain is red, and the DNA double helix is shown in blue. The wild-type and mutant amino acids stand out as yellow stick models.

domain, A194V does not interact with the DNA helix, and A221E appears to interact only marginally with DNA (Fig. 1B).

Nuclear Localization of *Msx1* Mutants

Msx1 protein without the homeodomain is mainly localized in the cytoplasm, while the *Msx1* homeodomain by itself is located in the nucleus (Fig. 2C). These findings indicate that nuclear localization is mediated by the homeodomain of *Msx1*. All 5 mutant *Msx1* proteins are found primarily in the nuclear compartment of transfected cells.

Physical Interactions between Pax9 and *Msx* Proteins

Co-transfection of pCMV-Myc Pax9 and pCMV-FLAG *Msx1*, followed by co-immunoprecipitation, showed that all of the mutant proteins are able to interact physically with wild-type Pax9 (Fig. 2D). The homeodomain of the *Msx2* protein differs from *Msx1*'s homeodomain by only 2 conservative amino acid replacements, but *Msx2* as well as the *Dlx* proteins did not co-immunoprecipitate with Pax9 (see Appendix Fig.). Co-IP does not coincide with transcriptional potentiation of Pax9 (see below).

Transcriptional Activation in the Presence of Pax9

Wild-type *Msx1* represses transcription of *Bmp4* and *Msx1* promoter constructs *in vitro*, but in the presence of Pax9, it synergistically up-regulates transcription from both promoters

Figure 2. Characterizations of Msx1 protein. **(A)** Msx1 protein half-life: Cos7 cells were transfected with Flag-Msx1 expression vectors and treated with 90 mM cycloheximide (CHX) for 1 to 6 hrs. Western blot of cell lysates with anti-FLAG was used to assess the protein degradation speed; actin served as a control. **(B)** DNA-binding abilities: Electrophoretic mobility shift assay (EMSA) was used to test DNA-binding activity of nuclear extracts from Flag-Msx1-transfected COS7 cells with a double-stranded oligonucleotide probe containing a consensus binding-site for Msx1. Specificity of the complexes was confirmed by super-shift induction with anti-FLAG antibody. To verify that equal amounts of Msx1 protein were used for the EMSA, we performed a Western blot of nuclear extracts (lower panel). (Note that R196P migrates more slowly than the other proteins.) **(C)** Nuclear localization: YFP-Msx1 fusion proteins were expressed in COS7 cells. None of the missense mutations affected nuclear localization, in contrast to the YFP-Msx1 construct without homeodomain, which is found in the cytoplasm (YFP-delHD). The homeodomain by itself suffices for translocation to the nucleus (YFP-HD). YFP-R196P showed densely clumped distribution within the nuclei. These protein aggregates could be the consequence of protein interactions or of a secondary modification which also causes the R196P mutant to migrate more slowly than the other mutants and wild type Msx1. **(D)** Protein interaction: Co-immunoprecipitation with COS7 cells co-transfected with Myc-tagged Pax9 proteins and FLAG-tagged Msx1. *Upper panel:* Western blot analyses of immunoprecipitates of whole-cell lysates pulled down with anti-FLAG antibody and probed with an anti-Pax9 antibody. *Middle panel:* Whole-cell lysates probed with anti-Pax9 antibody or (*lower panel*) anti-Flag antibody. (Note that the Msx1-R196P migrates more slowly in both immunoprecipitations and input cell lysate.)

(Ogawa *et al.*, 2006; Wang *et al.*, 2009a). Evaluating the 5 mutants, we found that M61K and A219T exhibited reduced synergistic cooperation with Pax9 on the *Bmp4* promoter (Fig. 3A); on the *Msx1* promoter construct, A219T even repressed Pax9's activity on transcription (Fig. 3B). However, A194V, R196P, and A221E could synergistically interact with Pax9 on both promoters even more effectively than wild-type Msx1.

We also tested Msx2 and the Dlx1 and 2 proteins in this assay. While Msx2 behaved like Msx1, the Dlx proteins either had no effect or inhibited Pax9's transactivation ability (see Appendix Fig.).

DISCUSSION

It has been known for more than a decade that Msx1 and Pax9 are required for *Bmp4* expression in tooth bud mesenchyme, but the molecular basis for this inter-relationship is largely unidentified. Potentiation of Pax9 is currently the only known molecular

mechanism of *Bmp4*-activation by Msx1 in tooth bud mesenchyme, since Msx1 by itself cannot activate the proximal *Bmp4* promoter, and other potential mediators are presently unknown. Homeodomain transcription factors usually acquire their selective functional properties through interactions with a variety of other co-factors, including paired domain transcription factors; thus, it could have been possible that Pax9 was one of these co-factors.

Here we analyzed naturally arising, human hypodontia-causing missense mutations in *MSX1* to examine their interaction with Pax9 and *Bmp4* on a molecular level. Most of the mutant Msx1 proteins could still potentiate Pax9-induced *Bmp4* and *Msx1* promoter activation, suggesting that Msx1's ability to cause human tooth agenesis may be independent of any synergism with Pax9. The severity of the families' phenotypes cannot be reasonably explained by the relatively mild variations observed in our functional tests, suggesting again that a different molecular mechanism must be responsible for Msx1-caused

tooth agenesis. Even the A194V mutation which showed incomplete penetrance and behaved like wild-type protein in all our tests cannot yet be excluded as a disease-causing mutation.

In conclusion, we infer from the functional evaluation of the 5 *MSX1* missense mutations that disturbances in the interaction with Pax9 are probably not the basis of the human phenotypes caused by *MSX1* mutations, unless another molecular mechanism for the observed dependence of Bmp4 expression on combined Msx1 and Pax9 activity is discovered. A recent study by Chandler *et al.* (2009) described the existence of an enhancer element far upstream of the *Bmp4* promoter that drives Bmp4 expression in tooth bud mesenchyme. This enhancer could be located and tested for responsiveness to wild-type Msx1 and the Msx1 mutants, with and without Pax9 as co-activator.

Alternatively, an altogether different pathway could be involved. Early on, it had been shown that Msx1-deficient mice displayed reduced expression of not only Bmp4 but also of Lef1, syndecan-1, Runx2, and Fgf3 (Chen *et al.*, 1996; Bei and Maas, 1998). In the dental mesenchyme, Fgf3 is another well-established signaling molecule besides Bmp4 that is known to promote the tooth morphogenetic process. Fgf3 appears to be expressed downstream of Wnt/Lef1 and of Runx2 (D'Souza *et al.*, 1999). The Msx1 pathway resulting in Bmp4 expression seems to operate in parallel with the pathway that leads to Msx1-dependent Fgf3 expression in tooth bud mesenchyme. In each of these two pathways, Msx1 is likely to cooperate with different partner factors targeting different promoters. The fact that one of the tooth agenesis *MSX1* mutations (M61K) is located in a Gro/Tle binding site points to a disturbance in transcriptional repression mechanisms.

Surprisingly, Msx1 may even be completely dispensable for odontogenesis: Activation of the Wnt pathway through Apc depletion (Wang *et al.*, 2009a), but not β -catenin stabilization (Liu *et al.*, 2008), could bypass Msx1-dependent signaling pathways for the formation of supernumerary tooth-like structures with enamel knots, normal mineralization, and signs of root growth. However, these teeth were rather small and dysmorphic, suggesting that the mesenchymal Msx1-dependent pathways play a significant role in determining proper size and shape of the normal endogenous tooth organ, possibly by delaying premature differentiation.

Translating experimental evidence from mice to humans is still a challenge, especially when the organ system in question shows several remarkable differences: The mouse dentition is monophyodont and consists of only molars and continually growing incisors, while tooth agenesis resulting from Pax9 and Msx1 mutations affects mostly the secondary dentition in humans. Also, a study with human embryonic tissue revealed a few differences in gene expression patterns—for example, PAX9 is not exclusively expressed in tooth bud mesenchyme but is also found in the dental epithelium (Lin *et al.*, 2007). Ultimately, we will have to find valid *in vitro* assays that may help to interpret mouse data on a human background.

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Figure 3. Luciferase reporter assays of synergistic promoter up-regulation. (A) *Bmp4* promoter-reporter constructs (p2.4 *Bmp4*-luciferase) or (B) *Msx1* promoter-reporter constructs (p3.5 *Msx1*-luciferase) were co-transfected with the different Msx1 expression vectors and the wild-type Pax9 expression vector at a 1:2 ratio. (Error bars represent standard error; * $p < 0.05$, ** $p < 0.01$.)

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