REVIEW ARTICLE

Genes affecting tooth morphogenesis

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Structured Abstract

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The development of dentition is a fascinating process that encompasses a complex series of epithelial–mesenchymal interactions involving growth factors, transcription factors, signal receptors and other soluble morphogens. It is not surprising that such a complex process is prone to disturbances and may result in tooth agenesis. Initial discoveries indicating that the homeo-domain protein MSX1 and the paired-domain transcription factor PAX9 are causative genes in tooth morphogenesis were made in mice. Both genes are co-expressed in dental mesenchyme and either one, when homozygously deleted, results in an arrest at an early developmental stage. Heterozygous Pax9 or Msx1 mice have normal teeth, however, double heterozygous Pax9/Msx1 mice show a phenotype of arrested tooth development which can be rescued by transgenic expression of Bmp4, a very influential signaling factor in many developmental processes. We have obtained mounting evidence for a partnership between PAX9 and MSX1 within the tooth-specific Bmp4 signaling pathway. In humans, unlike in mice, a heterozygous mutation in either *PAX9* or *MSX1* suffices to cause tooth agenesis of a predominantly molar or more premolar pattern, respectively. Our laboratory and others have identified several PAX9 and MSX1 mutations in families with non-syndromic forms of autosomal dominant posterior tooth agenesis. We have also identified families with tooth agenesis in whom PAX9 and MSX1 mutations have been excluded opening up the possibilities for the discovery of other genes that contribute to human tooth agenesis.

Key words: BMP4; MSX1; PAX9; protein-protein; signaling pathways; tooth development

Introduction

The formation of mammalian dentition is one of the most remarkable processes in development and provides a powerful model for studying the epithelial–mesenchymal interactions that control patterning and morphogenesis of a variety of developmental processes. The application of a multitude of both *in vivo* and *in vitro* strategies in the mouse has greatly enabled our understanding of the intricate molecular mechanisms that influence the patterning of dentition. What has emerged is the realization

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that tooth development involves a complex series of genetic interactions involving growth factors, transcription factors, signal receptors and diffusible morphogens that interact within independent signaling pathways (1). That the patterning of dentition is under strict genetic control is further proven by the condition of human tooth agenesis, one of the most commonly inherited disorders which affects up to 20% of the population and imposes significant functional, psychosocial and financial burdens on patients.

To date, data obtained from mouse genetic and molecular studies have been indispensable for unraveling the genetic etiology of human tooth agenesis. For instance, the initial discoveries of *MSX1* and *PAX9* as causative genes for human tooth agenesis were guided by earlier studies performed in mice (2, 3). Subsequently, numerous *PAX9* and *MSX1* mutations in families with non-syndromic forms of autosomal dominant posterior tooth agenesis have been identified. Despite these advances, the precise mechanisms by which these disease-causing mutations exert their effects are largely unknown.

Tooth initiation involves regional specification of dental ectoderm and mesenchyme

In mice, tooth development has been characterized in much detail. The first morphological sign of tooth development is the formation of the dental lamina, a thickening of the oral epithelium, which appears around embryonic day 11.5 (E11.5). Classic transplantation experiments first demonstrated that morphogenetic fields for tooth development are present at this time (4, 5). At this stage, tooth-forming potential resides in the regionally specified dental ectoderm (6). After E11.5, the odontogenic potential shifts from the epithelium to the mesenchyme which itself is now able to induce tooth formation when combined with a non-dental epithelium, whereas the epithelium has lost this ability. Subsequently, the dental lamina grows into the underlying mesenchyme of the first branchial arch, thereby forming epithelial buds (bud stage). Around E14.5, odontogenesis is directed by a specific group of signaling epithelial cells, known as the enamel knot. Thus, the potential to dominate tooth development shifts back and forth between epithelium and mesenchyme. However, the precise nature and role of the molecules involved in the transfer of inductive potential from the epithelium to the mesenchyme and back to the epithelium is poorly understood.

In recent years significant advances have been made in understanding the molecular mechanisms that determine the site of tooth initiation (7, 8). Several studies indicate that synergistic and antagonistic interactions of signaling molecules are recursively utilized in tooth development. This leads to the local activation or inhibition of transcription factors in tooth epithelium and mesenchyme (7). Msx1 and Pax9 are among the best studied tooth mesenchymal transcription factors that appear to have key regulatory functions in the early phases of odontogenesis (7–9).

Pax9 belongs to a family of Pax genes that play highly tissue-specific functions in development and disease

Pax genes are comprised of a family of nine transcriptional regulators isolated through sequence homology to the DNA binding domain of the Drosophila segmentation gene *paired*. They encode proteins that share a 128-amino acid DNA binding domain (paired box) and are important regulators of numerous developmental processes (10, 11). The paired domain, which exhibits the highest level of sequence conservation among Pax proteins, is structurally composed of two distinct helix-turn-helix motifs that mediate sequence-specific interaction with DNA, primarily with target genes containing the core GTTC motif (12). In addition to the paired domain, the protein possesses another functionally distinct domain that likely functions as the transactivation domain. Although detailed characterization of the transactivation domain of PAX9 is currently unavailable, sequence homology with potent transactivator proteins localizes the putative transactivation function to the proline-, serine-, and threonine-rich (PST) carboxyl terminal domain of PAX9 (13).

Pax9 plays important roles during tooth development, as indicated by its expression pattern, the phenotype of transgenic mice lacking both copies of the gene, and by the association of agenesis of posterior dentition with *PAX9* mutations in humans (3, 14). In mouse embryos, *Pax9* is an early marker of tooth development, appearing at E10 in the mesenchyme before ectodermal thickening and before the expression of other tooth signaling genes. High levels of *Pax9* expression are subsequently maintained throughout the initiation (E11.5), bud, and cap stages and are down regulated at the bell stage (E16)

(14). Mesenchymal expression of *Pax9* is initially regulated by antagonistic signals between Bmp4 and Fgf8. While Bmp4 signaling is inhibitory, that of Fgf8 is activating and the coupled antagonistic interaction mediated by these two epithelial signaling molecules restricts the expression of *Pax9* to prospective sites of tooth development (14). Bmp4 is also expressed in the mesenchyme, but there its expression is downstream of Pax9, as will be discussed below.

Similar to other Pax family members that act in a highly tissue-specific manner, Pax9 is likely to mediate its tooth-specific functions through its interactions with other proteins. Several of our studies point to an important partnership between the Pax9 paired domain protein and the Msx1 homeoprotein in regulating gene expression in dental mesenchyme. Recent studies from our laboratory suggest that Pax9 alone can transactivate the Msx1 and Bmp4 promoters and that Pax9 interaction with Msx1 on the protein level modulates this transactivation. Hence, Pax9 appears to be integrated with Msx1 in a feedback loop to regulate Bmp4 expression in the mesenchyme. This is critical for the advancement of the tooth bud since Bmp4 is involved in downstream signaling events that are required for the formation of the enamel knot, a transient signaling center of the epithelium that directs progress to the next developmental stage (cap stage). We hypothesize that a key function of Pax9 and Msx1 is the maintenance and regulation of mesenchymal Bmp4 expression and that this regulation involves not only the DNA binding/transcription factor activity of Pax9, but also an interaction on the protein level of Pax9 with Msx1 and other homeo-domain proteins that are expressed in dental mesenchyme.

Human tooth agenesis

The fact that the formation of human dentition is under strict genetic control is proven by the condition of tooth agenesis, where teeth are congenitally missing. The following summary characterizes what is currently known about the clinical manifestations and etiology of tooth agenesis.

Phenotypic manifestations and inheritance patterns

Tooth agenesis is classified as a clinically heterogeneous condition that affects various combinations of teeth. It is the most common developmental anomaly in man, reported to occur in 2–20% of the population, excluding third molars (15–19). This disorder is most often bilaterally symmetrical and affects permanent dentition at a much higher rate than primary teeth. Tooth agenesis can occur as a part of a syndrome affecting multiple organ systems, or it may present in an isolated familial manner. The most commonly diagnosed non-syndromic form, hypodontia, is defined by less than six congenitally missing permanent teeth while the rare form, termed oligodontia is defined by more than six missing permanent teeth (excluding third molars). The non-syndromic form of tooth agenesis can be sporadic or familial. Familial tooth agenesis is typically inherited in an autosomal dominant manner, but autosomal recessive and X-linked forms have also been reported (20, 21).

Clinical implications

The unavoidable dental consequences of tooth agenesis include malocclusion due to improper position of the teeth, deficient growth of the alveolar processes associated with the missing teeth (22), and excess space within the dental arches. The availability of space results in drifting, tipping, and supra-eruption of the adjacent or opposing teeth (23). In posterior tooth agenesis, the functional atrophy in bone height is easily recognizable. Although clinicians have long observed tooth agenesis, the early diagnosis, preventive or interceptive dental measures and treatment options for this condition have been extremely limited. Therapy is phasic, complicated and lengthy, and involves at least two dental specialists. When several posterior teeth are missing, orthodontic correction is followed by bone augmentation procedures to increase the bone mass before the placement of implants. With the exception of syndromic cases of anodontia, tooth agenesis involving molars and premolars manifests with the most severe of dental complications.

Genetics of human tooth agenesis

Genes implicated in epithelial-mesenchymal interactions by studies in the mouse serve as potential candidates for tooth agenesis in humans. To date, the mutation spectra of non-syndromic tooth agenesis in humans has revealed defects in two such genes that encode transcription factors, *MSX1* and *PAX9*. More recently, *AXIN2*, a Wnt-signaling receptor was identified as responsible for a non-syndromic form of tooth

agenesis (24). When compared with a fairly mixed pattern of tooth agenesis seen in individuals with a non-sense mutation in *AXIN2*, the phenotypes reported in *MSX1* and *PAX9* affected families are more restricted to posterior dentition.

To date, we and others have identified 11 distinct disease-causing mutations in the *PAX9* gene and five mutations in the *MSX1* gene that result in posterior tooth agenesis. Most of these mutations are located in the paired box domain of Pax9 or the homeodomain of Msx1 (2, 3, 25–32). They range from missense mutations that change just one amino acid in the entire protein to premature stop codons that result in truncation of the protein products. Given that the mode of inheritance in all of these cases is autosomal dominant, the resulting phenotype may be due to haploinsufficiency, a dominant-negative activity, or a novel activity of the mutant protein. In support of haploinsufficiency as the causative mechanism is a unique family affected with severe hypodontia involving agenesis of all primary and permanent posterior teeth. Fluorescence *in situ* hybridization (FISH) analysis showed the presence of a >57 kb deletion encompassing the *PAX9* locus (29). Thus, human *PAX9* is a dosage-sensitive gene; true haploinsufficiency results in a severe form of tooth agenesis. This, however, fails to fully explain the mechanisms underlying the other disease-causing mutations that result in less severe and variable phenotypes where not all posterior teeth are affected. It is possible that the mutant allele may be hypomorphic, in which case the combined activities of the wild type and mutant alleles do not reach the threshold level necessary for normal tooth development. Alternatively, relatively milder phenotypes may be the result of a defective allele that generates an aberrant protein that acts in a dominant-negative manner or has a novel function. Besides Bmp4 downregulation, mutations in *PAX9* could result in a selective reduction in PAX9 binding to sites that regulate *MSX1* expression levels. With *PAX9* mutations, the absence of premolars in addition to molars may reflect a secondary down regulation in MSX1. Equally compelling is the hypothesis that mutations in either *PAX9* and/or *MSX1* can lead to defective protein–protein interactions that disrupt normal downstream functions important for tooth morphogenesis. Our data showing that Pax9 interacts with Msx1 at both gene and protein levels lend strong support to both mechanisms (see below).

Experimental Evidence

Pax9 and Msx1 proteins interact stably in COS7 cells

To begin to address the mechanisms underlying the potential actions and interactions of Pax9 and Msx1, we analyzed the ability of the proteins to physically associate *in vivo*. Briefly, physical interaction between Pax9 and Msx1 was evaluated by immunoprecipitation of epitope-tagged proteins expressed in a mammalian cell line, COS7. Our co-immunoprecipitation analysis demonstrated that both proteins interact stably within cells. Independent co-immunoprecipitation assays performed in the Peters lab also confirmed that Pax9 and Msx1 can form a protein complex (33).

Pax9 is needed for the expression of Msx1 and Bmp4 in mesenchyme during tooth morphogenesis

The expression of mesenchymal genes that are known to be co-expressed with *Pax9* during tooth morphogenesis was evaluated in molar organs of homozygously mutated Pax9 embryos using *in situ* hybridization with riboprobes to Msx1 and Bmp4 (14). The tooth organs in Pax9 (−/−) mice showed considerable growth retardation when compared with wild type and mesenchymal expression of Msx1 and Bmp4 was significantly reduced, suggesting an upstream epistasis of Pax9 with Msx1 and Bmp4 during tooth morphogenesis.

Pax9 X Msx1 double heterozygote mice show tooth agenesis that is significantly rescued by transgenic Bmp4 expression

Elegant studies performed by Dr Heiko Peters (University of Newcastle, UK) revealed that mice heterozygous for both Pax9 and Msx1 demonstrated significant defects. In contrast to the single Pax9 or Msx1 heterozygous mice, which have no observable phenotype, *Pax9*/*Msx1* double heterozygote mutant mice show absence of lower incisors and third molars. On the basis of the finding of a down-regulation of Bmp4 expression in Pax9 (−/−) tooth organs, it was examined whether the tooth agenesis phenotype seen in the *Pax9*/*Msx1* (+/−) mice could be rescued *in vivo* by increasing the level of Bmp4 expression in dental mesenchyme. Indeed, the Pax9/Msx1 (+/−) phenotype was rescued by two transgenic copies of the human Bmp4 gene resulting in a complete rescue of third molar formation. The incisor phenotype was partially rescued, as they appeared hypoplastic with delayed ameloblast

differentiation. These data provide compelling evidence that deregulation of Bmp4 expression is a key event underlying the tooth agenesis caused by the heterozygous loss of Pax9 and Msx1.

Interactions between Pax9 and Msx1 proteins are synergistic for the activation of the Msx1 and Bmp4 promoters

To determine the transcriptional activities of Pax9 and Msx1, DNA co-transfection assays of Pax9 and/or Msx1 expression vectors with a 3.5 kb Msx1 or 2.4 kb Bmp4 promoter element linked to a luciferase reporter gene were performed. Transfection of the Pax9 expression plasmid alone showed a two-fold activation of the p3.5Msx1-Luciferase reporter plasmid, whereas transfection of the Msx1 expression plasmid alone resulted in a slight repression below basal level. However, co-transfection of Msx1 and Pax9 expression vectors in this assay enhanced Pax9-mediated activation of the reporter construct almost four-fold, while further increase of the concentration of Msx1 expression plasmid resulted in a two-fold activation of the Msx1–Luc reporter construct. Reporter assays using the Bmp4 construct p2.4Bmp4-Luc containing 2.37 kb of sequence upstream of the translation initiation site showed that transfection of the Pax9 expression plasmid alone resulted in a seven-fold activation of luciferase activity, whereas transfection of Msx1 expression plasmid alone failed to regulate this promoter. Co-transfections with Pax9 and Msx1 expression plasmids showed a fourteen-fold increase in Bmp4 promoter activation, but doubling the concentration of Msx1, while keeping the concentration of Pax9 the same resulted in a decrease of activation to approximately five-fold (33). The functional consequences of Pax9–Msx1 protein interaction could hence involve the localized modulation of Bmp4 activity in dental mesenchyme. Importantly, these assays corroborate *in vivo* data from mouse genetic studies and support reports of Msx1-dependent expression of Bmp4 in dental mesenchyme (34).

A natural Pax9 mutant (L21P) that causes human tooth agenesis can bind to Msx1 but is unable to upregulate either Msx1 or Bmp4 expression

We evaluated the functional effects of one human PAX9 missense mutation (30) which is located in the amino terminal portion of the paired box and known to cause posterior tooth agenesis. A plasmid encoding the nucleotide change (T62C) was generated and the myc-tagged mutant protein, L21PPax9, studied in transfected COS7 cells.

Immunolocalization studies performed with anti-c-Myc antibody show that L21PPax9 protein, like the wild type (wt) protein is stable in mammalian cells and localizes in the nucleus. Immunoprecipitations performed on cells co-expressing combinations of wild type and mutant Pax9 and Msx1 proteins demonstrate that the interaction of L21PPax9 protein with the Msx1 protein is indistinguishable from wild type Pax9/MSX1 protein interaction. The functional consequences of the L21P mutation on promoter activation was assessed by transient transfection assays using reporter plasmids with either a 3.5 kb Msx1 promoter element or a 2.4 kb Bmp4 promoter sequence upstream of a luciferase reporter gene. Transcriptional activation of these promoter constructs by L21Pax9 was compared with activation by wild type Pax9. While wild type (wt) Pax9 lead to a four-fold and 40-fold activation of Msx1 and Bmp4 promoters, respectively, the L21Pax9 mutation completely abolished this promoter activation and co-expression of Msx1 with L21Pax9 did not achieve any synergistic transcriptional activation which is usually seen with co-expression of Msx1 and wt Pax9. Furthermore, it was shown with electromobility shift assays (EMSA) that the L21PPax9 protein unlike wt Pax9 does not bind to the paired domain recognition sequences e5 and CD19-2(A-ins). Thus, our data suggests that this specific region of the N-terminal paired box subdomain of Pax9 is critical for Bmp4 activation and is not involved in interaction with the Msx1 protein (33).

We have also identified families with tooth agenesis who carry PAX9 paired domain mutations that do not lead to a loss of DNA binding capability as shown by strong interaction with e5 and CD19-2(A-ins) in gel mobility shift assays. Studies are currently ongoing to determine if the impaired function of these mutations may involve protein–protein interactions with Msx1.

Studies on human tooth agenesis will further our understanding of tooth morphogenesis

DNA from 16 families with posterior tooth agenesis from our registry was characterized by sequencing and linkage analysis for *PAX9* and *MSX1* mutations. Only six of these families showed pathogenic sequence changes (missense or insertion/deletion mutations) in *PAX9* or

MSX1. We conclude that additional genes are responsible for tooth agenesis and it is conceivable that several of these genes also belong to the large group of genes that are so far known to be expressed during tooth morphogenesis.

Conclusion and Future Perspectives

The cumulative data obtained from mouse and human genetics studies, as well as molecular assays indicate that *Pax9* directly and/or indirectly exerts control over early events in tooth development, especially the transition from bud stage to cap stage. This would encompass: A) the complex interplay of Pax9 with other transcription factors which are co-expressed in dental mesenchyme, B) the direct transcriptional regulation of molecules that are downregulated in Pax9 deficient tooth organs, and C) the regulation of a target effector molecule, Bmp4, through the interaction of Pax9 with candidate transcription factors. The following discussion describes experiments which will contribute insights into integrating components of the *Pax9* signaling pathway to understand tooth formation.

It is clear that the functions of Pax9 and Msx1 are essential for the establishment of the odontogenic potential of the mesenchyme through the maintenance of mesenchymal Bmp4 expression. However, the relationship between the three genes on the molecular level remains unknown. As a logical next step of an inquiry into the tooth-specific biological effects of Pax9, this molecular relationship between Pax9, Msx1, and Bmp4 must be examined more closely. Based on the hypothesis that the selective functions of Pax9 in early tooth development involve specific protein-protein interactions with Msx1, as well as the direct modulation of Pax9-responsive elements within *Msx1* and *Bmp4* regulatory regions, studies to understand the functional specificity of Pax9 must be performed to further explain how defects in these regions may contribute to the pathogenesis of human tooth agenesis.

It is widely accepted that the tissue or cell-specific actions of transcriptional regulatory proteins, in particular, homeoproteins, are mediated through selective interactions with other protein factors. Indeed, our laboratory has demonstrated that Pax9 is able to form a heterodimeric complex with Msx1. Based on this and previous studies that have shown that the paired domain of Pax proteins and the homeodomain of Msx1 participate in protein–protein interactions, the first step is to address potential interactions of Pax9 with other homeodomain-containing transcription factors that are co-expressed in developing dental mesenchyme. Of all the candidate genes that are expressed in developing tooth organs, *Lef1*, *Dlx1*, *Dlx2*, *Barx1*, and *Lhx6*, and *Lhx7* represent suitable candidates based upon knowledge of their function and specific expression patterns and because tooth agenesis phenotypes were observed in transgenic null mice (see website: http://bite-it.helsinki.fi/). Interactions between Pax9 and the candidate proteins can be evaluated using co-immunoprecipitation experiments with full-length and truncated proteins.

To identify additional Pax9-dependent genes expressed in dental mesenchyme, it would be logical to first analyze those genes whose expression is downregulated in the absence of *Pax9*. These genes must first be delineated to determine whether *Pax9* maintains expression of those genes selected by direct activation of transcription or through the activity of intermediate regulators. Next, these novel genes must be integrated into the Pax9-dependent signaling pathway using methods like ChIP-on-chip, *in situ* hybridization and further analysis in *Pax9*-deficient mice.

While it is has been shown that mutations in *PAX9* and *MSX1* are involved in human tooth agenesis, it is becoming increasingly evident that other genes also play a role. This is clearly supported by the following: first, current literature classifies human tooth agenesis as occurring in specific patterns: a posterior pattern of missing molars and premolars; anterior patterns involving agenesis of cuspids and/or incisors and mixed patterns with missing premolars and lateral incisors (35). Therefore, clinical presentations of tooth agenesis clearly suggest that different genes are involved in the formation of each family of teeth (Fig. 1). In support of this theory are results from multiple studies performed in mice suggesting that spatially and molecularly distinct signaling pathways involving unique combinations of transcription factors and growth factors create patterning domains of dentition (36, 37). Second, our laboratory has shown that many families with a pattern of posterior tooth

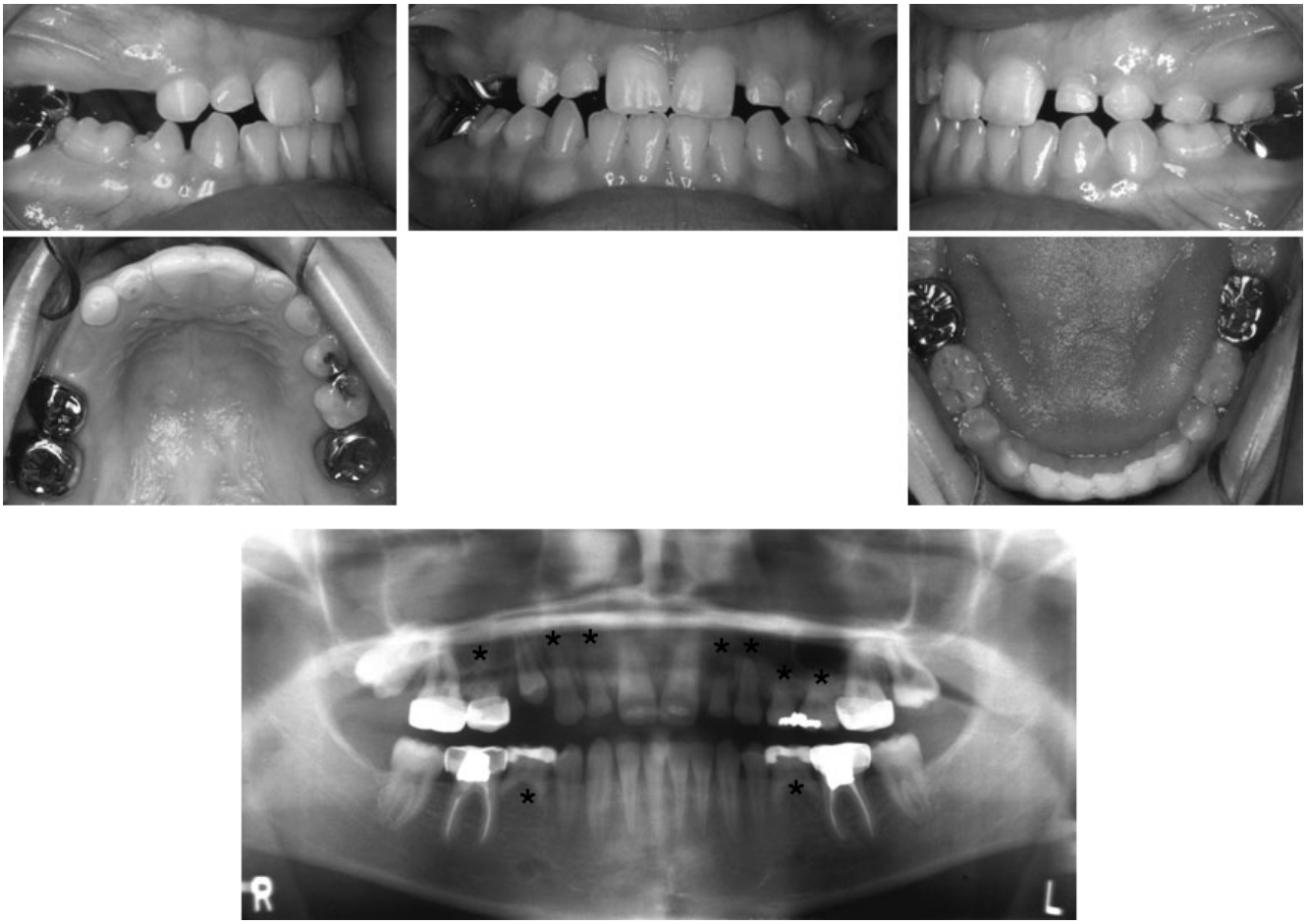

Fig. 1. Intraoral photos and panoramic radiograph of an individual with both anterior and posterior tooth agenesis. The missing teeth are indicated by an asterisk (*).

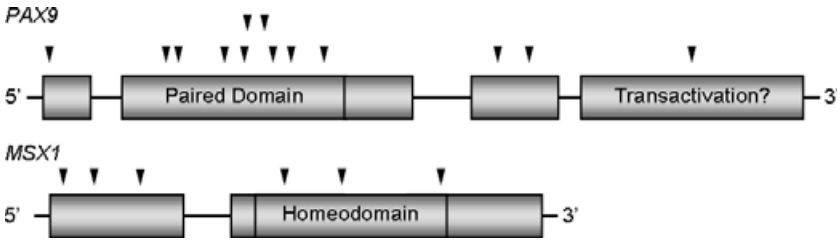

Fig. 2. Schematics representing the coding regions for *PAX9*and *MSX1* and known mutations in patients with tooth agenesis (represented by inverted triangles). This does not include the two mutations which have been described for *AXIN2* (38).

agenesis closely resembling that seen with *PAX9* or *MSX1* mutations do not have changes in these two genes. For 10 of the 16 families, we identified in our laboratory, bi-directional sequencing of *PAX9* and *MSX1* revealed no pathogenic mutations and linkage testing excluded *PAX9* and *MSX1*. This strongly suggests that other unidentified genes contribute to posterior tooth agenesis. Therefore, another direction is to identify 'novel' genes and mutations responsible for posterior tooth agenesis in these families by genetic mapping. The identification of additional genes would contribute valuable insights that cannot be gained through the mouse model alone (Figs 2 and 3).

The studies described in this review are of significance because they will contribute to a broader understanding of how the activities of transcription factors and growth factors are regulated and how they can be modulated as an approach to therapy. Furthermore, a better fundamental understanding of how transcription factors achieve their context-dependent or tissue-specific functions can be anticipated. Therefore, the information gained is equally

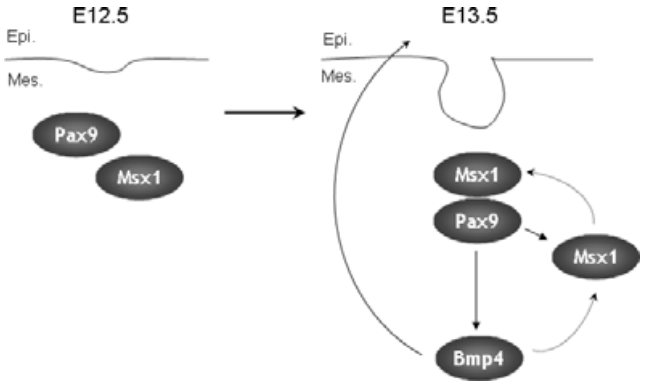

Fig. 3. Schematic view of our current understanding of the roles of Pax9, Msx1, and Bmp4 in early tooth morphogenesis. Pax9 is not a transcriptional regulator of Msx1 at the time of tooth initiation, but the presence of Msx1 is critical for advancing of tooth morphogenesis. At E12.5, Pax9 cannot induce Msx1 in dental mesenchyme (*Mes.*). At 13.5, Pax9 begins to induce Msx1 expression, and the heterodimer of wild-type Pax9 and wild-type Msx1 proteins can induce and enhance Bmp4 expression in dental mesenchyme. *Epi.*, epithelium (33).

applicable to research in emerging areas of tooth bioengineering and regenerative dental medicine as it is in developmental research.

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