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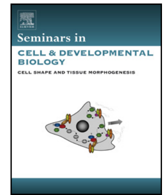

Review

Molecular patterning of the mammalian dentition

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ABSTRACT

Four conserved signaling pathways, including the bone morphogenetic proteins (Bmp), fibroblast growth factors (Fgf), sonic hedgehog (Shh), and wingless-related (Wnt) pathways, are each repeatedly used throughout tooth development. Inactivation of any of these resulted in early tooth developmental arrest in mice. The mutations identified thus far in human patients with tooth agenesis also affect these pathways. Recent studies show that these signaling pathways interact through positive and negative feedback loops to regulate not only morphogenesis of individual teeth but also tooth number, shape, and spatial pattern. Increased activity of each of the Fgf, Shh, and canonical Wnt signaling pathways revitalizes development of the physiologically arrested mouse diastemal tooth germs whereas constitutive activation of canonical Wnt signaling in the dental epithelium is able to induce supernumerary tooth formation even in the absence of Msx1 and Pax9, two transcription factors required for normal tooth development beyond the early bud stage. Bmp4 and Msx1 act in a positive feedback loop to drive sequential tooth formation whereas the Osr2 transcription factor restricts Msx1-mediated expansion of the mesenchymal odontogenic field along both the buccolingual and anteroposterior axes to pattern mouse molar teeth in a single row. Moreover, the ectodermal-specific ectodysplasin (EDA) signaling pathway controls tooth number and tooth shape through regulation of *Fgf20* expression in the dental epithelium, whereas Shh suppresses Wnt signaling through a negative feedback loop to regulate spatial patterning of teeth. In this article, we attempt to integrate these exciting findings in the understanding of the molecular networks regulating tooth development and patterning.

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1. Introduction

Cell–cell interactions through signal transduction pathways are crucial for the development of all multicellular organisms. Despite the enormous number of distinct cell types and a wide variety of tissue structures and patterns in the animal kingdom, a few conserved cell–cell signaling pathways, including the fibroblast growth factor (Fgf), hedgehog, transforming growth factor-beta (Tgfβ) and wingless-related (Wnt) signaling, are used repeatedly to regulate most of the developmental programs within individual animals and throughout vertebrate evolution [1]. Whereas decades of genetic and biochemical studies have identified many of the molecular components of each of these signaling pathways and revealed extensive cross-talk among them, the detailed mechanisms regarding how they are modulated and how new components are integrated into existing signaling networks to control morphogenesis and patterning during mammalian organogenesis remain to be elucidated.

Teeth, like many organs, form through sequential and reciprocal inductive interactions between the adjacent epithelium and mesenchyme [2–5]. Tooth development is largely independent from the rest of the body, and isolated, even dissociated and recombined, mammalian tooth germs can continue to develop to mineralized teeth upon transplantation to ectopic sites in adult animals [6,7], such as the renal capsule. Thus, tooth development has long been used as a model for studying inductive interactions regulating organogenesis. In addition to allowing detailed analysis of the mechanisms regulating initiation, morphogenesis, and maturation of the individual organ, teeth exhibit species-specific number, shape, and patterns, and therefore provide a general paradigm for the studies of molecular mechanisms of developmental patterning and of evolution [8,9]. Through combinations of experimental embryological manipulations and transgenic and gene knockout studies in mice, research in the past 20 years have investigated the roles of each of the major signaling pathways in tooth organogenesis [2–5,10–13]. Whereas many mutant mice exhibit tooth developmental arrest phenotypes and revealed requirements of particular genes and pathways for specific steps of tooth organogenesis [10], several transgenic or gene-knockout mutant mouse strains exhibit alterations in the number, shape, and/or pattern of teeth, of which recent studies have provided fascinating new insights into the integration of signaling networks regulating tooth organogenesis and dentition patterning [13]. Many review articles published previously provide excellent references on the progress in the studies of the molecular mechanisms of tooth development [2–5,9–13]. In this review, we highlight the integration of the actions of networks of activators and inhibitors of the Bmp (bone morphogenetic proteins, members of the Tgfβ superfamily), Fgf, Shh, and Wnt signaling pathways in the regulation of tooth morphogenesis and spatial patterning of the dentition.

2. The Bmp, Fgf, Shh, and Wnt signaling pathways are each repeatedly required for tooth initiation and morphogenesis

Whereas most of our understanding of the molecular mechanisms of tooth development has been derived from studies using mouse models, the basic steps of tooth organogenesis are similar in all vertebrates [8,9]. In mice, tooth development begins as a thickening of the oral epithelium, termed dental lamina (Fig. 1A), at 11 days of gestation (E11). The dental lamina proliferates and buds into the underlying neural crest-derived mesenchyme and induces the mesenchyme to condense around the epithelial bud from E12 to E13 (Fig. 1B). The dental mesenchyme in turn induces formation of an epithelial signaling center in the distal region of the epithelial bud, termed primary enamel knot, which drives tooth morphogenesis through the "cap" and "bell" stages (Fig. 1C and D). As development proceeds, the epithelial cells in contact with the dental papilla mesenchyme differentiate into ameloblasts and the adjacent mesenchymal cells differentiate into odontoblasts (Fig. 1E) [14]. The ameloblasts and odontoblasts deposit enamel and dentin matrices, respectively back-to-back and subsequent mineralization of these matrices forms the hard tissues of the tooth [6]. Thus, formation of each individual tooth, from its initiation through morphogenesis to cytodifferentiation, involves an extensive series of reciprocal interactions between the dental epithelium and the neural crest derived mesenchyme.

2.1. Regulation of tooth initiation and tooth bud formation

At the beginning of tooth development, multiple members of the Bmp, Fgf, and Wnt families, including *Bmp2*, *Bmp4*, Bmp7, *Fgf8*, *Fgf9*, *Wnt4*, *Wnt6*, *Wnt10a*, and *Wnt10b*, and *Shh* are expressed in the presumptive dental epithelium [15–22]. Blocking each of these four signaling pathways at the beginning of tooth development genetically or in explant culture causes tooth developmental arrest at the dental lamina or early bud stage [4,5,7,10–12]. Bmp and Fgf signaling is necessary for activation of expression of the Msx1 and Pax9 transcription factors, respectively, in the presumptive tooth mesenchyme [17,19,22,23]. Mice lacking either *Msx1* or *Pax9* function exhibit tooth developmental arrest at the bud stage [24,25]. Expression of *Bmp4* shifts from the presumptive dental epithelium to the developing tooth mesenchyme at the early bud stage during normal tooth development and is significantly reduced in the developing tooth mesenchyme in either *Msx1*$^{-/-}$ or *Pax9*$^{-/-}$ mutant mice [17,25,26]. In addition, Fgf8 induces *Fgf3* expression in the dental mesenchyme in an Msx1-dependent manner [27]. Although teeth develop nearly normally in *Fgf3*$^{-/-}$ mutant mice [28,29], mice homozygous for null mutations in both *Fgf3* and *Fgf10*, which are both expressed in the developing tooth mesenchyme, exhibit tooth developmental arrest at the bud stage [29]. Fgf8 also induces expression of *Inhibin-βA* (*Inhba)* (also known as *Activin-βA*), another member of the Tgfβ superfamily, in the developing tooth mesenchyme [30]. Mice lacking *Inhba* function exhibit early developmental arrest of incisors and mandibular molar tooth germs [30,31]. In addition, tissue-specific inactivation of the Bmp receptor gene *Bmpr1a* in either the neural crest lineage or the oral epithelium caused tooth developmental arrest at the bud stage [32–34]. Mice with a deletion of the epithelial isoform of the type-2 Fgf receptor also exhibit tooth developmental arrest at the bud stage [35]. Thus, both Bmp and Fgf signaling are critical for the reciprocal interactions between the epithelium and mesenchyme during early tooth development. On the other hand, although expression of the Wnt ligands is mostly restricted to the dental epithelium, with exception of expression of *Wnt5a* in the dental mesenchyme [21], tissue-specific inactivation of β-catenin, the obligatory intracellular mediator of the canonical Wnt signaling pathway, in either the dental epithelium or the dental mesenchyme also caused tooth developmental arrest at the bud stage [36,37].

Recently, O'Connell et al. [38] analyzed properties of the gene regulatory networks mediating the reciprocal epithelial–mesenchymal interactions during early mouse molar development through systematic analyses of previously reported gene expression data together with more than one hundred new microarray-based gene expression profiling datasets from isolated early tooth epithelial and mesenchymal tissues. They identified the Wnt and Bmp pathways as the two major mediators of epithelial–mesenchymal signaling in early tooth development. The Wnt and Bmp pathways collectively control the production of signaling molecules in all major pathways, including Bmp4, Shh, Fgfs, and Wnts in the epithelium and Fgfs, Bmp4, and Inhba in the mesenchyme of the early tooth germs [38]. Whereas a simple

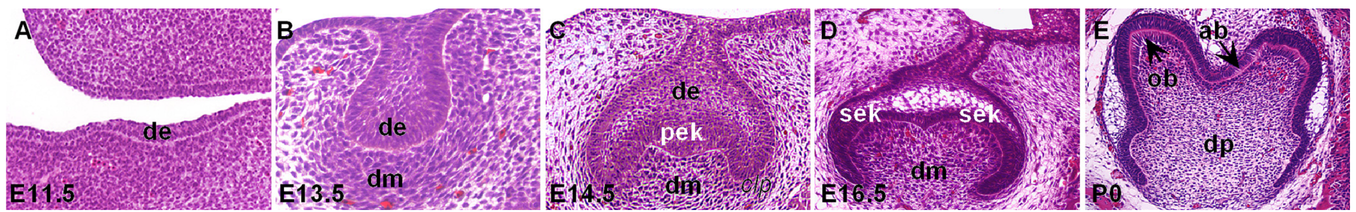

Fig. 1. Histology of first molar tooth development in mice. (A–E) Selected coronal sections of developing first molar tooth germs at dental lamina (E11.5, A), bud (E13.5, B), cap (E14.5, C), early bell (E16.5, D), and late bell (P0, E) stages are shown. ab, ameloblast; de, dental epithelium; dm, dental mesenchyme; dp, dental pulp; ob, odontoblast; pek, primary enamel knot; sek, secondary enamel knot.

ordinary differential equation model shows that the structure of a Wnt–Bmp feedback circuit recapitulates key features of the observed sequential and reciprocal epithelial–mesenchymal signaling [38], the exact mechanisms that control the cross-regulation and integration of the Bmp, Fgf, Shh, and Wnt signaling pathways remain to be elucidated.

2.2. Formation of the primary enamel knot and tooth morphogenesis

Just prior to transition of the tooth bud to the cap stage, the primary enamel knot forms at the tip of the tooth bud and exhibits restricted expression of multiple members of the Bmp, Fgf, and Wnt families, including Bmp2, Bmp4, Bmp7, Fgf3, Fgf4, Fgf9, Fgf20, Wnt3, Wnt6, Wnt10a, Wnt10b, as well as Shh [4,10–12,17–21]. The primary enamel knot has a central role in patterning the tooth crown by regulating growth and folding of the dental epithelium as well as regulating secondary enamel knot formation during molar development [3,10,39,40]. Interestingly, while the enamel knot expresses multiple Fgf ligands, expression of Fgf receptors is dramatically down-regulated and, concomitantly, expression of the cyclin-dependent kinase inhibitor gene *p21* (also known as *Cdkn1a*) is highly activated in the enamel knot [40,41]. Thus, the enamel knot stimulates proliferation of the surrounding epithelial cells but cells within the enamel knot do not proliferate, causing epithelial folding to form the "cap" and subsequently "bell" shaped tooth germs [12].

Induction of the enamel knot depends on a combination of signals from the epithelium and mesenchyme. Of the mesenchymally expressed signals, Bmp4 is able to induce expression of several primary enamel knot markers, including *p21* in isolated dental epithelium [40] as well as to rescue the bud-to-cap transition of the *Msx1*$^{-/-}$ mutant molar tooth germs in explant culture [26,42]. On the other hand, Wnt/β-catenin signaling in the dental epithelium plays a critical role in the induction and function of the enamel knot. Constitutive stabilization of β-catenin in the oral epithelium through direct overexpression using the human *keratin-14* (*K14*) gene promoter, as well as epithelial-specific deletion of *Apc*, resulted in continuous formation of enamel knots in the dental epithelium and subsequently supernumerary tooth formation [43,44]. Deletion of *Bmpr1a* together with *Apc* in the oral epithelium, however, blocked enamel knot formation and resulted in tooth bud developmental arrest similar to that in mice lacking epithelial *Bmpr1a* alone [38], indicating that both Bmp and Wnt signaling activities are required for enamel knot formation.

Integration of Bmp and Wnt signaling during the bud-to-cap transition is mediated in part by Lef1, a transcription factor that interacts with β-catenin to regulate expression of Wnt target genes [45]. *Lef1* is expressed in both the dental epithelium and mesenchyme at the bud stage, with the epithelial expression restricted to the primary enamel knot by the cap stage [20,46]. Both Bmp4 and Wnt10b are able to induce *Lef1* mRNA expression in E10.5 mouse embryonic mandibular explants [20,26]. Lef1 function is required for *Fgf4* expression in the primary enamel knot and exogenous Fgf4 is able to rescue *Lef1*$^{-/-}$ mutant tooth germs from a bud stage developmental arrest [46,47]. Thus, the Bmp, Fgf, and Wnt signaling pathways are all required for tooth development through the bud-to-cap transition. Further growth and morphogenesis of the tooth germ through the cap and bell stages largely depend on Fgf signaling, with the different Fgf family members acting partially redundantly [11,48]. Shh signaling also plays a critical role in proliferation of both the dental epithelium and mesenchyme [49,50].

Taken together, the reiterative use of the Bmp, Fgf, Shh, and Wnt signaling pathways during tooth development, combined with a large number of mutant mouse strains for their detailed analysis in tooth initiation and morphogenesis, makes mouse tooth development an excellent model for further studies of the molecular mechanisms integrating these and other signaling networks in mammalian organogenesis.

3. An integrated network of activators and inhibitors of the Bmp, Fgf, Wnt, and Shh pathways regulates tooth number and pattern along the tooth row

Mammals have a single row of teeth along the oral margin of the upper and lower jaws. The ancestral tooth formula of placental mammals consists of three incisors, one canine, four premolars, and three molars in each half of the jaw [51]. In comparison, humans have a reduced number of teeth but still a full complement of tooth types, whereas mice have only one incisor and three molars, separated by a toothless diastema, in each jaw quadrant. The lack of teeth in the mouse diastema region is not due to lack of tooth initiation during embryogenesis, however. Careful 3D reconstruction analyses of histological sections and molecular marker studies showed that at least two rudimentary buds, which exhibit transient *Shh* expression, form in the diastema region prior to first molar morphogenesis in each quadrant of the embryonic mouse jaws [51–54]. In the maxilla, both diastemal tooth buds, called R1 and R2, regress by apoptosis. In the mandible, whereas the anterior bud regresses, the posterior bud, also called R2, is only transiently affected by apoptosis and subsequently incorporated into the anterior part of the first molar [51–55]. These transient tooth buds anterior to the first molar tooth germs have been interpreted as evolutionary remnants of premolars that were lost during rodent evolution [51–56]. Remarkably, premolar-like supernumerary teeth anterior to the first molars have been reported in mice carrying distinct mutations that affect each of the Bmp, Fgf, Shh, and Wnt signaling pathways [55–60], which provide new insights into the complex cross-regulation and integration of these pathways in the regulation of tooth morphogenesis and patterning.

3.1. Regulation of tooth number in mice by the Sprouty family of antagonists of Fgf signaling

The *sprouty* (*spry*) gene was first identified in *Drosophila* for its role in negatively regulating Fgf induction of tracheal branching [61]. Fgf signaling induces *spry* expression, but the Spry protein acts in a competitive fashion to block Fgf signaling [61]. Of four spry

homologs in mice, three are expressed during tooth development, with *Spry2* abundantly expressed in the tooth bud epithelium, *Spry4* in the tooth mesenchyme whereas *Spry1* is expressed in both tooth epithelium and mesenchyme [56]. Most *Spry2*-null mice exhibit a supernumerary tooth anterior to the first molar in each half of the mandible. Histological and 3D reconstruction analyses of the developing tooth germs suggest that the supernumerary teeth result from revitalization of the diastemal R2 buds [55,56]. Whereas the wildtype mouse mandibular R2 buds never express *Fgf4* and only transiently express *Shh*, the *Spry2*-null mouse embryos show robust expression of both *Fgf4* and *Shh* mRNAs in each mandibular R2 bud. Heterozygosity of either *Fgf3* or *Fgf10* suppressed the supernumerary tooth phenotype in *Spry2*-null mice, indicating that the revitalization of the diastemal tooth germs is due to increased Fgf signaling [56]. Whereas explant culture assays showed that Fgf3 and Fgf10 could induce *Shh* expression in the dental epithelium [47,56], however, the mechanisms underlying the induction of *Fgf4* expression and of enamel knot function in the *Spry2*-null diastemal tooth buds have not been resolved.

Some *Spry4*-null mice also develop supernumerary diastemal teeth in the mandible, which appeared to correlate with ectopic activation of *Fgf3* expression in the R2 bud mesenchyme [56]. In addition, although the *Spry1*$^{-/-}$ mice do not have supernumerary teeth, the frequency of supernumerary tooth formation in *Spry4*$^{-/-}$ mutant mice is increased 3-fold by *Spry1* heterozygosity [56]. Dose-dependent genetic interactions between *Spry2* and *Spry4* as well as between *Spry1* and *Spry4* have also been shown to regulate the number of incisors, with *Spry2*$^{+/-}$*Spry4*$^{-/-}$ mice having duplicated upper incisors and *Spry2*$^{-/-}$*Spry4*$^{-/-}$ mutant mice showing duplicated upper and lower incisors [62]. Thus, the Spry family proteins play critical roles in antagonizing the reciprocal Fgf signaling between the developing tooth epithelium and mesenchyme to regulate the number of teeth in the mammalian dentition.

3.2. Supernumerary tooth formation resulting from increased Shh signaling in the mouse embryonic diastema mesenchyme

Mice homozygous for a transgenic insertion *Tg737*^{orpk}, which causes partial loss of function of the *Ift88* gene encoding the intraflagellar transport (IFT) protein Polaris, exhibit a supernumerary tooth in the diastema in all four quadrants [58,63]. IFT proteins are essential for the formation of cilia, hair-like appendages on the cell surface. Most mammalian cells have a single primary cilium, which plays essential roles in processing the Gli2 and Gli3 transcription factors, effectors of the Hedgehog signaling pathway [64]. In the *Tg737*^{orpk} mutant embryos, expression of both *Ptc1* and *Gli1*, transcriptional target genes of Hedgehog signaling, was apparently increased in the diastemal tooth mesenchyme, suggesting that increased Shh signaling underlies the revitalization of the diastemal teeth in these mutant mice [58]. Whereas Shh signaling plays critical roles in regulating proliferation and differentiation of the developing dental epithelium [49,65], tissue-specific inactivation studies showed that the formation of diastemal teeth occurred in mice lacking Polaris function in the neural crest derived dental mesenchyme but not in those lacking Polaris in the oral epithelium [58]. Since Shh is the only hedgehog signal expressed in the developing tooth germs, these results suggest that a mesenchymal factor(s) downstream of increased Shh signaling is responsible for stimulating the continued development of the diastemal tooth buds in the Polaris-deficient mouse embryos. Mice homozygous for a targeted disruption of *growth arrest specific-1* (*Gas1*), which encodes an inhibitor of Shh signaling and is expressed in the diastema mesenchyme [66,67], also exhibit supernumerary diastemal teeth in all four quadrants [58], further supporting the hypothesis that increased Shh signaling activates a mesenchymal factor(s) which in turn induces/maintains a functional primary enamel knot in the diastemal tooth buds to stimulate continued tooth morphogenesis. The responsible mesenchymal factor(s) has not been identified, however.

3.3. Sostdc1 and Lrp4 function as a ligand–receptor pair to regulate tooth number and tooth shape via suppression of canonical Wnt signaling

Whereas a positive feedback circuit between Wnt and Bmp4 signaling appears to be the major mediator of the reciprocal epithelial–mesenchymal interactions during early molar tooth development [38], Bmp signaling also induces expression of Sostdc1 (also known as ectodin, Wise, and USAG1), a secreted protein that can antagonize both Bmp and canonical Wnt signaling [68–70]. During early tooth development, *Sostdc1* is expressed in the epithelium and mesenchyme surrounding the developing tooth bud but its expression is excluded from the enamel knot and adjacent epithelial and mesenchymal cells [57,59,69]. Targeted disruption of *Sostdc1* in mice resulted in multiple tooth developmental defects including molar fusions, supernumerary incisors, and a supernumerary tooth anterior to the first molar in each quadrant [59,60,71,72]. The *Sostdc1*$^{-/-}$ mutant tooth germs show increased canonical Wnt signaling, as demonstrated by using the *Top-Gal* transgenic reporter [60]. Reduction in gene dosage of *Lrp5* (*low density lipoprotein receptor related protein 5*) and *Lrp6*, which encode co-receptors for canonical Wnt signaling, showed dose-dependent rescue of the tooth anomalies whereas heterozygosity of *Fgfr1*, *Fgfr2*, *Fgf10*, or *Bmpr1a* did not affect diastemal tooth development in the *Sostdc1*$^{-/-}$ mutant mice [60], suggesting that supernumerary tooth formation in these mutant mice primarily resulted from increased canonical Wnt signaling.

Remarkably, mice homozygous for a hypomorphic mutation in the *Lrp4* gene exhibit tooth phenotypes almost identical to the *Sostdc1*$^{-/-}$ mice [57]. Lrp4 shares extensive structural similarity in its extracellular domain with those of Lrp5 and Lrp6 [73,74]. Although Sostdc1 has been shown to physically interact with both Lrp6 and Lrp4 *in vitro* [57,67,70], recent genetic analysis showed that Lrp4 is required for Sostdc1 to exert its function in regulating epidermal appendage development *in vivo* [75], suggesting that Sostdc1 and Lrp4 act as a ligand–receptor pair to antagonize Lrp5/6-mediated canonical Wnt signaling [57,75].

Paradoxically, whereas *Shh* mRNA expression and Shh signaling activity are significantly increased in the R2 tooth germs in the *Sostdc1*$^{-/-}$ mouse embryos [60], *Shh*$^{+/-}$*Sostdc1*$^{+/-}$ double heterozygous mice exhibited high penetrance of diastemal teeth while no tooth defects were detected in either *Shh*$^{+/-}$ or *Sostdc1*$^{+/-}$ mice [60]. Reducing the gene dosage of either *Ptch1* or *Lrp6* by 50% significantly reduced the frequency of the supernumerary tooth phenotype in *Shh*$^{+/-}$*Sostdc1*$^{+/-}$ mice [60], suggesting that Shh and Sostdc1 act synergistically to antagonize canonical Wnt signaling in the diastemal tooth buds. Furthermore, tissue-specific deletion of *Shh* in the oral epithelium caused significantly increased and expanded the domain of Top-Gal reporter expression in the diastemal tooth buds, indicating that Shh inhibits canonical Wnt signaling. Ahn et al. further showed that *Dkk1* mRNA expression was significantly reduced in the E13.5 *Shh*$^{+/-}$*Sostdc1*$^{+/-}$ tooth germs in comparison with the *Sostdc1*$^{+/-}$ tooth germs [60]. These data uncover a Wnt–Shh negative feedback loop as part of the tooth developmental regulatory network [60] (Fig. 2).

Consistent with the previous finding that Fgf4 acts downstream of Lef1-mediated canonical Wnt signaling at the bud-to-cap transition [47], expression of several Fgf ligands, including Fgf3 and Fgf4, is significantly increased in the E13.5 *Sostdc1*$^{-/-}$ tooth germs [60]. Thus, in *Sostdc1*$^{-/-}$ as well as *Lrp4*-deficient mice, increased canonical Wnt signaling activates the expression of multiple Fgf ligands in the tooth bud epithelium and the increased Fgf signaling

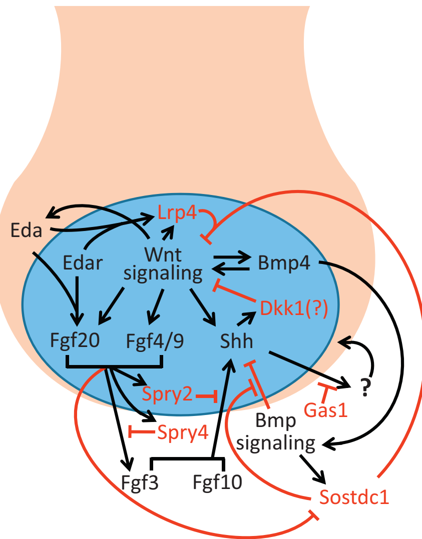

Fig. 2. The Bmp, Fgf, Shh, Wnt, and Eda signaling pathways form an integrated network of positive and negative feedback loops to regulate early tooth development through the bud-to-cap transition. The tooth bud epithelium is represented in light cinnamon color whereas the primary enamel knot (not drawn to scale) is represented in blue color. The mesenchyme surrounding the tooth bud is not colored. Black arrows indicate positive input and red indicates repressive input. A question mark is placed next to Dkk1 because it has not been experimentally confirmed as the mediator of repression of Wnt signaling by Shh. The mesenchymal signal downstream of Shh is represented with a question mark because it has not been identified and it is not clear how increased Shh signaling in the tooth mesenchyme stimulates primary enamel knot function.

overcomes Spry2/4 inhibition to stimulate continued development of the R2 tooth buds [60] (Fig. 2).

Whereas the increased Wnt signaling resulted in significantly increased *Shh* mRNA expression in the R2 tooth buds at E13.5, *Shh* mRNA expression was significantly reduced in the first molar tooth germs at E14.5 in *Sostdc1*$^{-/-}$ mouse embryos in comparison with control littermates [57,60]. Similarly, *Shh* mRNA expression was significantly reduced in the first molar tooth germs in *Lrp4*-deficient mouse embryos at E14.5 [57]. Tissue-specific inactivation of either *Shh* or *Smo* in the oral epithelium caused molar tooth fusions similar to that in *Sostdc1*$^{-/-}$ and *Lrp4*-deficient mice [49,57,60,65]. Molar fusion was also induced in the mouse pups by injecting pregnant mice with the Shh function-blocking antibody 5E1 between E10 and E16 [76]. These results suggest that the molar fusion phenotype in all of these mutant or experimentally treated mice is due to reduced Shh signaling.

Why is *Shh* expression significantly increased in the diastemal tooth buds but significantly reduced in the molar tooth germs in the *Sostdc1*$^{-/-}$ and *Lrp4*-deficient mouse embryos? The answer may lie in the finding that Sostdc1 is an antagonist of both Wnt and Bmp signaling [68–70]. Both *Sostdc1*$^{-/-}$ and *Lrp4*-deficient tooth germs showed increased Bmp signaling activity, as demonstrated by increased accumulation of phosphorylated Smad1/5/8 [57,72]. In addition, *Sostdc1*$^{-/-}$ mutant molar tooth germs showed markedly increased sensitivity to Bmp4 in explant culture [59]. It was shown previously that transgenic overexpression of *Bmp4* in the tooth mesenchyme suppressed *Shh* expression in the tooth epithelium [77]. Thus, increased Bmp signaling activity might have contributed to the molar fusion phenotype of the *Sostdc1*$^{-/-}$ or *Lrp4*-deficient mice by suppressing *Shh* expression in the molar tooth epithelium. However, Ahn et al. [60] showed that deleting both copies of *Lrp5* and one copy of *Lrp6* suppressed both the supernumerary teeth and molar fusion phenotypes in *Sostdc1*$^{-/-}$ mice. It is not known whether the rescuing effects of loss of Lrp5 and Lrp6 on the *Sostdc1*$^{-/-}$ mutant tooth germs occurred upstream or downstream of Shh signaling. Canonical Wnt signaling is also required for *Bmp4* expression in the developing tooth germs [36]. Thus, the genetic rescue of *Sostdc1*$^{-/-}$ tooth phenotypes by inactivation of *Lrp5* and *Lrp6* does not exclude the possibility that Sostdc1-mediated inhibition of Bmp signaling may play a primary role in preventing molar tooth fusion. Further investigation is needed to clarify the detailed biochemical mechanisms involving Sostdc1 and Lrp4 in tooth development and patterning.

3.4. The ectodysplasin (Eda) signaling pathway regulates tooth number, size, and shape through Fgf20

The *EDA* gene was first identified by positional cloning of the gene responsible for X-linked hipohidrotic/anhidrotic ectodermal dysplasia (XLHED) in humans, characterized by missing or abnormally shaped teeth, sparse hair, and impaired exocrine glands [78–80]. A classical spontaneous mouse mutation, *Tabby*, causes similar ectodermal developmental defects, including missing teeth, smaller teeth, and reduced molar cusps, and was shown to be due to a mutation in the mouse *Eda* gene [81–83]. Eda is a signal of the tumor necrosis factor (TNF) superfamily [80]. During early tooth development, expression of Eda and its receptor Edar are both restricted to the epithelium, with *Eda* mRNAs broadly expressed in the oral and tooth bud epithelium while *Edar* mRNAs restricted to the epithelial signaling centers, the placodes and enamel knots [84]. *Eda* mRNA expression in the early tooth epithelium depends on *Lef1* function and is induced by exogenous Wnt6 [84], indicating that Eda acts downstream of Wnt signaling during early tooth development. Interestingly, overexpression of either Eda or an active form of Edar in the oral epithelium resulted in development of supernumerary teeth anterior to the first molars, most likely due to revitalization of the R2 diastemal tooth germs [48,85,86]. Recently, Häärä et al. [48] identified *Fgf20* as a downstream target gene of Eda signaling in the developing tooth epithelium. Mice lacking Fgf20 exhibit tooth phenotypes similar to the *Tabby* mice. Paradoxically, reduction in *Fgf20* gene dosage increased the frequency of supernumerary teeth in the *K14-Eda* transgenic mice: while 50% of *K14-Eda* mice had supernumerary teeth, 76% of *K14-Eda;Fgf20*$^{+/-}$ and 88% of *K14-Eda;Fgf20*$^{-/-}$ mutant mice had supernumerary teeth. Häärä et al. [48] further showed that Fgf20 was able to induce *Spry2* and *Spry4* expression in the tooth epithelium and mesenchyme, respectively, and that decreased levels of expression of these Fgf signaling antagonists likely underlie the increased frequency of supernumerary tooth development in the compound transgenic mice. Additional studies have shown that Eda signaling also regulates expression of *Shh*, *Dkk4*, *follistatin*, and *Lrp4* in epidermal placode development [87,88], suggesting that Eda signaling may modulate multiple components of the integrated signaling network that regulates the balance of activators and inhibitors of tooth morphogenesis and patterning (Fig. 2).

4. The Bmp4–Msx1 positive feedback loop propagates mesenchymal odontogenic potential for sequential tooth formation

Mammalian molars form sequentially in an anterior-to-posterior direction but the molecular mechanisms regulating sequential tooth formation are not well understood. Kavanagh et al. [89] showed that mouse mandibular first molar tooth germ inhibited second molar development and proposed an inhibitory cascade model, in which initiation of posterior molars depends on a balance between intermolar inhibition and mesenchymal activation, to account for sequential molar initiation in mammals. The

candidate mesenchymal activators include Inhibin-βA and Bmp4 whereas Sostdc1 is an excellent candidate intermolar inhibitor [89].

During mouse tooth development, *Bmp4* is first expressed in the tooth epithelium at about E11 and its expression shifts to the dental mesenchyme by E12, coincident with the transfer of the tooth inductive potential from the epithelium to mesenchyme [6,17]. Exogenous Bmp4 protein induced *Msx1* and *Bmp4* gene expression in cultured mandibular mesenchyme explants [17,18]. *Msx1* gene knockout mouse embryos exhibit developmental arrest of all tooth germs at the bud stage, with significantly reduced *Bmp4* mRNA expression in the dental mesenchyme [24,26]. Remarkably, exogenous Bmp4 protein partly rescued morphogenesis of the *Msx1*$^{-/-}$ mutant mandibular molar tooth germs in explant culture [26,42], suggesting that Bmp4 and Msx1 function in a positive feedback loop to regulate early tooth morphogenesis. Maas and Bei [90] proposed that the Bmp4–Msx1 positive feedback loop functions as a molecular amplifier to rapidly propagate the Bmp4 signal throughout the developing tooth mesenchyme during the transfer of the odontogenic potential from the epithelium to mesenchyme. In contrast to *Msx1*$^{-/-}$ mutant mice, however, the *Bmp4*$^{f/f}$*;Wnt1Cre* mice, which lack functional *Bmp4* mRNA expression in the tooth mesenchyme, showed only developmental arrest of the mandibular molar but the mutant maxillary first and second molars as well as all incisors continued to develop to mineralized teeth [91] (Fig. 3A, B, F and G). The mandibular molar developmental arrest in the *Bmp4*$^{f/f}$*;Wnt1Cre* embryos correlated with a more significant decrease in *Msx1* mRNA expression in the mandibular molar mesenchyme than in the maxillary molar mesenchyme [91], confirming a critical role for mesenchymal Bmp4 in the maintenance of *Msx1* expression and suggesting that other Msx1-dependent mesenchymal odontogenic factors can partially complement for loss of Bmp4 in driving tooth morphogenesis through the bud-to-cap transition. Reducing *Msx1* gene dosage by 50% in the *Bmp4*$^{f/f}$*;Wnt1Cre* mice enhanced the maxillary molar developmental defects such that the first molar tooth germ was significantly retarded and the second molar failed to develop (Fig. 3C). These results indicate that the Bmp4–Msx1 positive feedback loop plays a critical role in propagating the mesenchymal odontogenic signals to drive sequential tooth formation.

Cho et al. [76] recently showed that development of the second molar was accelerated in mouse embryos by the Shh function-blocking antibody 5E1 and proposed a Wnt–Shh–Sostdc1 negative feedback loop, in which Wnt signaling induces Shh and Shh suppresses Wnt/β-catenin pathway indirectly via Sostdc1, as a candidate mechanism for spatial tooth patterning. Although mathematical simulation of this model could generate the molar tooth patterns of wildtype and several mutant mouse conditions [76], experimental evidence points to inability of Shh in inducing *Sostdc1* expression in the intact tooth germ [69,76]. Laurikkala et al. [69] showed that Shh antagonized Bmp induction of *Sostdc1* expression. Although exogenous Shh protein could bring about weak *Sostdc1* mRNA expression in isolated tooth mesenchyme [76], Bmp ligands induced much more robust *Sostdc1* expression in whole tooth germ explants [69]. Moreover, reducing *Shh* gene dosage further enhanced the molar fusion phenotype in *Sostdc1*$^{-/-}$ mice [60], indicating that Shh acts downstream of *Sostdc1* in preventing molar tooth fusion. Ahn et al. [60] suggest that Shh antagonizes Wnt signaling through upregulation of *Dkk1*.

Taken together, the mechanism underlying sequential formation and spatial patterning of teeth involves integration of the Bmp4–Msx1 positive feedback loop for propagation of mesenchymal odontogenic activators, the Bmp–Wnt inter-tissue feedback circuit driving tooth morphogenesis, Bmp induction of the Sostdc1 inhibitor, and the feedback repression of Wnt signaling by Shh.

5. Osr2 patterns mammalian molar teeth into a single row by antagonizing Msx1-mediated expansion of the mesenchymal odontogenic field

Mammals have teeth in a single row whereas many vertebrates have multirowed dentitions. The *Osr2*$^{-/-}$ mutant mice were found to develop supernumerary molar teeth from oral epithelium lingual to the normal tooth row [92]. *Osr2* is expressed in a lingual-to-buccal gradient in the developing tooth mesenchyme surrounding the early molar tooth buds, with higher levels on the lingual side (Fig. 4A). Tissue recombination assays demonstrate that the mesenchymal odontogenic potential is expanded to the mandibular mesenchyme lingual to the molar tooth germs in the *Osr2*$^{-/-}$ mutant embryos [92]. Remarkably, *Bmp4* mRNA expression exhibits a complementary pattern to that of *Osr2* along the buccolingual axis of the developing molar tooth mesenchyme in wildtype mouse embryos even though Msx1 is expressed throughout the tooth mesenchyme (Fig. 4B and C). *Bmp4* expression is significantly increased and expanded into the lingual side of the developing tooth mesenchyme in the *Osr2*$^{-/-}$ mutant embryos [92]. Moreover, whereas *Msx1*$^{-/-}$ mutant embryos exhibit tooth bud developmental arrest accompanied by significantly reduced *Bmp4* expression in the tooth mesenchyme, *Msx1*$^{-/-}$*Osr2*$^{-/-}$ compound mutant embryos show partial restoration of *Bmp4* expression and their first molar tooth germs continue to develop beyond the bud stage [92]. Together with biochemical studies showing physical interaction between Msx1 and Osr2 proteins [93], these data suggest that Osr2 patterns the buccolingual axis of the developing tooth mesenchyme by antagonizing Msx1-mediated activation of mesenchymal odontogenic signals, including *Bmp4* expression, in the developing tooth mesenchyme to restrict mammalian molar tooth development in a single row (Fig. 5).

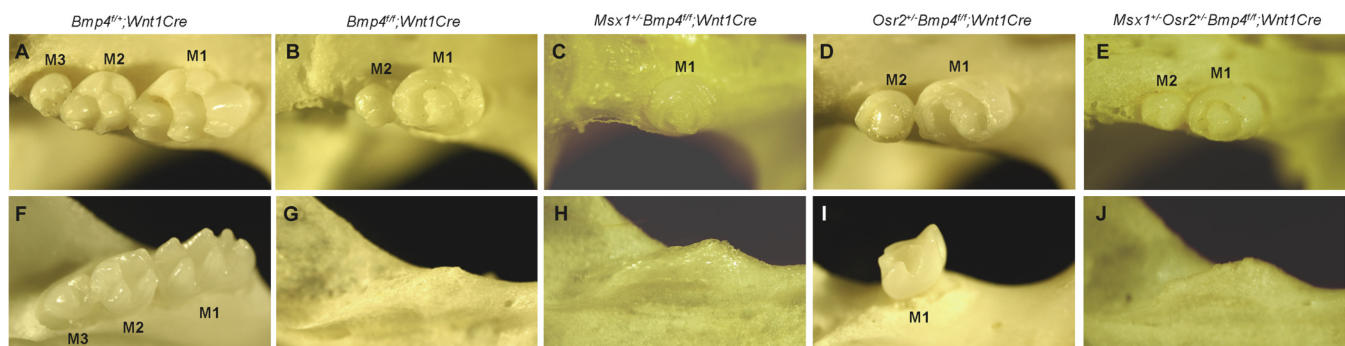

Fig. 3. Genetic interactions between Bmp4, Msx1, and Osr2 regulate sequential molar development in mice. (A–J) Skeleton preparations showing the maxillary (A–E) and mandibular molar (F–J) regions of *Bmp4*$^{f/+}$*;Wnt1Cre* (A, F), *Bmp4*$^{f/f}$*;Wnt1Cre* (B, G), *Msx1*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* (C, H), *Osr2*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* (D, I) and *Msx1*$^{+/-}$*OSR2*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* (E, J) mice at 21 days after birth. M1, M2, and M3 indicate first, second, and third molars, respectively.

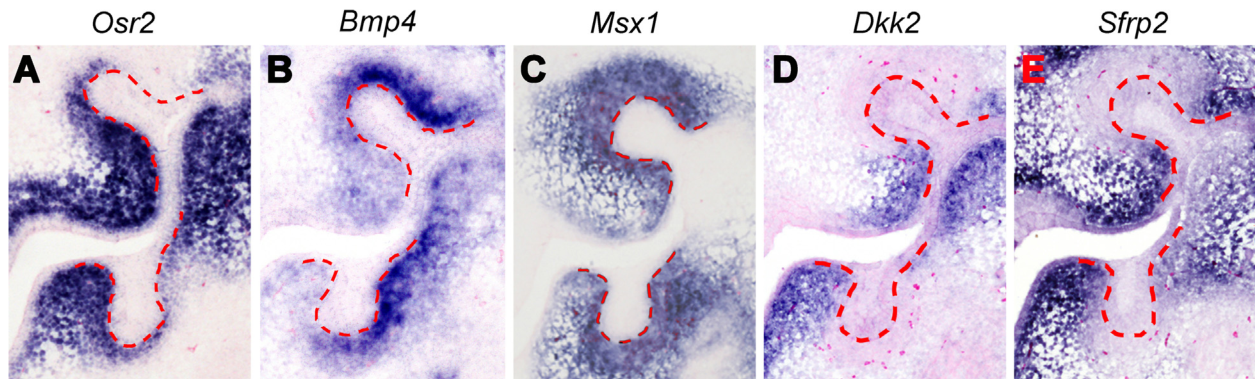

Fig. 4. Differential gene expression along the buccolingual axis of the developing mouse tooth bud mesenchyme at E13.5. (A–E) Spatial patterns of *Osr2* (A), *Bmp4* (B), *Msx1* (C), *Dkk2* (D), and *Sfrp2* (E) mRNAs are detected by *in situ* hybridization of coronal sections of E13.5 mouse first molar tooth germs. The mRNA signals are shown in blue color. Lingual side is to the left for all panels. Red dashed lines mark the boundary between the tooth bud epithelium and mesenchyme. Each panel shows both the maxillary (top) and mandibular (bottom) first molar tooth germs.

Whereas the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ compound mutant mice showed nearly normal morphogenesis of the first molars, they failed to develop either the supernumerary teeth or the second and third molars, in contrast to the *Osr2*$^{-/-}$ single mutants [92], suggesting that, in addition to patterning the buccolingual axis of the developing tooth mesenchyme, Osr2 interaction with Msx1 also regulates sequential tooth formation along the tooth row. This is confirmed by further analysis in the *Bmp4*$^{f/f}$*;Wnt1Cre* mice. Whereas the *Bmp4*$^{f/f}$*;Wnt1Cre* mice exhibit mandibular molar developmental arrest at the bud stage, reducing *Osr2* gene dosage by 50% rescued mandibular first molar tooth germs to small mineralized teeth in the *Osr2*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* mice [91] (Fig. 3H and I). Moreover, whereas the maxillary second molars failed to develop in the *Msx1*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* mice, they were rescued in the $^{-}$*Msx1*$^{+/-}$*Osr2*$^{+/-}$*Bmp4*$^{f/f}$*;Wnt1Cre* mice (Fig. 3E). Together, these data indicate that Osr2 patterns the mesenchymal odontogenic field along both the buccolingual and anteroposterior axes by restricting the domain of Msx1-mediated propagation of mesenchymal odontogenic potential (Fig. 5).

Another important finding by Jia et al. [91] is that the mandibular molar mesenchyme expresses significantly higher levels of Wnt antagonists, including *Dkk2* and *Wif1*, than the maxillary molar mesenchyme during normal tooth development. Previous studies of the *Dlx1*$^{-/-}$*Dlx2*$^{-/-}$ and *Inhba*$^{-/-}$ mutant mice [30,94], showing maxillary-only and mandibular-only molar developmental arrest, respectively, suggested that maxillary and mandibular molar development involved distinct genetic pathways [94,95]. The findings of Jia et al. [91] suggest that normal mandibular molar morphogenesis through the bud-to-cap transition requires higher levels of mesenchymal activators than the maxillary molar tooth germs to counteract the higher levels of Wnt antagonists. Expression of *Dkk2* and *Wif1* mRNAs were significantly increased in the *Bmp4*$^{f/f}$*;Wnt1Cre* mutant tooth mesenchyme [91], suggesting that Bmp4 signaling might cross-regulate Wnt signaling activity by regulating expression of the Wnt antagonists in the developing tooth mesenchyme. Remarkably, *Dkk2* mRNA expression in the developing molar tooth mesenchyme exhibits a strong lingual bias [96] (Fig. 4D). Another Wnt antagonist Sfrp2 also exhibits preferential expression on the lingual side of the developing molar tooth mesenchyme (Fig. 4E). Recently, we carried out quantitative real-time RT-PCR analysis of microdissected E13.5 *Msx1*$^{-/-}$ and control tooth bud mesenchyme and found that expression of both *Dkk2* and *Sfrp2* is significantly upregulated in the *Msx1*$^{-/-}$ molar mesenchyme. Thus, the antagonistic actions of Osr2 and Msx1 might pattern the buccolingual axis of the molar tooth developmental field through modulation of both Bmp4 and Wnt signaling (Fig. 5).

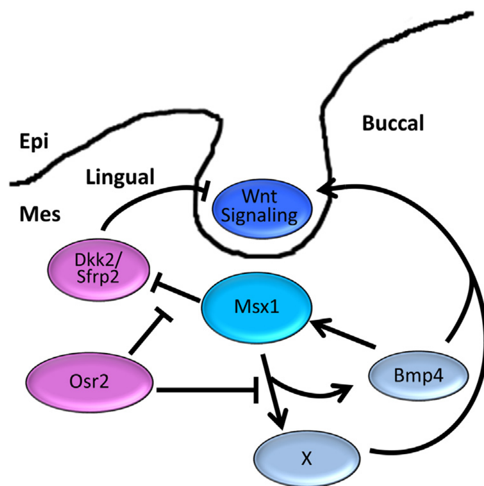

Fig. 5. Schematic diagram depicting antagonistic interactions between Msx1 and Osr2 in patterning the molar tooth developmental field. Msx1 and Bmp4 act in a positive feedback loop in the tooth mesenchyme. Osr2 restricts Msx1-mediated activation of Bmp4 and other mesenchymal odontogenic signals (represented by X) to the buccal side of the tooth mesenchyme. Expression of *Dkk2* and *Sfrp2* are negatively regulated by Msx1 and restricted to the lingual side, possibly resulting from Osr2-mediated repression of Msx1 function. Epi, epithelium; Mes, mesenchyme.

6. Concluding remarks

Research in the past 20 years has revealed that development of both the individual teeth and the whole dentition is controlled by coordinated actions of only a few conserved intercellular signaling pathways. Whereas each of the Bmp, Fgf, Shh, and Wnt signaling pathways is repeatedly used throughout tooth organogenesis and regulated by a number of activators and inhibitors, the revitalization of the same physiologically arrested diastemal tooth germs in mice due to mutations affecting distinct pathways demonstrate that these signaling pathways act in highly integrated networks regulating tooth development (Fig. 2). These data suggest that tinkering with the activity of one pathway has the potential to overcome activator insufficiency in another to rescue morphogenesis of arrested tooth germs. This is further supported by the findings that constitutive activation of the canonical Wnt signaling pathway in the dental epithelium is able to induce supernumerary tooth development even in mice lacking Msx1 or Pax9 [38,44]. Although much of the molecular mechanisms mediating the coordination and integration of the signaling pathways and gene regulatory networks in tooth organogenesis remains to be elucidated, these findings have

great implications for development of therapeutic strategies for tooth developmental abnormalities, particularly for congenitally missing or hypoplastic teeth.

Approximately 20% of people do not develop all third molars and 5% lack some of the other permanent teeth, with agenesis of six or more permanent teeth (apart from the third molars) occurring in about 1 in 1000 [97]. Mutations in *MSX1*, *PAX9*, *AXIN2*, *EDA*, and *WNT10A*, have been detected in a large number of nonsyndromic tooth agenesis patients [97–99]. Most patients with tooth agenesis exhibit relatively normal primary tooth development and missing only some, but not all, of the permanent teeth, indicating that the phenotype is caused by perturbation of the activator–inhibitor balance, such as reduced Wnt signaling activity, rather than complete disruption of the tooth developmental gene regulatory network. The revitalization of the physiologically arrested diastemal tooth germs in mice by increased activity in Eda, Fgf, or Wnt signaling, suggests that it is possible to rescue the missing permanent teeth if specific and safe pharmacological agonists of these pathways can be developed and administered at the appropriate developmental stage. Following an experimental study that demonstrated effective correction of tooth and other ectodermal defects in *Tabby* mice with recombinant EDA protein [100], short postnatal treatment of XLHED dogs with recombinant EDA protein showed remarkable rescue of development of permanent teeth as well as other affected ectodermal organs [101,102]. These data provide proof of concept for development of a pharmacological cure of congenital tooth defects.

Because humans and most other mammals replace their teeth only once, tooth loss due to decay or accident is a major health problem. Development of strategies for biological replacement of lost teeth requires better understanding of the detailed molecular mechanisms controlling tooth induction and morphogenesis. Classic tissue recombination experiments demonstrated that, while tooth initiation signals first arise in the presumptive dental epithelium, the odontogenic potential shifts into the tooth mesenchyme at the early bud stage such that mouse molar mesenchyme from E12 through E16 could induce complete tooth formation when recombined with embryonic non-dental epithelium [6,103–108]. More recently, it was reported that the E13 mouse molar tooth mesenchyme instructed tooth formation when recombined with cultured human primary keratinocytes, with the human cells differentiating into enamel-secreting ameloblasts and mouse tooth mesenchyme to dentin-secreting odontoblasts [109]. Thus, understanding the genetic program controlling the mesenchymal odontogenic potential will facilitate development of strategies for tooth bioengineering. The findings that the Bmp4–Msx1 pathway drives expansion of, whereas the Osr2 transcription factor suppresses, the inductive potential of the dental mesenchyme [91,92] provide an excellent opening to uncovering the molecular network regulating mesenchymal odontogenic potential.

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