Transformation of Tooth Type Induced by Inhibition of BMP Signaling

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Mammalian dentitions are highly patterned, with different types of teeth positioned in different regions of the jaws. BMP4 is an early oral epithelial protein signal that directs odontogenic gene expression in mesenchyme cells of the developing mandibular arch. BMP4 was shown to inhibit expression of the homeobox gene *Barx-1* and to restrict expression to the proximal, presumptive molar mesenchyme of mouse embryos at embryonic day 10. The inhibition of BMP signaling early in mandible development by the action of exogenous Noggin protein resulted in ectopic *Barx-1* expression in the distal, presumptive incisor mesenchyme and a transformation of tooth identity from incisor to molar.

BMP4 that is produced from the early oral epithelium is able to activate expression of the homeobox gene (*Msx-1*) in the underlying ectomesenchyme cells of the mandibular arch, which is initially expressed in the distal ectomesenchyme that marks presumptive incisor cells (*1*). The proximal extent of *Msx-1* expression coincides with the distal extent of another homeobox gene (*Barx-1*), which is restricted to the proximal mesenchyme that marks presumptive molar cells (Fig. 1A) (*2*). The boundary between cells that express *Msx-1* and *Barx-1* thus demarcates the distinction between presumptive incisor (*Msx-1* positive:*Barx-1* negative) and molar (*Msx-1* negative:*Barx-1* positive) tooth-forming regions in mouse embryos.

The boundary between *Msx-1* and *Barx-1* expression was found to correspond to the junction between *Fgf-8* and *Bmp-4* expression in the overlying oral epithelium (schematically represented in Fig. 1A) (*3*, *4*). The ability of these signaling proteins to regulate the spatial expression of *Barx-1* was investigated by implanting protein-soaked beads into mandibular arch explants (*5*). At embryonic day 10 (E10), the removal of the epithelium resulted in a dramatic down-regulation of *Barx-1* expression (*6*). Fgf8 beads that were placed in mesenchymal explants from which the epithelium had been removed resulted in a reexpression of *Barx-1* (Fig. 1B). In contrast, when BMP4 beads were implanted into intact mandible explants, BMP4 was found to have a marked inhibitory effect on the endogenous *Barx-1* expression (Fig. 1C). The ability of endogenous BMP4 to inhibit *Barx-1* expression was investigated by implanting Fgf8 beads into the distal (*Barx-1* negative) mesenchyme of intact explants at E9.5. In the presence of endogenous BMP4, exogenous Fgf8 was unable to induce ectopic *Barx-1* expression (Fig. 1D). However, if the endogenous source of BMP4 (the epithelium) was removed, Fgf8 beads were able to induce *Barx-1* expression in the medial region at E9.5 (Fig. 1E). At E10.5, Fgf8 beads were no longer able to induce *Barx-1* expression in this presumptive incisor region, indicating a change in competence of the distal neural crest-derived ectomesenchyme cells (*6*). In order to determine whether the BMP4 inhibition of *Barx-1* was direct or through the activation of *Msx-1*, which might repress *Barx-1* expression, the expression of *Barx-1* was investigated in *Msx-1*$^{-/-}$ embryos at E10 (*7*). No change in the medial-lateral boundary of *Barx-1* was detectable in the *Msx-1*$^{-/-}$ mandibular mesenchyme, which indicates that the inhibitory action of BMP4 on *Barx-1* does not occur through the activation of Msx-1 (Fig. 1, F and G).

To investigate the outcome of inhibiting BMP signaling from the oral epithelium to the mandibular arch mesenchyme, we implanted Noggin beads into the distal, presumptive incisor mesenchyme of mandibular arch explants at E10 (*5*). Noggin protein binds BMP4 with high affinity and can abolish BMP4 activity by blocking binding to cell surface receptors (*8*). After a culture period of 24 hours, *Barx-1* expression was considerably extended distally into the presumptive incisor domain and, as shown previously, *Msx-1* expression was lost (Fig. 2, A through C) (*3*). The consequence of ectopic expression of *Barx-1*, a molar marker, in presumptive incisor regions was investigated by examining the effect that *Barx-1* had on incisor marker *Tlx-1*. *Tlx-1* (*Hox-11*) is expressed specifically in the mandibular arch incisor tooth epithelium at E10 and is not expressed in the molar tooth epithelium (Fig. 2D) (*9*). Noggin beads that were implanted into the distal mesenchyme of E10 mandibular arch explants produced a marked repression of *Tlx-1* expression (Fig. 2, G and H). BMP4 beads were not able to induce *Tlx-1* expression in the epithelium, which indicates that the Noggin-induced loss of expression in the presumptive incisor epithelium is not due to the loss of a direct inducer (BMP4) of expression (Fig. 2, I through K).

To determine whether these Noggin-induced changes in gene expression in the distal part of the mandibular arch had any affect on incisor tooth development, we dissected mandibular explants [which had been cultured with Noggin or bovine serum albumin (BSA) beads] into molar and incisor regions and transplanted these explants into renal capsules (*10*). This technique allows for the full morphological development of teeth after E10 (*11*). Normal molar and incisor teeth developed from control explants (Fig. 3, A,

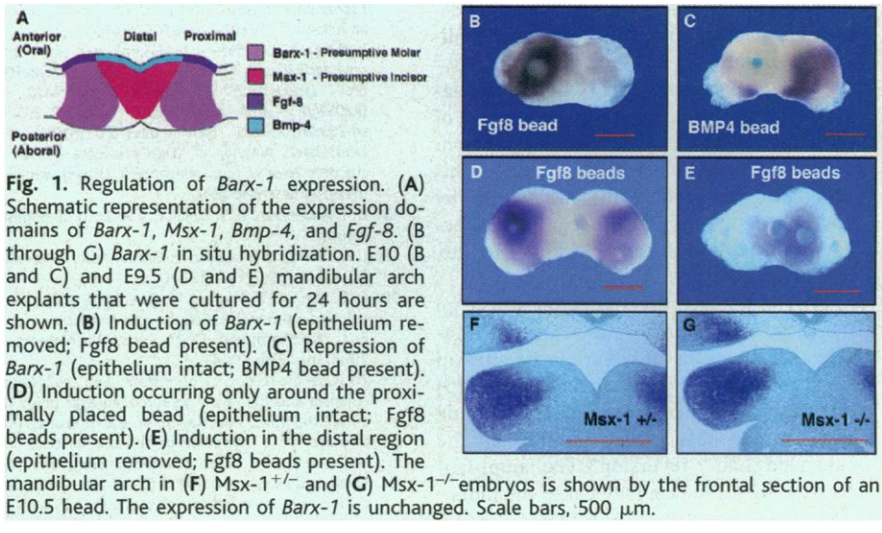

Fig. 1. Regulation of *Barx-1* expression. (**A**) Schematic representation of the expression domains of *Barx-1*, *Msx-1*, *Bmp-4*, and *Fgf-8*. (B through G) *Barx-1* in situ hybridization. E10 (B and C) and E9.5 (D and E) mandibular arch explants that were cultured for 24 hours are shown. (**B**) Induction of *Barx-1* (epithelium removed; Fgf8 bead present). (**C**) Repression of *Barx-1* (epithelium intact; BMP4 bead present). (**D**) Induction occurring only around the proximally placed bead (epithelium intact; Fgf8 beads present). (**E**) Induction in the distal region (epithelium removed; Fgf8 beads present). The mandibular arch in (**F**) Msx-1$^{+/-}$ and (**G**) Msx-1$^{-/-}$embryos is shown by the frontal section of an E10.5 head. The expression of *Barx-1* is unchanged. Scale bars, 500 μm.

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D, E, and H), but in Noggin-treated explants at E10, multicuspid molar teeth developed from incisor explants at the expense of incisor teeth (Table 1 and Fig. 3, B, C, F, and G). In these treated explants, the expected number of molar teeth developed from the molar explants, indicating that molars had indeed formed from incisor explants. The same experiment (when repeated with E11 explants) showed no effect on tooth identity (Table 1), indicating that the ability of Noggin beads to affect tooth patterning is limited to a small temporal window of development. This temporal window was further confirmed by showing that, at E11, Noggin had no effect on *Barx-1* expression but still inhibited *Msx-1* expression (Fig. 3, I and J). All the molars obtained from the incisor regions were morphologically and histologically normal, except for one that had a molar-incisor hybrid appearance (Fig. 3, C and G). Macrodontia and microdontia are relatively common human craniofacial abnormalities, and macrodontia of premolars has frequently been described as molarized premolars. True transformations of incisors to molars have rarely occurred in humans (*12*).

The distinct expression boundaries of homeobox genes in the mandibular ectomesenchyme that are found before the tooth-germ initiation resulted in the suggestion that these domains provide the positional information for tooth patterning (*13*). This suggestion was supported by mutations in the *Dlx-1* and *Dlx-2* genes, which resulted in a restricted loss of *Barx-1* expression and the specific arrest of maxillary molar development (*14*). We found that ectopic expression of *Barx-1* in the distal, presumptive incisor tooth ectomesenchyme results in a change in morphogenesis such that molar teeth develop in place of incisors. The observation that this change of tooth type is not always complete, with molar-incisor hybrid teeth also being formed occasionally, suggests that a transformation of incisor tooth germs to molar tooth germs has taken place. Although the observed phenotype is entirely consistent with the proposed role of *Barx-1* in molar development, we cannot exclude the possibility that ectopic expression of *Barx-1* alone is not sufficient for transformation and may need to be accompanied by other molecular changes to the ectomesenchyme cells (such as a loss of *Msx-1* expression), which also occur after the inhibition of BMP signaling. A loss of *Msx-1* alone, however, is not sufficient to produce a transformation of tooth type because a loss of *Msx-1* expression at E11, which is not accompanied by a change in *Barx-1* expression, does not result in a transformation of tooth identity (Fig. 3, I and J). A loss of the function of genes that are involved in tooth patterning (such as *Dlx1/2* and *Activin* β*A* knockout mice) results in the early arrest of tooth development rather than in a transformation of tooth type, which appears to require misexpression (*11*, *14*).

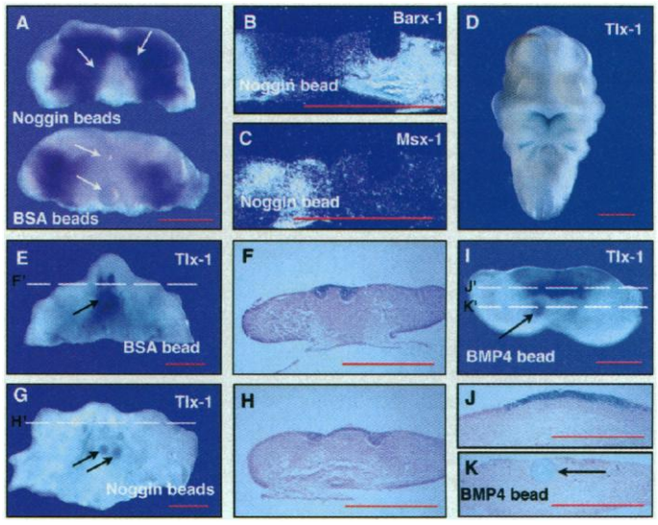

Fig. 2. Inhibition of Bmp-4 signaling by Noggin beads. E10 mandibular arch explants that were cultured for 48 hours are shown in (A) through (C) and in (E) through (H). (**A**) DIG in situ hybridization showing the extension of the domain of *Barx-1* into the distal regions of the arch after the addition of Noggin beads (blue) and BSA beads (white). (**B** and **C**) Radioactive in situ hybridization showing serial sections through an arch that was cultured with a Noggin bead. (B) The expression of *Barx-1* extends toward the distal region, in comparison with the control side with no bead. (C) The expression of *Msx-1* is inhibited in the distal region, in comparison with the control side with no bead. (**D** through **K**) β-galactosidase–stained *Tlx-1*$^{+/-}$ embryos. Expression in the presumptive incisor epithelium and the mesoderm core of the first and second branchial arches is shown by the E10.5 head (D). The mandibular arch explant and BSA control beads are shown (E). In (F), early tooth buds expressing *Tlx-1* are shown in a section through the incisor region of the explant at the level of F′ (dashed white line) in (E). A loss of *Tlx-1* expression is shown by the mandibular arch explant and Noggin beads (G). In (H), a section through the incisor region of the explant at H′ in (G) is shown. The E10 mandibular arch explant that was cultured for 24 hours with BMP4 beads is shown in (I) through (K). BMP4 beads cannot induce expression of *Tlx-1* in the epithelium (I). Sections through the explant at the level of J′ and K′ (white dashed lines) show endogenous expression in the epithelium (J) but show no new expression in the epithelium near to the bead (K). Arrows indicate the position of beads. Scale bars, 500 μm.

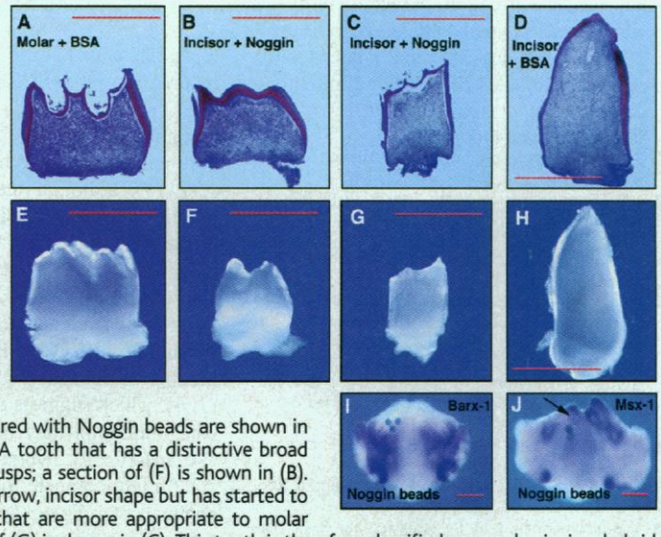

Fig. 3. Transformation of tooth identity from incisor to molar shape. (**A** through **H**) Teeth obtained from E10 tooth germs that were cultured for 2 days in vitro and implanted under host kidney capsules. A section of the multicuspid tooth shown in (E) that was formed from a molar tooth germ cultured with a BSA bead is shown in (A). Multicuspid teeth that were formed from incisor tooth germs cultured with Noggin beads are shown in (B), (C), (F), and (G). (F) A tooth that has a distinctive broad molar shape and three cusps; a section of (F) is shown in (B). (G) A tooth that has a narrow, incisor shape but has started to develop multiple cusps that are more appropriate to molar development; a section of (G) is shown in (C). This tooth is therefore classified as a molar-incisor hybrid. (H) A conical-shaped tooth that was formed from an incisor tooth germ cultured with a BSA bead; a section of (H) is shown in (D). (**I** and **J**) E11 mandibular arch explants that were cultured for 2 days with Noggin beads and were subjected to DIG whole-mount in situ hybridization. The proximal-distal boundary of expression of *Barx-1* is not effected by Noggin beads at this stage (I). At E11 onward, the expression of *Msx-1* expands proximally to include the condensing mesenchyme immediately underneath the developing incisor and molar tooth germs (*2*) (J). At this stage, Noggin beads are still able to inhibit expression, as can be seen by the loss of *Msx-1* under the incisor bud that is nearest to the beads (arrow). Scale bars, 500 μm.

Table 1. Transformation of incisors to molars. Incisors do not develop as well as molar tooth germs in kidney capsules, despite being cultured as pairs, with two out of three (70%) forming cysts at E10 and E11. After E11, incisor tooth germs develop well in kidney capsules. Some secondary molars also developed alongside primary molars in the Noggin/BSA presumptive molar regions.

Gestation	Presumptive incisor region		Presumptive molar region treated with Noggin or BSA
	Treated with Noggin	Treated with BSA	
E10	1 incisor and 4 molars out of 12 pairs	4 incisors and 0 molars out of 9 pairs	35 primary molars out of 36
E11	5 incisors and 0 molars out of 16 pairs	8 incisors and 0 molars out of 15 pairs	29 primary molars out of 32

At E9.5, Fgf8 beads can induce ectopic *Barx-1* expression in distal regions of the mandible, indicating that all neural crest–derived ectomesenchymal cells of the mandibular arch are equally responsive to epithelial signals. This implies that the neural crest cells that populate the mandibular arch are not prespecified but are patterned by contact with epithelial signals. The evidence for a prepatterning of cranial neural crest cells is limited to the proximal cells of the mandibular arch; our results are consistent with data showing that distal mandibular arch cells have a different axial origin than proximal cells (*15*).

The antagonistic effects of Fgf8 and Bmp4, which act to establish the distal boundary of *Barx-1* expression, are similar to those reported for *Pax-9* (*16*). *Barx-1* is, however, expressed earlier than *Pax-9* and, unlike *Pax-9*, is not induced at localized sites that underlie all developing teeth. This antagonistic signaling interaction thus has at least two roles in the specification of mandibular mesenchymal fates: an early role in proximal-distal axis specification, which determines whether mesenchymal cells have presumptive molar or incisor fates, and a later role in determining the sites of tooth bud initiation.

References and Notes

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5. Mandibular arch explants were dissected and cultured as described (*3*). Beads were also prepared as described (*3, 4*), with Fgf8 and BSA (white) and BMP4 and Noggin (blue) beads at a concentration of 100 μg/mg. Whole-mount digoxigenin (DIG) and sectioned ^{35}S in situ hybridization were carried out as described (*3, 4*).
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10. E10 and E11 mandibular arch explants were cultured with beads that were placed in the distal (presumptive incisor) mesenchyme for 48 hours in vitro. These were then dissected into incisor and molar regions; the incisor regions were dissected as pairs. Single incisor and molar regions from each mandibular arch were transferred into opposite ends of a kidney, under the kidney capsule, in adult male mice. These regions were then left to develop for 14 days in vivo. The resulting teeth were photographed, placed in Bouin's fixative for 3 days, dehydrated, and embedded. Sections were stained with alcian blue and chlorontine fast red.
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Membrane Phospholipid Control of Nucleotide Sensitivity of K_{ATP} Channels

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Adenosine triphosphate (ATP)–sensitive potassium (K_{ATP}) channels couple cell metabolism to electrical activity. Phosphatidylinositol phosphates (PIPs) profoundly antagonized ATP inhibition of K_{ATP} channels when applied to inside-out membrane patches. It is proposed that membrane-incorporated PIPs can bind to positive charges in the cytoplasmic region of the channel's $K_{ir}6.2$ subunit, stabilizing the open state of the channel and antagonizing the inhibitory effect of ATP. The tremendous effect of PIPs on ATP sensitivity suggests that in vivo alterations of membrane PIP levels will have substantial effects on K_{ATP} channel activity and hence on the gain of metabolism-excitation coupling.

K_{ATP} channels (*1*) are formed from a sulfonylurea receptor (SURx) and an inward rectifier ($K_{ir}6.x$) subunit (*2*). Evidence is accumulating that the $K_{ir}6.x$ subunit forms the pore and controls the hallmark inhibition by ATP (*3*), although the mechanism of this inhibition remains elusive. Recent reports indicate that membrane PIPs bind K_{ATP} and other K_{ir} channels, stabilizing them in an active conformation (*4, 5*). Both the inhibitory effect of adenosine nucleotides and the stimulatory effects of PIPs increase with the number of phosphate groups in the molecule (*1, 6*), which suggests that PIP activation and ATP inhibition may be related phenomena. We specifically hypothesized that PIPs and ATP might compete for binding to the K_{ATP} channel, stabilizing open and closed channels, respectively.

To test this hypothesis, we recorded currents in inside-out patches from COSm6 cells expressing cloned ($K_{ir}6.2$+SUR1) K_{ATP} channels (*7*). After patch excision, a slow run-down of channel activity occurred over the following minutes, and this run-down could be avoided by addition of phosphatidyl inositol-4,5-bisphosphate (PIP_2) (Fig. 1A). Open probability in the absence of ATP (P_{open}) was ~0.4 before application of PIP_2 but increased to ~0.85 (Fig. 1C), which is consistent with the approximate doubling of current in macroscopic patches that followed treatment with PIP_2 (Fig. 1E) and demonstrates that PIP_2 activates K_{ATP} current by increasing P_{open}. ATP sensitivity (*7*) immediately after patch excision could be fit by a sigmoid relation with a half-maximal inhibition concentration ($K_{1/2}$) of 12.1 μM and a Hill coefficient (H) of 1.3 (n = 26), which is similar to previous reports (*2*), but it decreased by orders of magnitude after PIP_2 application (Fig. 1, A and B). The rate of this decrease was variable from patch to patch (Fig. 1D), probably because of variable diffusion distances (*8*), but correlated with the time course of increase in ATP-independent

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