Deletion of BMP7 Affects the Development of Bones, Teeth, and Other Ectodermal Appendages of the Orofacial Complex

VASILIKI ZOUVELOU1, HANS-ULRICH LUDER2, THIMIOS A. MITSIADIS2*, AND DANIEL GRAF1*
1Biomedical Sciences Research Center “Alexander Fleming”, Institute of Immunology, Vari, Hellas, Greece
2Department of Orofacial and Structural Biology, Institute of Oral Biology, ZZMK, Faculty of Medicine, University of Zurich, Zurich, Switzerland

ABSTRACT Sequential and reciprocal epithelial–mesenchymal interactions govern the development of most tissues and organs of the craniofacial region. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β family of secreted signaling molecules that have long been implied to have a significant contribution in this process. However, evidence for such a role during craniofacial development is largely missing. Using a lacZ reporter mouse we mapped the spatiotemporal expression of BMP7 in the developing craniofacial region. The observed pattern suggested a potential involvement of BMP7 in epithelial–mesenchymal interactions and thus a direct role for this molecule in the development of ectodermal appendages (teeth, hair follicle, lachrymal and sweat glands, taste buds) and, furthermore, palatal formation. To correlate the expression to function we analyzed germline deleted conditional BMP7-deficient embryos for malformations. We found developmental defects in many craniofacial structures such as teeth, eyes, whiskers, hair follicles, salivary glands, and palate. These findings place BMP7 as a central mediator of epithelial–mesenchymal interactions that are necessary for the correct development of structures belonging to the orofacial complex. J. Exp. Zool. (Mol. Dev. Evol.) 312B:361–374, 2009. © 2009 Wiley-Liss, Inc.


Reciprocal interactions between epithelium and mesenchyme underlie the development of many embryonic tissues and organs. Organs such as lung, pancreas, kidney, intestine, hairs, and mammary glands develop through interactions of mesenchyme with either endoderm or ectoderm. Tissues developing through epithelial–mesenchymal interactions that involve the ectoderm are termed as ectodermal appendages. Many ectodermal appendages such as teeth, hair follicles, whiskers, taste buds, lachrymal glands, salivary glands, and sweat glands develop in the orofacial complex (Shuler et al., 91, 92). Although quite different in morphology, all these organs have in common that their morphogenesis is regulated by a series of sequential and reciprocal interactions between epithelial and mesenchymal tissues, which require a tightly regulated gene expression program (Millar, 2002). Numerous growth factors and signaling pathways have been reported to control this process. Disturbances to this tightly controlled molecular network often result in malformed structures. Bone morphogenetic proteins (BMPs) are among these important signals as they have widely been implied to control the development of these organs (Chuong and Noveen, ’99; Chuong et al., 2000; Jernvall and Thesleff, 2000; Fuchs et al., 2001; Millar, 2002). Previous

Grant sponsor: BSRC; Grant number: LSHG-CT-2005-005203; Grant sponsors: European B2i COST Action; Aristea, Greek Ministry of Development.

*Correspondence to: Daniel Graf, Biomedical Sciences Research Center “Alexander Fleming”, Institute of Immunology, 34 Alexander Fleming Street, 166 72 Vari, Hellas/Greece. E-mail: graf@fleming.gr or Department of Orofacial & Structural Biology, Institute of Oral Biology, ZZMK, Faculty of Medicine, University of Zurich, Plattenstrasse 11, 8032 Zurich, Switzerland. E-mail: thimios.mitsiadis@zzmk.uzh.ch

Received 11 November 2008; Accepted 14 November 2008

Published online 6 January 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21262
studies have shown that the BMP2, BMP4, and BMP6 genes are expressed during embryogenesis in developing ectodermal appendages in a spatially and temporally specialized pattern, suggesting that a coordinated expression of BMPs is needed for the proper formation of these organs (Lyons et al., '89a,b, '90; Jones et al., '91). In particular, it has been shown that BMP4 mediates epithelial–mesenchymal interactions during early tooth development (Vainio et al., '93). In addition, the developmental arrest of several ectodermal organs prior to the placode stage reported in Msx1/Msx2 double-mutant mice, two genes thought to be downstream of BMP signaling, indicates a role of BMP signaling in inductive events (Bei and Maas, '98; Bei et al., 2000; Zhang et al., 2002; Alappat et al., 2003).

BMPs form a family of phylogenetically conserved secreted signaling molecules belonging to the transforming growth factor-β (TGF-β) superfamily. BMP7 belongs, together with BMP5 and BMP6, to the 60A/glass bottom boat subgroup of BMPs (Zhao, 2003). Upon binding to its heterodimeric receptor, BMP7 activates the “canonical” pathway leading to phosphorylation of Smad1, Smad5, and Smad8. The interaction of these three Smad molecules with Smad4 leads to their translocation into the nucleus, where they regulate gene expression. In addition to the Smad pathway, other signal transduction pathways can be wired to the BMP signaling such as MAPK and PI3K/Akt (Hassel et al., 2003; Sugimori et al., 2005). BMP activity is regulated in the extracellular domain by secreted BMP antagonists that bind to BMPs, thus preventing their interaction with the appropriate BMP receptors (Canalis et al., 2003). The importance of this type of regulation in craniofacial development was revealed by the ectopic expression of Noggin, which is a BMP antagonist (Plikus et al., 2005). Noggin is capable of binding with BMP2, BMP4, and BMP7, and thus neutralizes their activity. In addition, Noggin has a strong affinity to heparin sulfate proteoglycans, which are abundantly expressed on the surface of all adherent cells and, furthermore, they constitute the major component of the extracellular matrix (Paine-Saunders et al., 2002). Therefore, the extracellular matrix could restrict the sequestering of Noggin and keep its activity localized, bringing additional complexity to BMP fine-regulation. Furthermore, it has been reported that BMPs can induce the expression of Noggin, thereby forming a negative feedback loop (Gazzarrotto et al., '98). Thus, it is safe to postulate that during embryogenesis BMPs, BMP antagonists, and BMP receptors must have a tight spatiotemporal expression pattern in order to accomplish the proper regulation of cell proliferation, differentiation, and apoptosis in different body tissues.

In order to be able to understand the role of BMP7 in craniofacial development and organogenesis in vivo, it is necessary to identify BMP7-expressing tissues and cells. In this study we have examined the expression patterns of the gene using BMP7-lacZ reporter mice (Godin et al., '98). We present an inventory of its expression, which provides a rational basis for understanding its role in the development of craniofacial structures. We find BMP7 expression in teeth and all developing ectodermal appendages of the orofacial complex and show that the development of many of these tissues is affected in the absence of BMP7, thus illustrating the critical role that BMP7 plays in the development of teeth, ectodermal appendages, and palate.

MATERIALS AND METHODS

Mouse strains

BMP7-lacZ mice (Godin et al., '98) and BMP7A mice obtained by germline deletion of a conditional BMP7 allele (Zouvelou et al., 2008) were used in this study. All mice were backcrossed to C57Bl6/J for at least six generations. Embryos were obtained by timed mating, and E0.5 was considered as the morning when the vaginal plug was seen. The Animal Care and Use Committee of the Biomedical Sciences Research Center “Alexander Fleming” approved the animal experiments.

Whole-mount LacZ staining

Embryos obtained from timed matings were washed briefly in phosphate-buffered saline (PBS) and immediately fixed in cold fixative solution (2% formaldehyde, 0.2% gluteraldehyde, 0.01% sodium deoxycholate, 0.02% NP-4 in PBS) for 5–15 min, washed several times with PBS/2 mM MgCl2, and stained for several hours to overnight in x-gal staining solution (0.1 M phosphate buffer pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 1 mg/ml x-gal) at room temperature in the dark. Following staining, embryos were washed in PBS, postfixed in 1% paraformaldehyde (PFA), dissected for macroscopic analysis, or processed for sectioning.
Histological analysis of whole-mount-stained embryos

Whole-mount lacZ-stained embryos were processed for histology by embedding into OCT or Epon. For Tissue-Tek O.C.T compound (OCT) (Miles Laboratories, Elkhart, IN) embedding, embryos were equilibrated in 25% sucrose in PBS before embedding in OCT. Blocks were cut at 10μm on a Micron cryotome and sections were mounted in Mowiol. For Epon embedding embryos were treated as follows: whole E11.5–E12.5 embryos and dissected maxillae and mandibles of E12.5 embryos were fixed for 2 days at room temperature in a mixture of 2.5% glutaraldehyde, 2% PFA, and 0.025% Ca-chloride in 0.02 M Na-cacodylate buffer (pH 7.4; half-strength Karnovsky’s fixative). After an overnight wash in 0.185 M Na-cacodylate buffer (pH 7.4), specimens were dehydrated in ascending grades of alcohol, transferred to propylene oxide and increasing concentrations of Epon in propylene oxide, and finally embedded in pure Epon. Serial or step-sagittal longitudinal (sagittal) or frontal, 2μm thick sections were cut with a histo-diamond knife (Diatome, Biel, Switzerland) in a Reichert Ultracut E microtome (Leica Microsystems, Herbrugg, Switzerland) and stained with periodic acid-Schiff. Light micrographs were taken with a resolution of 2,592 × 1,944 pixels using a Leica DM6000 B microscope equipped with a DFC420 C camera (Leica Microsystems).

LacZ staining on sections

Embryos obtained from timed mating were fixed in 4% PFA, equilibrated in 25% sucrose, and embedded in OCT. Eight to ten micrometer sections were cut and stained for LacZ following the same protocol as for whole-mount staining with the difference that the fixation step was kept to 5 min and staining was performed at 37°C. Sections were postfixed in 1% PFA and mounted in Mowiol.

Histological analysis

For histological analysis, embryos were directly fixed in formalin and embedded in paraffin. Paraffin blocks were cut at 4μm and sections were stained with H/E before mounting. Light micrographs were taken with a Nikon Eclipse 800 microscope equipped with a DMX1200F camera (Nikon, Tokyo, Japan).

RESULTS

BMP7 expression in developing craniofacial structures

As revealed by whole-mount lacZ staining, BMP7 was expressed at embryonic day 9.5 (E9.5) at the anterior—most dorsal—roof of the telencephalon, the region of lamina terminalis, the olfactory and otic placodes, the cranial notochord, and the ectoderm of the first branchial arch in the region of future oral epithelium. Additionally, we observed expression in the ectoderm covering the second branchial arch and in a part of the mesenchyme (Fig. 1A, C). At E10.5 expression persisted in the areas described for E9.5, but in addition we observed clear expression in the mesenchyme of the forming medial and lateral nasal processes and in the posterior part of the oral epithelium (Fig. 1B, D). Additionally, a continuous dorsal midline expression was apparent, which extended from the telencephalon through the spinal cord. At E12.5 (Fig. 1E–H) whole-mount examination showed that BMP7 continued to be expressed in the same regions, but also became evident at the naso-lachrymal groove, the epithelium of the forming palate, and in the epithelium of the incisor and first molar buds of the maxilla and mandible. Strong expression was also observed at the midline where the hemimandibles fuse and give rise to the Symphysis menti.

To identify the BMP7-expressing cells within the developing orofacial region, we analyzed sections of E11.5–E12.5 mouse embryos. BMP7 expression was obvious at the medial and lateral nasal processes with more prominent expression in the mesenchyme; however, lacZ staining was also clearly detected in the cells of the aboral epithelium covering the nasal process (Fig. 2A–D). In some areas of the roof of the oral cavity expression was clearly restricted to the epithelium (Fig. 2E). Staining was also detected in cells of the thickening oral epithelium (dental placodes) where the future incisors (Fig. 2F, G) and molars (Fig. 2I, M) will develop. More lateral sections revealed lacZ staining in the epithelium of the developing Rathke’s pouch and the lateral wall of the neural component of the developing pituitary gland (Fig. 2H). Furthermore, staining was obvious in the mesenchyme that will condense to form the lachrymal gland, and at the site of the ocular part of the trigeminal nerve (Fig. 2I). Expression in the developing eye was obvious at
Fig. 1. BMP7 expression in E9.5–E12.5 whole-mount lacZ-stained embryos. Whole-mount x-gal staining of BMP7-lacZ embryos at various developmental stages: E9.5 (A, C), E10.5 (B, D), E12.5 (E–H). (C) and (D) represent higher magnifications of figures (A) and (B), respectively. (G) and (H) represent views on dissected maxilla and mandible, respectively. e, eye; fb, forebrain; fl, forelimb; h, heart; hb, hindbrain; hl, hindlimb; i, incisor; m, molar; mb, midbrain; md, mandibular process; mg, mammary glands; mx, maxillary process; n, nose; nc, nasal cavity; np, nasal process; oe, oral epithelium; on, optic nerve; op, otic placode; p, palate; t, tongue; tg, trigeminal ganglion; tl, tail; red arrows indicate placodes, where the facial trigeminal nerves terminate, and green arrows indicate placodes of mammary glands. BMP, bone morphogenetic protein; E9.5, embryonic day 9.5.

Fig. 2. BMP7 expression in orofacial structures of E11.5 and E12.5 lacZ-stained embryos. Longitudinal (sagittal) sections of Epon-embedded whole-mount lacZ-stained E11.5–E12.5 BMP7lacZwt embryos for high-resolution expression analysis. (A–D) Views of the nasal process. Figures (B) and (D) represent close-ups from (A) and (C), respectively. (E–G) Views of the oral cavity showing BMP7 expression in oral epithelium (E) and placodes of the maxillary (F) and mandibular (G) incisors. (H) View of the developing pituitary. (I–L) Views showing the expression in the developing eye region. Figure (J) represents a close-up from Figure (I), and figure (L) is a higher magnification of figure (K). (M) Expression in the developing trigeminal nerve. (N) A close-up from figure (M). (O) Expression in the developing facio-acoustic ganglion complex. de, dental epithelium; e, eye; eie, epithelium of inner ear; ep, epithelium; epc, eye pigment cells; fb, forebrain; md, mandible; mes, mesenchyme; mx, maxilla; n, nose; nc, nasal cavity; np, nasal process; oc, oral cavity; oe, oral epithelium; oep, olfactory epithelium; on, optic nerve; pit, pituitary gland; tgg, trigeminal ganglion; red arrows indicate dental placodes (or dental epithelium) and the green arrow indicates pigment cells of the eye. BMP, bone morphogenetic protein.

J. Exp. Zool. (Mol. Dev. Evol.)
MALFORMATIONS IN BMP7-DEFICIENT MICE

J. Exp. Zool. (Mol. Dev. Evol.)
several sites: the perioptic mesenchyme that will differentiate to form the sclera, the inner layer of the optic cup that will form the nervous layer of the retina, the outer layer of the optic cup giving rise to the pigmented layer of the retina, and finally the primitive hyaloid vessels within the hyaloid cavity (Fig. 2I–L). Staining was also visible in the developing trigeminal nerve characteristic- 

ally located laterally to the primary head vein (Fig. 2M, N). LacZ staining was also seen at the epithelium of the developing otic vesicle and the condensating mesenchyme, which will form the facio-acoustic ganglion complex (Fig. 2O).

Inspection of embryos at stages E13.5–E18.5 revealed BMP7 expression in the developing palatal shelves, cranial and facial bones, and several orofacial ectodermal appendages such as teeth, taste buds, whiskers, hair follicles, as well as nasal and salivary glands (Fig. 3). Strong BMP7 expression was obvious by whole-mount staining in the developing eye, the trigeminal nerve and its ganglion, the maxillary incisors, nose, whiskers and hair follicles, and the palatal rugae of E18.5 embryos (Fig. 3A, B). Expression at the rugae was first detected at E14.5 and persisted until E18.5, the last time point of our analysis. BMP7 was also expressed in structures forming bone (e.g. cranial bones, mandibular, and maxillary bones).

In the developing tongue and the taste buds, expression of BMP7 was evident from E13.5, where it was observed in the epithelium of the most anterior part of the tongue, the dorsal and ventral grooves (median sulcus), and the region where the two lingual swellings will fuse. At more advanced developmental stages, BMP7 expression expanded and by E15.5 was prominent throughout the epithelium covering the tongue and the taste buds (Fig. 3C, D). After E16.5 the expression regressed and by E18.5 BMP7 was only expressed at the epithelium of the most posterior part of the tongue and around the median circumvallate papilla (not shown).

BMP7 expression in the epithelium of the developing salivary glands was evident from E12.5 to E18.5 (Fig. 3E) and at all stages of whisker and hair follicle development (Fig. 3B, F–H). In the hair follicles, BMP7 was first detected at E13.5, when the expression became evident in the inner and outer root sheaths, hair shaft and dermal papilla, as well as the mesenchyme that surrounds the follicles (Fig. 3G, H). At more advanced developmental stages, BMP7 was detected in the dermal papilla and outer root sheath, showing an intense expression in the lower half of each follicle (the bulb of hair follicle) and at the bulge region (not shown).

LacZ staining was observed at the olfactory epithelium, the epithelium of the nasopharynx and nasal glands, the perichondrium of the nasal cartilages (Fig. 3I, J), and parts of the epithelium covering the semicircular canals of the inner ear (Fig. 3K).

**Expression pattern of BMP7 in developing teeth**

BMP7 expression was found in developing teeth from the initiation (placode) stage (E11.5) to the late bell stage (E18.5) in both incisors (Figs. 1G, H, 2F, G, and 4A–F) and molars (Figs. 1G, H, 2I, M, 3C, and 4G, H). From E11.5, expression of BMP7 was detected at various sites of the oral epithelium, where it was more intense in the thickening dental epithelium, whereas in the underlying mesenchyme the expression was very weak (Fig. 2F, G). The epithelial expression persisted during the bud stage of development (E12.5–E13.5), but the gene was not expressed in all epithelial cells (Fig. 4B, C). At the cap stage, BMP7 was detected in the enamel knot and weakly in the adjacent inner dental epithelium (data not shown). At the E15.5 incisors, LacZ staining was observed at the dental follicle that surrounds the dental epithelium, a part of inner dental epithelial cells at the labial side, and the dental papilla mesenchyme at the cervical loop region, with a more pronounced expression at the lingual part (Fig. 4D–F). This pattern was maintained in incisors until E18.5 (data not shown). In molars, during the bell stage of development (E16.5–E18.5), a strong and asymmetric BMP7 expression was detected in the inner enamel epithelium and stratum inter- 

medium (Figs. 3C and 4G, H). A weaker signal was detected in cells of the dental lamina and some cells of the stellate reticulum (Fig. 4G, H). BMP7 expression was also evident in dental papilla mesenchymal cells juxtaposing the inner enamel epithelium that will differentiate into odonto- 

blasts (Fig. 4G, H).

**BMP7-deficient embryos exhibit a tooth phenotype and defects in the orofacial region**

In order to assess whether the observed expression of BMP7 could be related to phenotypic changes, we analyzed BMP7-deficient embryos at various stages of development. The BMP7 null
Fig. 3. BMP7 expression in orofacial structures of E13.5–E18.5 lacZ-stained embryos. Whole-mount lacZ-stained heads of E14.5 (F) and E18.5 (A, B) BMP7-lacZ embryos and cryosections of E13.5 (G, H), E15.5 (I, J), and E18.5 (C–E, K) BMP7-lacZ embryos. (C, D, G, I, J) Frontal sections. (E, H, K) Longitudinal (sagittal) sections. br, brain; c, cartilage; cb, (calvaria) cranial bone; b, bone; e, eye; ep, epithelium; i, incisor; ie, inner ear; m, molar; md, mandible; mes, mesenchyme; mx, maxilla; n, nose; nc, nasal cavity; ng, nasal gland; olfe, olfactory epithelium; pe, palatal epithelium; pl, palate; pm, palatal mesenchyme; pr, palatal rugae; sg, salivary gland; t, tongue; te, tongue epithelium; gl, gland; gd, gland duct; tm, tongue mesenchyme; wfe, whisker follicle epithelium; wfp, whisker follicle papilla; wh, whisker; H, level of section in figure (H); red arrows in figure (K) indicate the hair cells of inner ear epithelium. BMP, bone morphogenetic protein.
allele (Bmp 7Δ) used here was derived from a conditional BMP7 allele (Bmp 7flx), in which exon 1 is flanked by loxP sites. Cre-mediated recombination in the germ line resulted in BMP7 heterozygous null mice (Bmp 7w1/Δ) that were used intercrossed to obtain BMP7 null embryos (Bmp 7Δ/Δ) (Zouvelou et al., 2008). For the histomorphological analysis, a total of ten BMP7-deficient embryos from E11.5 until E18.5 were embedded in paraffin and serially sectioned in frontal, longitudinal, and coronary orientations. Thereafter, the sections were stained with hematoxylin/eosin (H/E) and compared with the corresponding sections of wild-type littermates. We found various phenotypic alterations in craniofacial structures, mostly in ectodermal appendages, which appeared to be disorganized. The craniofacial malformations included absence of the eyes and maxillary teeth, cleft palate, deformed mandible and maxilla, as well as disrupted acoustic and
cranial bones. Examination of the cranial bones showed a microplastic mandible with porotic appearance and a smaller Meckel’s cartilage. The bones shaping the cranial cavity were disorganized and discontinuous in places, and this was easily noticeable in the basisphenoid bone. This could explain the exencephaly phenotype that was exhibited by some of the BMP7-deficient embryos (not shown). In addition, the development of the cartilage primordium of the hyoid bone was remarkably delayed. The thyroid cartilage was displaced and distorted, whereas the cricoid’s cartilage was either displaced or inexistent.

Analysis of the developing palatal shelves revealed a severe disturbance that created a cleft palate. Analysis of longitudinal and frontal sections revealed the retarded growth of the hard and soft palates (Fig. 5A–D). In detail, the palatal shelves of the left and right maxilla were not developed properly, leading to the failure of soft palate formation. As a result, the opening between the nasopharynx and the oropharynx persisted and was never closed. The expansion of the palate shelves stopped where the cartilage primordium of the basisphenoid bone forked to form the roof and floor shelves of the nasal cavity. Closer inspection clearly showed that the cartilage primordia of the basisphenoid and basiocipital bones had not fused, leaving a gap at the side of the pituitary gland. In addition, a stalled formation and ossification within the basiocipital bone was observed. The overall facial anatomy was also severely deformed. In particular, the precartilage primordium of the nasal capsule was not properly formed (the septal cartilage, the vomerina nasal cartilage, and the lateral nasal cartilage were not fused) and the cartilage primordium of the nasal septum was developmentally retarded (not shown), but the nasal septum and primary palate were able to fuse. Additionally, the cartilage primordium of a part of the temporal bone (petrous part) was disorganized, the semicircular canals and cochlea were enlarged, whereas the tubo-tympanic recess was occluded (not shown).

Specific phenotypic alterations were also observed in the developing teeth. In several mutant embryos, the maxillary incisors were missing (4/7 embryos) and the mandibular incisors were deformed and hypoplasic (Fig. 5A, B, E, F). Rarely (1/7 embryos), only one mandibular incisor was present (not shown). The development of the maxillary and mandibular first molars was delayed (Fig. 5C, D), and in some cases (3/7 embryos) these teeth were misplaced or missing (Fig. 5G, H).

Whiskers and hair follicles were also affected in BMP7-deficient embryos: vibrissae were reduced in number (not shown) and their morphology was disrupted on either side of the upper and lower lips. In detail, the structures of hair follicles and whiskers were severely malformed with enlarged epidermal root sheaths and gaps among the cells of the epidermis and mesenchyme that surrounds the developing follicles (Fig. 5I–J).

**DISCUSSION**

Craniofacial malformations comprise approximately one-third of all birth defects, thus indicating a need to determine the genetic and environmental factors that cause these anomalies. Wnts, sonic hedgehog, fibroblast growth factors, and BMPs have been identified during the past years as the key signals orchestrating the formation of the head, but a detailed understanding of the molecular, cellular, and tissue interactions that are interfered with still remains elusive. Although a number of BMPs are expressed in the developing orofacial complex, most of the functional studies have focused upon BMP4. In this report we describe the expression of BMP7 throughout embryonic craniofacial development using a lacZ reporter allele (Godin et al., ’98) and correlate the expression to phenotypic changes observed in homozygous, germline deleted, conditional BMP7-deficient mice (Zouvelou et al., 2008).

BMP7 expression in selected craniofacial tissues has been sporadically addressed in previous reports (Takahashi and Ikeda, ’96; Aberg et al., ’97; Helder et al., ’98; Rice et al., ’99; Jaskoll et al., 2002). In contrast to those reports, this study covers the whole spectrum of orofacial development and is not restricted to either individual structures or single time points. Overall, BMP7 expression is detected in both mesenchymal and epithelial structures of the developing head, whereas an epithelial expression pattern is observed in all ectodermal appendages of the orofacial region.

In the developing teeth, BMP7 expression is dynamic and often locally restricted. This is nicely shown in the developing molars where an asymmetric expression pattern is observed. This asymmetry becomes obvious when comparing consecutive sections encompassing the entire tooth. Asymmetric expression patterns of several other genes have been already described in the enamel organ of the developing molar and suggest the existence of planar signals for the formation of...
Fig. 5. Orofacial malformations in E17.5 BMP7-deficient mouse embryos. Hematoxylin/eosin-stained paraffin sections of wild-type (A, C, E, G, I) and Bmp7^−/− (B, D, F, H, J) E17.5 embryos. (A, B) Longitudinal sections showing nonfusion of shelves of the hard palate and a missing incisor (B). (C, D) Frontal sections showing arrest of soft palate shelf expansion in Bmp7^−/− embryos and the absence of the maxillary molar (D). (E, F) Longitudinal (sagittal) sections showing the lack of a maxillary incisor (green dotted circle in figure (F)). (G, H) Longitudinal sections showing the lack of a maxillary molar (green dotted circle in figure (H)). (I, H) Sagittal sections showing malformed hair follicle with enlarged epidermal root sheath and a gap between the cells of the epidermis and mesenchyme surrounding the developing hair follicle (J). b, bone; br, brain; c, cartilage; de, dental epithelium; e, eye; ep, epithelium; i, incisor; m, molar; md, mandible; mes, mesenchyme; mx, maxilla; n, nose; nc, nasal cavity; oc, oral cavity; oe, oral epithelium; p, dental papilla; pl, palate; t, tongue; wf, whisker follicle; wt, wild-type animal; Δ/Δ, mutant animal. BMP, bone morphogenetic protein.

J. Exp. Zool. (Mol. Dev. Evol.)
the characteristic tooth crown shape (Pouyet and Mitsiadis, 2000). The asymmetric expression of BMP7 could be connected to the asymmetric proliferation rate and the growth of the secondary enamel knots (Obara and Lesot, 2007); however, a more detailed analysis is clearly required to correlate differential BMP7 expression with dental morphogenetic processes.

Rodent incisors differ from molars in that they are continuously growing and are covered by enamel only on their labial side. The continuous eruption of incisors suggests the existence of a stem cell niche at their posterior part where the cervical loop is located (Harada et al., '99; Mitsiadis et al., 2007). To learn if there is a potential link of BMP7 to stem cells in teeth, we extended our analysis on BMP7 expression to the developing incisors. Interestingly, BMP7 is detected in mesenchymal cells of the dental follicle and dental papilla that surround the epithelial stem cell niche of the incisor. This finding suggests that the BMP7-expressing cells are actively involved in the eruption process of the incisor, either acting as modifiers of the epithelial stem cell niche microenvironment or by forming a pool of undifferentiated mesenchymal cells (probably stem cells) that are necessary for the continuous growth of the incisors.

Although most of the present findings are in agreement with previously reported results, several time- and space-related differences were observed. With respect to molar development, BMP7 expression is not only found at the tip of the epithelial bud at E12.5–E13.5 (Aberg et al., '97) but also in cells at the flank and the top of the bud. Similarly, in contrast to these previously reported results (Aberg et al., '97), transcripts are also detected at the cervical part of the inner enamel epithelium during the bell stage (E16.5–E18.5).

Discrepancies also exist concerning the time of initial expression of the gene in several developing organs/tissues; for example, previous studies have reported that BMP7 is first seen in hair follicles at E15 (Jaskoll et al., 2002), but these findings are in contrast with the present results. These discrepancies can most likely be attributed to the use of different techniques (gene reporting vs. RNA in situ hybridization) to detect gene expression. Gene reporting is a widely used approach to map the expression of a gene in time and space. In this method, a reporter gene, most often the bacterial β-galactosidase encoding LacZ, is stably introduced into the mouse genome in such a manner that the promoter (and hopefully also the regulatory elements) of the gene of interest drives the reporter expression. Staining for lacZ is very sensitive and allows the visualization of the expression in whole embryos or histological sections usually with single cell resolution (for a review, see Adams and Gale, 2006), particularly when combined with high-resolution histological techniques as used in this report. In addition, as a protein, the β-galactosidase is likely to be more stable than the RNA of the gene it reports. This enables the visualization of weak or very transient gene expression that might otherwise be missed. On the other hand, it should be noted that the increased stability of the β-galactosidase can lead to apparent expression in cells that have actually ceased producing the mRNA message. Thus, most of the differences observed between the present and previous reports can be attributed to the increased sensitivity of the technology used here.

The role of BMP7 signaling in craniofacial development has received little attention because the initial reports on BMP7-deficient mice did not mention malformations of orofacial structures (Dudley et al., '95; Luo et al., '95). Only a more recent study reported an abnormal salivary gland development in BMP7-deficient mice (Jaskoll et al., 2002). The present findings are in clear contrast to these previous reports as BMP7 deficiency affects the development of almost all structures expressing the gene. Two possible explanations could be advanced for the opposing craniofacial phenotypes: either a different design of the conditional allele or the use of mice with a different genetic background. Although differences in allele design cannot be formally ruled out, these are unlikely as conditional allele removes exon 1, which is the same exon removed in the obligatory BMP7-deficient mouse created previously (Dudley et al., '95). Differences in genetic background are more likely, as the dependence of genetic background on phenotype manifestation has been well documented for other members of the BMP signaling network such as BMP4 and twisted gastrulation (Dunn et al., '97; Nosaka et al., 2003; Petryk et al., 2004). Previous studies on BMP7-deficient mice have been performed on 129 or mixed 129/B6 background (Dudley et al., '95; Luo et al., '95), whereas our mice were backcrossed to the C57Bl/6J background, thus providing a likely explanation for the observed phenotypic differences.

Specific phenotypic alterations are found in the developing teeth of BMP7-deficient mice. In
approximately half of the embryos studied, the maxillary incisors were missing, whereas the mandibular incisors were deformed and hypoplastic. Rarely, only one mandibular incisor develops. The maxillary and mandibular first molars are developmentally delayed and in some cases misplaced or missing. Defects in odontogenesis have been reported in several mouse mutants for genes associated with BMP signaling. Deletion of Alk3 (BMPR1a) in the epithelium leads to tooth development arrest at the bud stage (Andl et al., 2004), indicating the importance of mesenchyme-derived BMP signals for the further development of the dental epithelium. The epithelial overexpression of Noggin, which is an antagonist of the BMP signaling, results in various phenotypic alterations including lack of mandibular molars, reduced number of maxillary molars, disrupted root size and pattern, as well as poorly mineralized enamel (Plikus et al., 2005). In Msx1-deficient mouse tooth development is arrested at the cup stage (Satokata and Maas, ’94), a phenotype that can be rescued by administration of BMP4 (Bei et al., 2000). In vitro, BMP4 and BMP7 can both induce the expression of Msx1 and Msx2 as shown by the implantation of BMP-releasing beads into the mouse molar mesenchyme (Vainio et al., ’93; Wang et al., ’99). The present report provides, for the first time, the direct functional evidence of a nonredundant role for BMPs in tooth initiation and development. The fact that the observed phenotypes are not fully penetrant could be explained by a partial redundancy where other BMPs (e.g. BMP4) or other signaling molecules compensate for BMP7. Therefore, it will be important to elucidate whether, and to what extent, the BMP7 signal differs from those induced by other BMP molecules and, furthermore, how this is reflected on target genes. As BMPs show different affinities for the various type I BMP receptors, a molecular discrimination between signals initiated by different BMPs under physiological conditions is expected. However, these differences could easily be masked when beads are used as delivery systems for BMPs, as the target cells are exposed to these molecules at nonphysiological concentrations.

An indication of the importance of BMP7 for aspects as variable as tooth induction, patterning, and development comes from observations showing a different degree of phenotype penetrance in incisors vs. molars as well as in maxillary teeth vs. mandibular teeth. The molecular networks that determine rodent tooth specification (i.e. molars and incisors, maxillary and mandibular teeth) involve genes such as the Islet1, Pitx1, Barx1, and Dlx (Thomas et al., ’97; Mitsiadis et al., 2003; Mitsiadis and Drouin, 2008), thus integrating BMP7 into their pathway.

BMP7-deficient embryos exhibit a cleft palate phenotype, and these findings link BMP7 with the growing number of genes that are related to this defect (for a review, see Thyagarajan et al., 2003). For example, knockout mice for TGF-β3, another member of the TGF-β superfamily, and Msx1, which is downstream of BMP7, exhibit a cleft palate (Satokata and Maas, ’94; Kaartinen et al., ’95; Proetzel et al., ’95). In addition, it has been shown that several BMPs, including BMP7, are misexpressed in the model of retinoic-acid-induced cleft palate (Lu et al., 2000; Ho et al., 2004). The etiology of the cleft observed in the BMP7-deficient mouse has not yet been investigated in detail, but it is tempting to speculate that Msx1 is involved in this malformation.

A more general involvement of BMP7 in the development of ectodermal appendages and thereby epithelial–mesenchymal interactions is indicated by its strong expression in developing hair follicles and salivary glands, and the concomitant morphological deformation of these two structures. With respect to the hair follicle, the early expression (E13.5) both at the epidermal layer and the underlying mesenchyme, as well as at most of the components of the immature follicle, suggests roles for BMP7 in the initiation, patterning, and growth of the follicle. The present findings reinforce the hypothesis that a BMP signaling network is involved in the development and homeostasis of these structures (for a review, see Botchkarev, 2003) through the activation and/or maintenance of stem cell populations (Zhang et al., 2006; Plikus et al., 2008).

In summary, this report establishes novel nonredundant roles for BMP7 in the development of the orofacial region. In particular, it places BMP7 as a central player in the development of ectodermal appendages and other structures involving mesenchymal–epithelial interactions such as the palate. In addition, the present findings suggest that BMP7 might regulate stem and/or progenitor cells in dental and nondental tissues. Additional experiments are needed to link BMP7 with already established molecular pathways governing tooth and facial development. It can be anticipated that, owing to the complex and partly overlapping phenotypes, tissue-specific gene ablation strategies will be empowered by the availability of a conditional BMP7 allele.
ACKNOWLEDGMENTS

The authors would like to thank Liz Robertson for generously providing the BMP7-lacZ reporter mouse that we used in this study and Mrs. Margrit Amstad for her skillful assistance in sectioning. This work was supported by MUGEN NoE 6FP (LSHG-CT-2005-005203) and the Greek Ministry of Development (Aristia) (DG), grants of the University of Zurich (H.-U. L. and T. A. M.) and a short-term mission grant (V. Z.) from the European B23 COST Action.

LITERATURE CITED


J. Exp. Zool. (Mol. Dev. Evol.)


