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Role of BMP-4 during tooth development in a model with complete dentition

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1. Introduction

The bone morphogenetic proteins (BMPs) are a group of molecules related to the transforming growth factor-β superfamily.  They were originally isolated from demineralized bone matrix and initially characterized by their ability to induce ectopic bone formation in vitro. Homozygous mice mutant BMP-4 embryos die between embryonic days 6.5 and 9.5 (E6.5 and E9.5) and show little or no mesodermal differentiation.

In relation to mouse tooth development, Bmp-4 transcripts are detected in the presumptive dental epithelium from its thickening to the early bud stage. During growth of the epithelial tooth bud (E12–13), Bmp-4 transcripts are present for a short period both in the epithelial cells and in the underlying mesenchymal cells. Subsequently, gene expression shifts to the mesenchyme, which coincides with the transfer of the odontogenic potential from epithelium to mesenchyme. Mesenchymal Bmp-4 regulates epithelial morphogenesis beyond E12. Blocking in vitro translation of BMP-4 gene results in lack of cusp formation and ameloblast differentiation.

Most of the data about BMP signalling underlying tooth development has come from studies in mouse embryos and comparable data from other vertebrates are extremely limited. Marsupials have been considered excellent models for ontogenetic studies due to its short intrauterine development. Moreover, the marsupials of the

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Didelphidae family have a full complement of teeth, with five upper and lower incisors, one canine, three premolars and four molars, on each side of the jaw.\textsuperscript{11}

Marsupials and placentals diverged in the evolutionary line in the early Cretaceous\textsuperscript{12} but maintained similar dental features that reflect their common ancestry more than 120 million years ago.\textsuperscript{13} Conserved molecular pathways may be responsible for these similarities. Gene expression patterns have been highly correlated with morphological features of dentition. As an example, the distinctions in molar pattern between mice and voles, the first murine group originated in the Middle Miocene and the second in the Early Pliocene, are the result of small changes in the expression of genes that determine the relative position of the secondary cusp.\textsuperscript{14} Similarly, differences in BMP-4 expression among species could be responsible for morphological differences in the dentition.

Considering that the same or similar genes may control the patterns of molar cusps between marsupials and placentals, the purpose of this work was to characterize the amino acid sequence and the expression pattern of BMP-4 during molar tooth development in Didelphis albiventris in order to search for similarities and differences between the opossum and the mouse.

2. Materials and methods

2.1. Animals and tissue preparation

Pregnant \textit{D. albiventris} females were captured in the nature during reproductive period (July-January, in Minas Gerais, Brazil) and maintained in captivity until the animals were born. Newborns were carefully removed from mothers’ nipples in the pouch, after immobilization of females, in a minimum number of three young animals for each postnatal day (P), from P0 (moment of birth) until P15, in order to collect samples of first molars in dental lamina, bud, cap and early bell stages. Following, the newborns were weighed, measured and decapitated. The heads were fixed in neutral 10% buffered formalin for 48 h at room temperature. After fixation, all samples were dehydrated through graded ethanol solutions, embedded in paraffin, serially sectioned at 7 μm in the frontal plane and placed on sylanized slides. The Ethics Committee in Animal Experimentation of UFMG (CETEA-UFMG and Brazilian Institute of Natural Environment and Renewable Resources (IBAMA) previously approved all procedures performed.

2.2. Cloning and sequencing of \textit{D. albiventris} BMP-4 cDNA

Adult brain cDNA library was generated from \textit{D. albiventris} total RNA extraction with Trizol reagent (Gibco BRL, Carlsbad, CA). BMP-4 cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) methods using a Super-Script First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). The oligonucleotides used in PCR were designed taking into account the BMP-4 cDNA sequence homology among human, mouse and rat sequences (GenBank-NCBI accession numbers NM_012827, X56848 and NM_012827, respectively). The forward primer was 5’-ATGATTCCCTGTTAAACCGAATGCTG-3’, and the reverse one 5’-TCAGCAGCAAYCCRCAYCC-3’. The amplified fragment was cloned into a pcR2.1-TOPO-vector (Invitrogen Life Technologies) and sequenced on MegaBACE DNA Analysis System (Amersham Biosciences, Buckinghamshire, England) using M13 primers. BMP-4 sequence homology analysis between \textit{D. albiventris} and other species was performed with the algorithms BLASTX\textsuperscript{15} and ClustalW\textsuperscript{16}, available in the Internet: http://www.ncbi.nlm.nih.gov and http://www.ebi.ac.uk/clustalw/, respectively.

2.3. Bacterial expression of BMP-4 recombinant protein

BMP-4 cDNA was removed from the TOPO vector through enzymatic digestion of EcoRI restriction sites. The fragment was inserted into the pGEX-4T-1 expression vector (Amersham Biosciences). Transformed \textit{Escherichia coli} BL21 (DE3) Star cells (Amersham Biosciences) were cultured in LB Broth media, induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) and then disrupted for protein separation by electrophoresis using 10% polyacrylamide gel under reducing conditions (SDS-PAGE). This expression system allowed production of the 26 kDa schistosomal glutathione-S-transferase (GST) fusioned to the BMP-4 recombinant protein.

2.4. Western blot analysis

Proteins collected from wild type and transformed \textit{E. coli} were separated by 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with PBS 2% casein for 1 h at room temperature, the membrane was probed for 1 h at room temperature with goat polyclonal primary antibodies against schistosomal GST (27-4577-01, Amersham Biosciences) and human BMP-4 (sc-6896, Santa Cruz Biotechnologies, Santa Cruz, CA). After incubation with peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO), visualization of staining was obtained by colorimetric detection with 6 mg of diaminobenzidine (DAB), 0.3% (w/v) NiCl₂ and 30% H₂O₂ in 0.15 M PBS solution. This step had two purposes: to confirm recombinant BMP-4 in vitro translation and to test the suitableness of the primary anti-human BMP-4 antibody for use in the immunohistochemistry.

2.5. Immunohistochemistry

After removing of paraffin, hydration, and PBS washing, opossum facial sections were incubated in 10% H₂O₂ in methanol to neutralize endogenous peroxidase activity. Non-specific binding sites were blocked with 2% BSA in PBS for 1 h. After overnight incubation of sections at 4 °C with the primary antibody anti-BMP-4 (sc-6896, Santa Cruz Biotechnology), sections were rinsed with PBS and incubated for 30 min at room temperature in biotinylated anti-goat antibody (LSAB+ System-HRP, Daku, Glostrup, Denmark). Following PBS washing, sections were incubated for 30 min at room temperature in streptavidin–horseradish peroxidase conjugate (LSAB+ System-HRP, Daku). Visualization of staining was obtained by incubating sections with 14 mg of DAB and 10% H₂O₂ in PBS
solution. Then, sections were counterstained with Mayer’s haematoxylin. Negative controls were performed by applying normal goat serum instead of the primary antibody. Developing opossum bone sections were used as positive control for the primary antibody.

3. Results

3.1. Cloning and sequencing of BMP-4 cDNA

BMP-4 cDNA is a 1233-base pair sequence comprising the entire BMP-4 gene open reading frame. Nucleotide sequence homology was 44.7% in relation to human, 43.8% in relation to mouse and 44.3% in relation to rat (NM_001202, X56848 and NM_012827, respectively). Translation resulted in a 410-residue polypeptide corresponding to the precursor protein with a predicted molecular mass of 46.7 kDa (ExPaSy Proteomics Server, http://au.expasy.org/). The amino acid sequence homology in relation to human was 86.3% of identity and 90.5% of similarity. In relation to mouse, the sequence identity was 86.8% and the similarity 91%. In relation to rat, the sequence identity was 86.3% and the similarity 90.7%. Precursor BMP-4 protein presents a TGF-β pro-peptide which represents the main part of the protein. The C-terminal region of the precursor protein contains the conserved central core of the TGF-β family and, after cleavage, functions as the mature BMP-4 protein and presents 116 residues and a molecular mass of 13.2 kDa. The opossum mature BMP-4 has 95.7% of sequence similarity in relation to mouse, rat and monkey (CAA40179, NP_0336959 and XP_0001084317, respectively) and 94.8% of similarity in relation to human (AAC72278). The cleavage site of the precursor protein has the conformation RAKR and is situated in the positions 291–294. In the

Fig. 1 – BMP-4 amino acid sequence homology among D. albiventris and other species through ClustalW (1.82) multiple sequence alignment: underlined, high-affinity type I receptor-binding sites (BMPR-IA); arrows, conserved cysteines of the TGF-β superfamily; boxes, presumptive heparin-binding sites (‘*’ identical residues; ‘:’ conserved substitution; ‘..’ semi-conserved substitution; ‘-’ gap; ‘ ’ no consensus).
C-terminal region, the seven conserved cysteines of the TGF-β family are localized at the residues 310, 339, 343, 374, 375, 407 and 409 (Fig. 1). In the opossum precursor BMP-4 there is a glutamic acid at the position 95 that is not present in the dog, rabbit, mouse and rat sequences (XP_547817, AAB97467, NP_031589 and AAH78901, respectively). In relation to the human sequence (P12644), there are two extra glutamic acids at residues 95 and 99 of the opossum sequence. The N-terminal region presents various triplets of basic residues (K, R and H), which are considered presumptive heparin-binding sites of BMP-4. The presumptive binding sites for the high-affinity receptor type I (BMPR-IA) are the residues C-310, R-311, V-322, G-323, W-324, D-326, W-327, I-328, P-344, F-345, P-346, L-347, T-348, D-349, H-350, Q-352, S-356, V-358, G-359, L-362, S-365, V-366, M-385, Y-387, L-388, K-397, Y-399, Q-400, E-401 and M-402.

D. albiventris BMP-4 cDNA was submitted to the GenBank database and has been assigned the accession number DQ192517.

3.2. Bacterial expression and Western blot analysis

In E. coli samples transformed with pGEX-4T-1/BMP-4 and probed with polyclonal anti-human BMP-4, the GST/BMP-4 fusion protein was resolved as a band of approximately 72.7 kDa, which is consistent with its predicted molecular mass (Fig. 2). Non-transformed E. coli samples did not show any specific reaction.

3.3. Histological analysis

In the moment of birth (P0), the first molars of D. albiventris were observed at the dental lamina stage with a condensation of the mesenchyme around the epithelial thickening (Fig. 3A and B). The bud stage occurred between P2 and P4 (Fig. 3C and D shows the dental organs at P2). The cap stage occurred between P4 and P8. At P5, the formative elements of the tooth (dental organ, dental papilla and dental follicle) were evident and the primary enamel knot was completely formed (Fig. 4A and B). The bell stage lasted from P8 to P15. At P13, the dental organs were still at the early bell stage (Fig. 4C and D).

3.4. Immunohistochemistry

In the developing bone sections, used as positive controls, BMP-4 was detected in the osteoblasts, osteocytes, and in the extracellular matrix adjacent to the sites of bone formation (Fig. 3F and F). Immunohistochemical data on BMP-4 expression was similar for both upper and lower first molars in D. albiventris. At the dental lamina stage, BMP-4 expression was evident at the dental epithelium (Fig. 3A and B). Subsequently, during the bud stage, the expression progressively shifted to the dental mesenchyme (Fig. 3C and D). During the cap stage, immunostaining was restricted to the enamel knot, inner enamel epithelium, outer enamel epithelium and dental follicle (Fig. 4A and B). There was no staining in the dental papilla. At the bell stage, the intensity of BMP-4 expression was significantly reduced. At this stage, staining was stronger at the dental follicle and enamel knots and there was no BMP-4 expression in dental papilla (Fig. 4C and D).

4. Discussion

The mechanisms involved in epithelial–mesenchymal interactions regulating tooth development have been studied in the mouse for decades. The mouse is a classical model for investigation of molecular patterning of odontogenesis because of the great conservation of signalling molecules among species and along evolution. However, the dentition of the mouse contains highly specialized incisors, reduced tooth
pattern and lack of enamel in molar cusp tips, and cannot be considered a typical representative of mammals. The opossum *D. albiventris* has been demonstrating a great suitableness to ontogenetic studies.\(^2\)\(^1\)

The great morphological differences of mammalian dentitions may be a result of distinctive patterns of gene expression.\(^1\)\(^4\) Fibroblast growth factors (FGFs) and BMPs are key regulators of dental morphogenesis\(^2\)\(^2\) and studying members of these families in the opossum is a good start point to understand possible correlation between species-dependent variations in molecular regulation of odontogenesis and morphological variations in dentitions. In a previous study, it was demonstrated that, despite of the great conservation of FGF-9 among species, its involvement in tooth development is apparently different between the opossum and rodents. While in rodents FGF-9 is involved in both tooth initiation and morphogenesis, in the opossum, there was evidence of its participation only in dental organ initiation.\(^2\)\(^1\) Such observation reinforces the necessity for further research on the regulation of odontogenesis in biological models with complete tooth pattern.

The sequence analysis of the *D. albiventris* BMP-4 showed its high degree of conservation among human, mouse and rat. In relation to tooth development, previous studies demonstrated the occurrence of *Bmp-4* expression in dental epithelium during tooth initiation in the mouse\(^4\)\(^,\)\(^2\)\(^3\) and rat.\(^2\)\(^4\) Similarly, opossum BMP-4 was preferentially expressed in the dental lamina epithelium than in the dental mesenchyme. In tooth initiation, epithelial BMP-4 is a regulator of mesenchymal transcription factors necessary to the proliferation of epithelial cells.\(^4\)\(^5\) Epithelial expression of BMP-4 in the opossum was transient. During growth of the epithelial bud, the expression was transferred to the dental mesenchyme as occurs in the mouse\(^4\)\(^,\)\(^2\)\(^3\) and rat.\(^2\)\(^4\) The transfer of BMP-4 expression from the epithelium to the mesenchyme coincides with the transfer of the odontogenic potential in the same direction.\(^7\)\(^5\)

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**Fig. 3 – Immunolocalization of BMP-4 in developing *D. albiventris* first molars.** (A) Epithelial expression of BMP-4 at the dental lamina stage (P0) of the lower first molar; (B) similar expression pattern for the upper first molar at the dental lamina stage; (C) strong staining for BMP-4 in the condensed mesenchyme around the lower first molar at the bud stage (P2); (D) similar expression pattern for the upper first molar at the bud stage; (E) negative control; (F) section of intramembranous ossification of maxilla was used as positive control (P2). Bars = 50 μm (DL, dental lamina; TB, tooth bud; OC, oral cavity; EC, ectomesenchyme; BN, bone).
During opossum tooth morphogenesis, BMP-4 expression was observed in the enamel knots and inner enamel epithelium. This data suggests that BMP-4 has a role in opossum tooth crown formation and ameloblast differentiation since these cellular structures are involved in cusps formation.26 While Bmp-4 expression is evident in the murine dental papilla cells4,7, there was no BMP-4 expression in the opossum dental papilla at the cap and early bell stages. This is the most significant difference in relation to mouse. There are few studies about the roles of BMPs and other signalling molecules in stages of tooth development later than the cap stage, but they suggest an implication of BMP-4 in the differentiation of dental papilla cells. In bovine adult pulp cells, BMP-4 is considered and important inducer of odontoblast differentiation and extracellular matrix proteins expression.27,28 In embryonic rat pulp, there are multiple receptors for BMPs, implicating diverse functions in cell differentiation and tissue repair.29 Opossum BMP-4 does not appear to play a role in early maturation of dental papilla cells but the data presented here cannot be extrapolated to the entire process of odontoblastic differentiation. Further studies on later stages of tooth development will help to clarify if BMP-4 may regulate or not dental papilla cells differentiation in the opossum. It was demonstrated here for the first time that BMP-4 is involved in tooth initiation and morphogenesis in the opossum molar tooth development.

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