Localization of *Bmp-4*, *Shh* and *Wnt-5a* transcripts during early mice tooth development by *in situ* hybridization

Localização de transcritos de *Bmp-4*, *Shh* e *Wnt-5a* durante as fases iniciais do desenvolvimento dentário de camundongos por hibridização *in situ*

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Abstract: A comparative nonisotopic *in situ* hybridization (ISH) analysis was carried out for the detection of *Bmp-4*, *Shh* and *Wnt-5a* transcripts during mice odontogenesis from initiation to cap stage. *Bmp-4* was expressed early in the epithelium and then in the underlying mesenchyme. *Shh* expression was seen in the odontogenic epithelial lining thickening, being stronger in the enamel knot area, during the cap stage. *Wnt-5a* transcripts were expressed only in the mesenchyme during the initiation, bud and cap stages, with strong expression in the dental mesenchyme during the bud stage. The present results showed that *Bmp-4*, *Shh* and *Wnt-5a* are expressed since the very early stages of tooth development, and they suggest that the *Wnt-5a* gene is expressed in different cell populations than *Bmp-4* and *Shh*.
Descriptors: Odontogenesis; Tooth germ; Wnt proteins; Bone morphogenetic proteins.

Resumo: No presente trabalho, realizou-se uma análise comparativa não isotópica por hibridização *in situ* a fim de se detectar a presença de transcritos de *Bmp-4*, *Shh* e *Wnt-5a* durante as fases iniciais da odontogênese em camundongos, desde a iniciação até o estágio de capuz. No estágio de iniciação, observou-se expressão precoce de *Bmp-4* no epitélio e no mesênquima subjacente, enquanto que a expressão de *Shh* ocorreu durante o estágio de capuz, na região de espessamento do revestimento epitelial odontogênico, tornando-se mais intensa na área de nó do esmalte. Os transcritos de *Wnt-5a* foram expressos somente no mesênquima durante os estágios de iniciação, botão e capuz, com intenso sinal na região no mesênquima na fase de botão. Estes resultados mostraram que *Bmp-4*, *Shh* e *Wnt-5a* são expressos desde os estágios mais precoces do desenvolvimento dentário, sugerindo que o gene *Wnt-5a* seja expresso em populações celulares distintas daquelas que expressam *Bmp-4* e *Shh*.
Descritores: Odontogênese; Germe de dente; Proteínas Wnt; Proteínas morfogenéticas ósseas.

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Introduction

The generation of a tooth relies upon a sequence of tightly regulated and reciprocal signaling interactions between the ectoderm lining the future oral cavity and neural crest-derived ectomesenchymal cells. Over 200 genes have now been demonstrated to be active in the developing tooth. In particular, members of the Fibroblast Growth Factor (*FGF*), Bone Morphogenetic Proteins (*Bmps*), Hedgehog (*HH*) and *WNT* families of signaling molecules induce regionally restricted expression of downstream target genes, such as homeobox, in the odontogenic ectomesenchyme.[4,17]

Bmps are active substances present in bone and dentin, capable of stimulating the formation of new bone and transmitting inductive signals during interactions between epithelial and mesenchymal tissues in developing organs.[23] *Bmp-4* expression was shown to be present during early tooth development and may be important to keep tooth identity.[24]

Sonic hedgehog (*Shh*) is a member of the *HH* family and its pathway is known to be a powerful signaling cascade in both embryonic and adult tissues.[9] Indeed, previous data have shown that *Shh* interactions occur within the dental epithelium and are necessary for cell proliferation, growth and polarization.[8]

Wnts are soluble glycoproteins thought to be involved in diverse embryological events and cellular processes such as gene expression, cell adhesion, proliferation and apoptosis.[25] According to Sarkar, Sharpe[20] (2000) interference with WNT signaling via addition of an antagonist (exogenous Mfrzb 1 protein) resulted in the formation of smaller teeth *in vivo*. *Wnt-5a* is a member of the *WNT* family that activates the Wnt-Ca^{2+} pathway and is involved with modulation of intracellular free Ca^{2+}.

Bmps, *HH* and *WNTs* family members encode secreted factors and can mediate autocrine or paracrine signaling to short- or long-range distances and regulate cell behavior.[25] Members of these three families mediate cell communication during tooth development, mostly between the ectoderm and the mesenchyme.[22] In view of this, the objective of the present study was to describe the co-localization of *Bmp-4*, *Shh* and *Wnt-5a* transcripts during early mouse tooth development, using *in situ* hybridization with nonisotopically labeled probes.

Material and Methods

Embryos

Wild-type C57BL mouse embryos were used for *in situ* hybridization (ISH). The day on which the plugs were detected was designated as embryonic day 1 (E1). The expression patterns of *Shh*, *Bmp-4* and *Wnt-5a* transcripts were mapped by ISH of mouse embryonic heads between E11.5 and E14.5. Two pregnant mice from each period were killed and the litters were collected according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals protocol. Embryos were individually staged according to Theiler[21] (1972) and treated in accordance with the NIH intramural guidelines as proposed in February of 1997.

Probes

All ISH riboprobes were generated by *in vitro* transcription labeling with digoxygenin-UTP according to the manufacturer's manual (Boehringer Mannhein). Probe size and yield were determined by electrophoresis on a 1.5% agarose gel with a RNA standard (Table 1). Hybridization with transcripts derived from the sense orientation of each probe resulted in no signal above background levels (Figure 1).

Table 1 - *In situ* hibridization probes according to orientation, RNA polimerase and restriction endonuclease.

Probes	Sense		Antisense		Product
	RNA polimerase	Restriction Endonuclease	RNA polimerase	Restriction Endonuclease	
*Bmp-4**	T7	AccI	SP6	EcoRI	540 bp
*Shh***	SP6	SmaI	T3	HindIII	640 bp
Wnt5a	T7	PstI	T3	NotI	381 bp

*a gift from Dr. Brigid Hogan;[10] **provided by Dr. Andrew McMahon.[7]

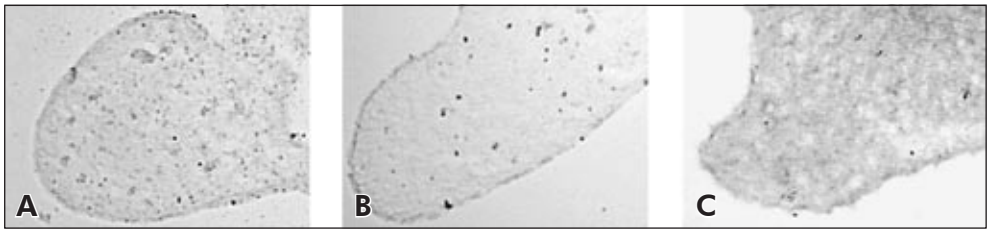

Figure 1 - Control sense probes for *Bmp-4* (**A**), *Shh* (**B**) and *Wnt-5a* (**C**) showed no signal.

Synthesis of DNA template

Total RNA was extracted from E9-E10 mice heads by using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Reverse transcription was performed with Superscript™ (Invitrogen) and oligoDT primers. cDNA was synthesized with 1 µg of total RNA treated with DNAse I in a volume of 20 µl. *Wnt-5a* primers were designed spanning intron-exon boundary using the human and mouse mRNA sequence (forward 5' GGAGAAGGCGCGAAGACAG 3'; reverse 5' GGGCGTCCACGAACTCCT 3') and GeneTool 1.0 Software (BioTools Incorporated, Edmonton, Alberta, Canada). PCR was conducted in 25 µl reactions using 50 pM of each primer, 1.5 mM $MgCl_2$, 1 X PCR Buffer, 100 µM of each dNTP, 20 ng cDNA, and 0.02 U/ µl Taq (Invitrogen). The thermal profile consisted of an initial denaturation step for 4 min at 93°C, followed by 25 cycles of amplification. Each round consisted of denaturation for 45 s/94°C, annealing for 1 min 30 s/55°C, extension for 2 min/72°C, and an additional 7 min/72°C for terminal elongation. Amplification products were analyzed on a 1% agarose gel with ethidium bromide, where a single band of 381 bp was visualized. Specificity of the amplicons was confirmed by cloning and sequencing.

ISH of frozen sections, alkaline phosphatase staining

Frozen sections of mouse embryos were processed for ISH as described previously[5,26] with some changes described bellow. Embryos from E11.5 to E14.5 were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight, dehydrated to 30% sucrose, embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA), and frozen at –80°C. Coronal plane serial sections of 10 µm were then collected on silane-coated glass slides. Sections were permeabilized with 10 µg/ml proteinase K for 2 min. Hybridizations were carried out in "seal-a-meal" bags, overnight/70°C, in 5 ml of hybridization solution (50% formamide, 5 X SSC (pH 4.5), heparin 50 µg/ml, yeast RNA 50 µg/ml, 1% SDS) with a probe concentration of ~0.2 µg/ml. Washes were as follows: three 15 min changes of 50% formamide, 30% 20 X SSC (pH 4.5) and 10% 10 X SDS at 70°C, and three 15 min changes of 50% formamide and 12% 20 X SSC (pH 4.5) at 65°C. Detection of bound probe was performed using anti-digoxigenin antibody and NBT/BCIP as color substrate. Slides were examined on a Nikon SMZ-2T microscope and digital pictures were taken with an Axiophot 2 Zeiss microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) and a 3CCD MTI camera (Dage-MTI, Michigan City, IN, USA). Images were captured and stored on a Macintosh computer using Adobe Photoshop 5.5 software.

Results

The results described below were separated according to the stage of tooth development.

Initiation stage of tooth development (IS)

During the initiation of odontogenesis the developing teeth can be visualized as localized thickenings of the oral epithelium. At E11.5, *Bmp-4* was expressed in the underlying mesenchyme (Figure 2A). When the dental lamina was formed and could be distinguished as an epithelial thickening, *Bmp-4* was expressed transiently in epithelial cells (data not shown) and the underlying mesenchyme (Figures 2A and 2B), in the same stage as *Shh* (Figure 2D) and *Wnt-5a* (Figure 2G). *Shh* expression was also seen and strongly maintained in the epithelial thickening, which represents the first morphological manifestation of the developing tooth (Figures 2C and

2D), although not uniformly throughout the epithelium. *Shh* transcripts were restrictedly expressed in dental epithelium, including incisor and molar germs (Figures 2C and 2D, respectively). *Wnt-5a* expression domains exhibited widespread expression in the mandibular and maxillary mesenchyme (Figure 2G).

Bud stage (BS)

By E12.5, the dental epithelium had invaginated to form the epithelial tooth bud. From this stage on, *Bmp-4* was expressed in the condensed mesenchyme around the bud (data not shown). *Shh* expression was not seen either in the epithelium or in the mesenchyme (Figure 2E), although signals could be seen

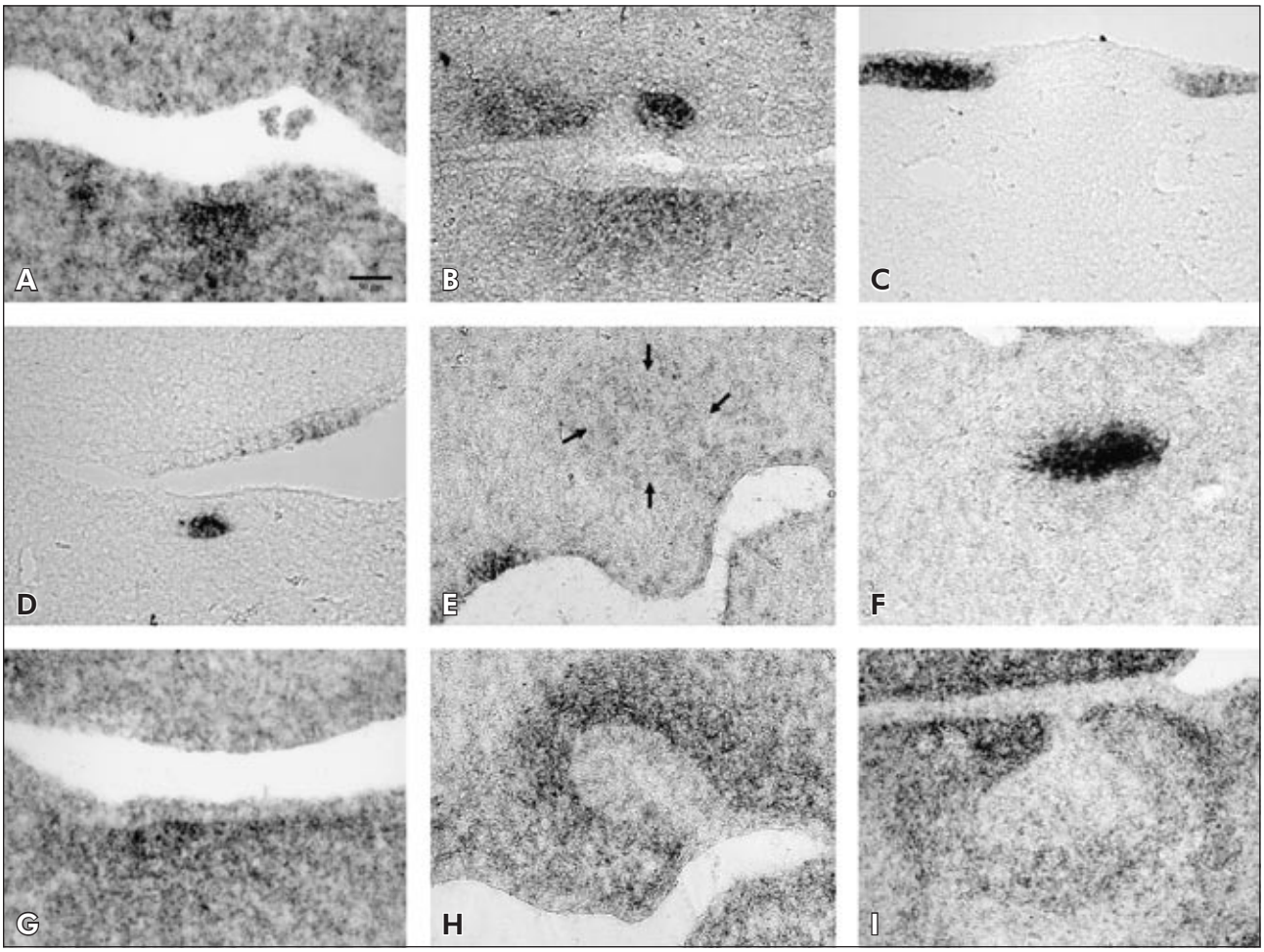

Figure 2 - *Bmp-4* (**A** and **B**), *Shh* (**C**, **D**, **E** and **F**) and *Wnt-5a* (**G**, **H** and **I**) expression during early tooth development. **A:** At E12.5, *Bmp-4* transcripts are expressed in the mandible mesenchyme before dental epithelium is thickened (initiation stage - IS). **B:** *Bmp-4* signal is broader and weaker than in A at the mandible mesenchyme of an E12.5 embryo. In the maxilla mesenchyme expression is adjacent to the thickened dental epithelium, where transcripts are seen in a small population of cells. **C:** *Shh* transcripts are localized in the mandible incisors dental epithelium at the IS. **D:** At E11.5, *Shh* transcripts are seen in a small population of cells in the dental epithelium. *Shh* signal in the maxilla epithelium is not related to odontogenesis. **E:** At 12.5, *Shh* signal is absent in the developing dental organ in the bud stage (delimited by arrows, to be compared to H). *Shh* medial signal in the maxilla epithelium is not related to odontogenesis. **F:** *Shh* is strongly expressed in a population of cells of the stellate reticulum in a developing dental organ in the cap stage (CS). **G:** An adjacent, more ventral section than A, showing broader *Wnt-5a* transcripts expression than *Bmp-4*. Signal is restricted to the mesenchyme and in a different population of cells when compared to *Bmp-4*. **H:** An adjacent, more dorsal section than E, *Wnt-5a* transcripts enclose a developing dental organ in the bud stage (BS). **I:** In an adjacent, more ventral section than F, *Wnt-5a* transcripts surround a developing dental organ in the CS. Signal is stronger at the lateral mesenchyme than at the dental papilla. Scale bar shown in A represents 50 μm and corresponds to the same amplification from A to I.

throughout the sections. Expression of *Wnt-5a* was now well localized around the mesenchyme of the bud (Figure 2H).

Cap stage (CS)

At the CS (E14.5), the developing dental papilla became visible, where a subset of epithelial cells formed the enamel knot, a transient cluster of non-proliferative epithelial cells supposed to act as a signaling center that directs further tooth development. *Shh* expression was seen as a strong signal in the tooth epithelium (Figure 2F) in the region corresponding to the enamel knot. *Wnt-5a* expression was localized in the mesenchyme around the dental follicle, and at the tip of the dental papilla (Figure 2I).

There was no noticeable difference between *Bmp-4*, *Shh* and *Wnt-5a* expression in incisors or molars germs in the developing maxilla or mandible.

Discussion

The present results showed that *Bmp-4*, *Shh* and *Wnt-5a* are expressed at a very early stage when the ectoderm thickens and forms a placode that buds to the underlying neural crest derived mesenchyme. Moreover, when analyzing serial sections, our data suggest that *Wnt-5a* is expressed in a different cell population than *Bmp-4*.

The results presented here revealed the expression of *Bmp-4* restricted to the underlying mesenchyme during the initiation of tooth development (E11.5), being transiently expressed in epithelial cells and the underlying mesenchyme when the dental lamina was formed. At the BS (E12.5), *Bmp-4* was preferably expressed in the condensed mesenchyme, in accordance with Aberg *et al.*[1] (1997). Conversely, Nadiri *et al.*[16] (2004), using immunohistochemistry, found that *Bmp-4* was immunolocalized both in the epithelium and mesenchyme at the BS of mouse first lower molar.

With regards to *Shh* expression, the signal was intense but restrictedly expressed in the epithelial thickening during the initiation of incisor and molar development, as well as in the tooth epithelium in the region corresponding to the presumptive developing cusps in the CS. In fact, there is evidence suggesting that *Shh* acts as a mitogen, inducing proliferation, growth and polarization[8] as those thickenings form a tooth bud.[2,19,20] The highly restricted expression of *Shh* at sites of tooth formation is likely to be essential for specifying the sites where tooth buds will invaginate and teeth will form.[15,19] According to our results, there was a lack of *Shh* signaling during the BS with subsequent increase in the CS, possibly regulating the shape of the tooth crown. Indeed, some authors have reported that the inhibition of *Shh* signaling in mandibular explants from E10.5 results in a failure of bud formation and an arrest of tooth development.[2,20]

Several authors have studied the complex regulation of the *Shh* signaling pathway during mice dental tissues development using *Shh* antagonists.[3,15] Although our study did not reveal different expression patterns between *Shh*, *Bmp-4* and *Wnt-5a*, in incisor or molar germs in the developing maxilla or mandible in the CS, according to Miletich *et al.*[15] (2005), Rab23 demonstrated contrasting expression domains in the incisor and molar mice dentition during the CS, restricted to the mesenchymal compartment of molar teeth and the epithelium of the enamel knot in incisor teeth. These findings provide the first evidence of distinct regulatory pathways for *Shh* in teeth of different classes, and suggest that the additional complexity of the molar dentition may require higher levels of *Shh* signaling activity.

In the CS, *Shh* transcripts were strongly expressed in the stellate reticulum, possibly including the enamel knot. Indeed, *Shh* has been previously shown to be expressed in the enamel knot in the CS.[6] Our results, however, showed a broader expression. This finding, in addition to the expression of *Shh* just prior to bud formation, is consistent with the statement that *Shh* has dual roles in early odontogenesis, first in bud formation by stimulating epithelial proliferation, and second in the development of cap-stage tooth germs by increasing epithelial cell survival.[2] Furthermore, during tooth development, this pattern of expression may become restricted to the stratum intermedium, as has been shown in bovine tooth germs.[12]

Wnt-5a signal initially exhibited widespread expression in the mandibular and maxillary mesen-

chyme, becoming well localized around the mesenchyme of the bud as well as around the dental follicle and at the tip of the dental papilla in the CS. Interactions between WNT and HH signaling pathways were first described as playing a role in establishing boundaries between ectodermal cells in *Drosophila* segmentation.[13] These molecules share the principle of keeping potent transcriptional activators in check in the absence of receptor ligand.[11] A relationship between *Wnt-5a* and *Shh* signals, as seen for WNT-7B and *Shh*,[19] cannot be suggested for now, although our data revealed that transcripts of both genes were present at the same period in the developing teeth. In other organs, however, this interaction is possible. Reddy *et al*.[18] (2001) identified *Wnt-5a* as a target of *Shh* in hair follicle morphogenesis. So, it is interesting to speculate that the absence of *Shh* transcripts in the dental epithelium during the BS may be related to *Wnt-5a* presence in the mesenchyme.

Very little is known about *Wnt-5a* and *Bmp-4* signaling pathways interactions. According to Li *et al*.[14] (2002) *Wnt-5a* may inhibit *Bmp-4* expression during lung morphogenesis in mice. These results may explain the present findings of *Wnt-5a* being expressed in a different cell population than *Bmp-4* during early tooth development.

Interactions between the ectoderm and underlying mesenchyme constitute a central mechanism regulating the morphogenesis of several organs.[23] Tooth development is considered an important model to study epithelial-mesenchymal interactions and, although many questions are still unanswered, genes that regulate tooth development are being identified with increasing speed. Understanding how these genes regulate tooth formation will help us to understand how specific genes cause dental defects, and possibly the mechanisms underlying odontogenic tumor formation.

Conclusion

The present results showed that *Bmp-4*, *Shh* and *Wnt-5a* are expressed since the very early stages of tooth development, and they suggest that *Wnt-5a* is expressed in a different cell population than *Bmp-4* and *Shh*.

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