CRISTIANE PEREIRA BORGES SAITO

ANÁLISE DO PADRÃO DE METILAÇÃO DA REGIÃO PROMOTORA DO GENE HUMANO PAX9 (-947 - +251) E ANÁLISE *IN VITRO* DA SUA ATIVIDADE TRANSCRICIONAL

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Biologia Buco Dental – Área de Concentração: Histologia e Embriologia

Orientador: Prof. Dr. Sérgio Roberto Peres Line

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Resumo

O gene PAX9 é um regulador chave durante o processo de desenvolvimento dentário e, particularmente em humanos, mutações neste gene estão associadas a fenótipos peculiares como agenesias dentárias. O objetivo deste estudo foi descobrir novas coordenadas acerca da atividade transcricional do promotor desse gene e de um possível padrão de metilação. Quanto ao estudo da metilação, foram avaliadas amostras de DNA genômico extraído da papila dentária de embriões humanos através de enzimas de restrição sensíveis à metilação e através da amplificação por Reação da Polimerase em Cadeia-PCR com oligos específicos. A extração de DNA de amostras incluídas em parafina foi padronizada no laboratório de Morfologia da Faculdade de Odontologia de Piracicaba, FOP-UNICAMP. Amostras de DNA extraídas de saliva humana foram usadas como controle. Para os ensaios de transcrição, foram amplificados através de ensaios com a transcriptase reversa, transcritos do gene Pax9 de Células Embrionárias de Rato, bem como de Células Odontoblastóides de Camundongo-MDPC-23 (*Mouse Odontoblast Cell-like*-23). Esses transcritos foram quantificados através de PCR semi-quantitativa. A região promotora do gene PAX9 humano íntegra (-947 - +251) e deletada (-485 - +22), recombinada com o vetor de expressão pGL3Basic, contendo o gene repórter luciferase após a região promotora, foi transfectada em cultura de Células Embrionárias de Rato. Todas as placas de cultura foram submetidas à ação de duas drogas: Ácido Retinóico (AR) e Dexametasona (Dex). Após a lise das células, os níveis relativos de expressão da proteína luciferase foram analisados, utilizando o kit Dual-Glo Luciferase (Promega), em um luminômetro. Os resultados mostram que: (1) O gene PAX9 encontra-se metilado *in vitro*; (2) O AR inibiu a síntese de RNA mensageiro transcrito pelas Células Embrionárias Rato. O promotor do gene humano PAX9 clivado nos sítios -485 e +22 e que não continha 507pb não foi afetado pelos receptores do AR. O Promotor PAX9 íntegro foi ativado na presença do AR na maior concentração da droga ; (3) A Dex estimulou a transcrição do gene Pax9 no

grupo de células MDPC-23 e influenciou positivamente ambas as versões do promotor do gene PAX9 com as concentrações: 0.1μM e 1nM. Esses resultados demonstraram que os receptores dos hormônios esteróides AR e Dex podem se ligar diretamente a sequências do gene Pax9 em ratos e camundongos e que a região contendo 507pb retirada do promotor do gene PAX9 humano pode conter sítios de ligação para receptores do AR. Além disso, o sítio de metilação encontrado na região promotora do gene PAX9 humano resultou em novas perspectivas acerca do seu padrão de funcionamento em células normais.

Palavras-chave: PAX9, metilação, transfecção, PCR semiquantitativo, humano.

Abstract

PAX9 is a key regulator during tooth development and plays an essential role in the patterning of murine and human dentition. In humans, mutations in PAX9 are associated with unique phenotypes of familial tooth agenesis that mainly involve posterior teeth. The objectives of this study were to gain new insights into the transcriptional activity and DNA methylation within the promoter region of human PAX9 gene *in vitro*. The methylation pattern was examined by studying PAX9 gene promoter in Dental Papilla of human Embryos through methylation-sensitive restriction enzyme and PCR amplified with specific oligos. DNA extractions from paraffin-embedded tissue sections were well established in Morphology Laboratory, Piracicaba Dental School. DNA samples from Buccal Epithelial Cells were used as control. In the present study, we have PCR amplified cDNAs encoding Rat Pax9 and Mouse Pax9 from Primary embryonic cell culture obtained from 13 day-old Wistar Rat and Mouse odontoblast cell–like culture-23 (MDPC-23) respectively and quantified by Semi-quantitative PCR technique. Furthermore, we examined the transcriptional activity of human Pax9 gene promoter: (-947 - +251) full Pax9 promoter and the promoter lacking 507bp (-485 - +22) through recombination into pGL3Basic expression vector and transfection in primary rat embryonic cells. Cell cultures were all submitted to selective regulation of both drugs: Retinoic Acid (RA) and Dexamethasone (Dex). Relative luciferase expression units were obtained by dual luciferase assay kit (Promega). Our results showed that: (1) human PAX9 promoter region is methylated *in vitro*; (2) RA inhibited Pax9 mRNA synthesis in Primary Rat embryonic cells while PAX9 promoter lacking 507bp (-485 - +22) was not activated by RA receptors. Human PAX9 full promoter activation was improved by RA treatment at the greater concentration; (3) Dex stimulated Pax9 mRNA activity in MDPC-23 and influenced positively both PAX9 promoter versions with 0.1µM and 1nM concentrations. The present experiments suggest that a 507bp region in PAX9 promoter may contain binding sites for RA receptors and that Dex and RA

steroid hormone receptors may be directly bind to the sequences of murine Pax9 gene. The methylation pattern finding give rise to new perspectives on PAX9 patterning in normal cells phisiology.

Keywords: PAX9, methylation, transfection, semi-quantitative PCR, human.

Sumário

Introdução

Os genes pertencentes à família dos fatores de transcrição caracterizados por uma região comum de domínio de ligação ao DNA são conhecidos por desempenhar papéis importantes nas interações indutivas dos tecidos, atuando durante a organogênese dos vertebrados (1).

O gene PAX9 pertence a uma família de fatores de transcrição com nove membros, chamados: genes de domínio pareado. Eles são importantes reguladores da embriogênese e do desenvolvimento dos vertebrados, funcionando como iniciadores da diferenciação celular ou mantendo a pluripotência das populações celulares em desenvolvimento. (2)

O presente estudo focaliza as variações da expressão gênica *in vitro* do gene PAX9. A verificação da possível metilação da região promotora, através da análise do DNA com enzimas de restrição, pode auxiliar na identificação de sítios metilados, o que por sua vez nos dá indícios do seu grau de ativação. Sítios metilados já foram encontrados, como relatam estudos prévios, na região promotora de genes envolvidos na embriogênese humana (3). A metilação dos resíduos de citosina é a alteração do DNA mais comum em eucariotos, e a metilcitosina aparece freqüentemente em regiões ricas em bases citosina e guanidina, ditas sítios CpG. A metilação do DNA pode estar, também, correlacionada com a inibição da atividade transcricional (4). Esse mecanismo parece ter um papel importante no desenvolvimento e diferenciação dos tecidos durante a embriogênese (5, 6, 7).

As amostras de DNA para essa análise foram amostras retiradas da papila dentária de fetos humanos com as seguintes idades: 10 semanas, 11 semanas, 12 semanas e 4 meses de vida intra-uterina. As amostras foram analisadas através de ensaios com enzimas de restrição *HpaII* e *HaeIII* sensíveis à metilação.

Para análise do padrão de transcrição, a região promotora inteira ou clivada do gene PAX9 humano, através de reação de restrição com a enzima

ApaI, foi recombinada ao vetor de expressão pGL3Basic. Sob influência de drogas que reconhecidamente influenciam na transcrição de alguns genes - dexametasona e ácido retinóico, células provenientes de embriões de rato sofreram transfecção com os recombinates supracitados, com a finalidade de avaliar o comportamento transcricional destes frente à ação das drogas.

A amplificação de amostras de cDNA provenientes da cultura de células embrionárias de rato e cultura de células odontoblastóides de camundongo (*Mouse Odontoblast-Like Cells*-MDPC-23), através de reação com a transcriptase reversa e posterior amplificação através da reação em cadeia da polimerase (PCR semi-quantitativa), forneceu dados complementares sobre o padrão de expressão gênica do Pax9 sob a ação do ácido retinóico e da dexametasona. Juntamente com a análise de metilação da região promotora do gene, este estudo visa contribuir para o melhor entendimento da função do gene PAX9 durante o período de diferenciação celular normal.

Capítulo 1

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Full Title: A methylation approach of PAX9 gene in microdissected dental papilla from human fetuses

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Abstract

Methylation is indispensable for vertebrate genome function and is involved in diverse genomic processes such as gene regulation, chromosomal stability, and parental imprinting. Waxed paraffin sections of dental papilla from human embryos were stained with 1% methylene blue and manually microdissected. Microdissected material was dewaxed with xylene and the DNA extracted with ammonium acetate. DNA was digested with restriction enzyme methylation-sensitive *HpaII* and *HaeIII* nucleases. A 230 base pair (bp) fragment from human PAX9 gene was PCR amplified. Restriction enzyme assay did not impair PCR amplification of PAX9 gene from human embryos. Through the protocol used for manual microdissection, PAX9 gene presented a methylation status in dental papilla of human embryos.

Key words: Human Fetuses, methylation, PAX9, restriction enzymes.

Introduction

Methylation is indispensable for vertebrate genome function and is involved in diverse genomic processes such as gene regulation, chromosomal stability, and parental imprinting (Bird 2002). Interest in the function of DNA methylation is further heightened by the various human diseases associated with epigenetic dysfunction, a notable example being cancer (Laird 2003). Aberrant hypermethylation of CpG islands presented in the promoters of genes is associated with gene silencing. Epigenetic modifications by DNA methylation are catalyzed by the enzyme DNA methyltransferase and results in the conversion of the cytosine to 5-methylcytosine (Teodoridis et al. 2004). Methylation of cytosine in CpG dinucleotides promotes transcriptional repression in mammals by blocking transcriptional factor binding and recruiting methyl-binding proteins, leading to the formation of a repressive chromatin structure, thus making DNA inaccessible to transcriptional factors (Ordway and Curran 2002). DNA methylation is carried out by DNA methyltransferases (DNMTs), which transfer a methyl group from S-Adenosyl methionine SAM to the 5′ position of cytosine residues, often within CpG dinucleotides (Klose and Bird 2006). There are two classes of DNMTs: (1) the de novo DNMT3a and DNMT3b, which are able to modify unmethylated DNA and (2) the maintenance DNMT DNMT1, which prefers to methylate hemimethylated DNA substrates, although it can also modify unmethylated substrates (Ting et al. 2006). DNMT1 is believed to be a component of the replication fork, where it may play a key role in propagating patterns of DNA methylation through multiple cell divisions (Rountree et al. 2000, Le Gac et al. 2006). The de novo enzymes function early in embryonic development to establish domains of methylation. Because these enzymes both establish and propagate methylation states that correlate with transcriptional activity, they represent factors that control stable epigenetic

transcriptional memory during development. Unlike restriction enzymes or DNA-binding proteins, the frequency of MTase target sites (usually CpG or GpC dinucleotides) is very high in most sequences. DNA methylation within the promoter region of human genes has been previously reported and it seems to be associated with transcriptional activity. Also, a recent study revealed the presence of high DNA methylation rates within the promoter region of PAX1 gene, a PAX9 homologue (Burden et al. 2005, Young et al. 2009). However, the DNA methylation profile of the human genome is still largely a mystery.

The study of methylation in human fetal tissue is hindered by the fact that methylation pattern is tissue specific and it requires the isolation of cells in tiny developing organs. Methylation analysis has been studied by PCR amplification of DNA from microdissected frozen or formalin fixed and paraffin embedded tissue samples. Although, laser capture microdissection has been increasingly used, manual microdissection techniques are still applicated (Moskaluk and Kern 1997, Huang et al. 2005). In most cases manual microdissection involves the direct staining of tissue sections. Staining of tissue sections, however, can decrease the yield of PCR amplification products (Serth et al. 2000, Huang et al. 2005). Huang and co-workers (2005) found that if the ethanol fixed tissue sections were stained with H&E and stored at room temperature, DNA was gradually degraded over time. Additionally, in our experience some tissues tend to powder or fragment during microdissection when scraped with the needle, which may cause some delay in collecting the material.

The aim of this study was use an improved protocol for manual microdissection of paraffin embedded tissue sections to obtain DNA from human fetal dental papilla. The DNA was used to investigate the methylation pattern of human PAX9 promoter gene.

Materials and methods

DNA was extracted from 4 maxillary dental papilla from human fetuses with ages (intra-uterine life) of 10, 11, and 12 weeks old (fixed with absolute ethanol) and 16 weeks old (formalin-fixed). Specimens were obtained from the Museum of Anatomy of the Faculty of Dentistry of Piracicaba – State University of Campinas, São Paulo, Brazil. Ethical approval for the study was granted by Piracicaba Dental School/Campinas University Ethical Committee for human Research. Before embedded in paraffin wax the specimen were decalcified in 5% EDTA for 7 days, dehydrated with ethanol and diaphanized in xylene. Fifteen — 15µm slices of about five hundred (500) paraffin wax embedded tissue sections were placed on glass slides and stained with 1% methylene blue. Before dewaxing dental papilla were microdissected with a sterile 22-gauge needle under a microscope. Microdissected material was collected in 2ml micro-tube and 1 ml of pre-heated xylene was added to each micro-tube and kept for 10 min at 65°C. The tubes were then centrifuged at 13620*g* for 5 minutes and the supernatant was discarded. This procedure was repeated 5 times to assure that no paraffin residues remained. The pellet was washed in a descending series of ethanol (absolute, 95%, 75%). Every change was preceded by homogenization and centrifugation at 13620*g* for 5 min. The pellet was diluted in 50µl Mili-Q water and washed in 1ml of TNE [17 mM Tris/HCl (pH 8.0), 50 mM NaCl and 7 mM EDTA]. The tubes were centrifuged at 17000*g* for 5 min. A volume of 1.3ml of lysis solution[10mM Tris-HCl (pH 8.0), 0.5% SDS, 5mM EDTA] and 10µl of proteinase K (Sigma) Chemical Co., St.Louis, MO, USA)(20mg/ml) was added in pellet and vortexed for 5 seconds, followed by an overnight incubation at 55°C. After incubation, 500µl of a solution containing 8M of ammonium acetate and 1 mM EDTA was added, followed by centrifugation at 17000*g* for 10 min. Nine hundred -900µl of supernatant were carefully

poured into two clean micro-tubes containing 540µl of isopropanol (2-propanol). The solutions were mixed by gently inverting the tubes 20 times and centrifuging at 17000*g* for 5 min. The supernatant was poured out, and 1 ml of 70% ethanol was added in pellet and centrifuged at 17000*g* for 5 min. The pellet was dried at 50°C for 15 min. The DNA was resuspended in 30µl of TE buffer [10 mM Tris-HCl (pH 7.8) and 1 mM EDTA].

Following DNA extractions, methylation status of PAX9 promoter region was checked through digestion with *HpaII* (5'-CCGG) and *HaeIII* (5'-GGCC) methylation-sensitive restriction enzymes. As a positive control, DNA extracted from buccal epithelial cells (Aidar and Line 2007) was methylated at all CpGs by using 4 units of CpG methyltransferase (M.SssI) following manufacturer's recommendation (New England Biolabs®) per 1µg of genomic DNA. The restricted DNA was amplified by PCR using specific primers CCCACCTATAGCCTTAACTT (forward), CTCTTTCAGGCTAGCTCCCC (reverse) on a Perkin-Elmer Thermocycler as follows: 1 cycle at 94^0C for 4 min for initial denaturation, 35 cycles at 94^0C- 1 min for denaturation, 55^0C - 1 min for annealing and 72^0C -1 min for extension, and 1 cycle at 72^0C- 7 min for final extension.

Results

Figure 1 shows a micrograph of methylene blue stained unwaxed section before (a) and after (b) microdissection. Note that dental papilla is clearly observed. Restriction analysis did not impair amplification PAX9 gene promoter region of any DNA extracted from human dental papilla (Figure 2), producing a fragment with the expected size of 230 bp, which is indicative of the presence of methylated DNA.

Figures

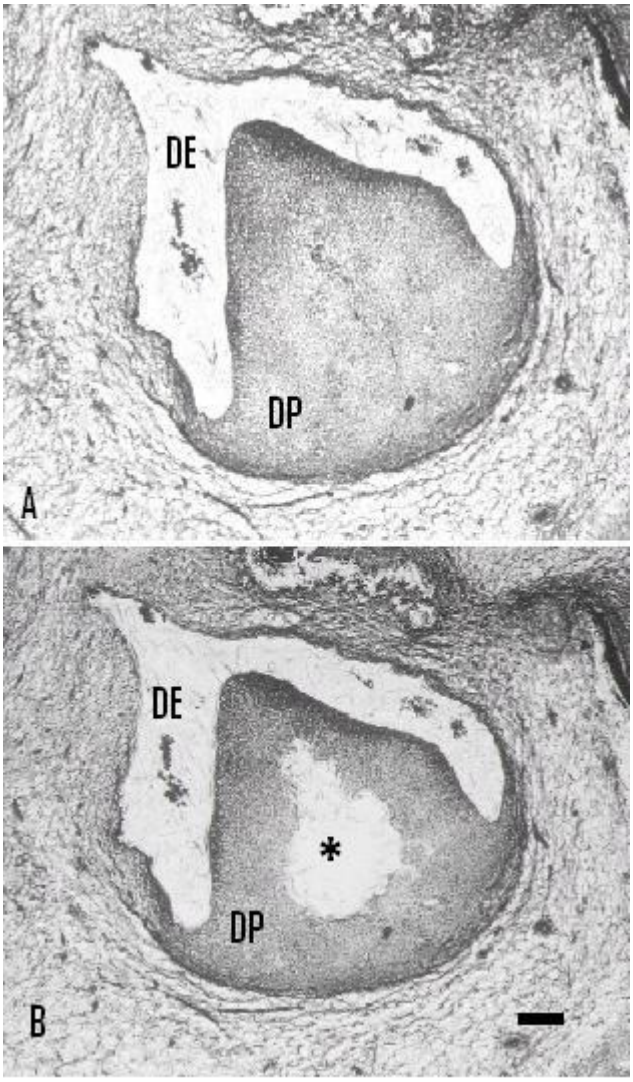

Figure 1. Methylene blue staining of a waxed section showing a tooth germ at the cap stage. **A** Before microdissection. **B**. After microdissection. The microdissected area is evidenced by *. DE = dental epithelium. DP = dental papilla. Barr = 100 um.

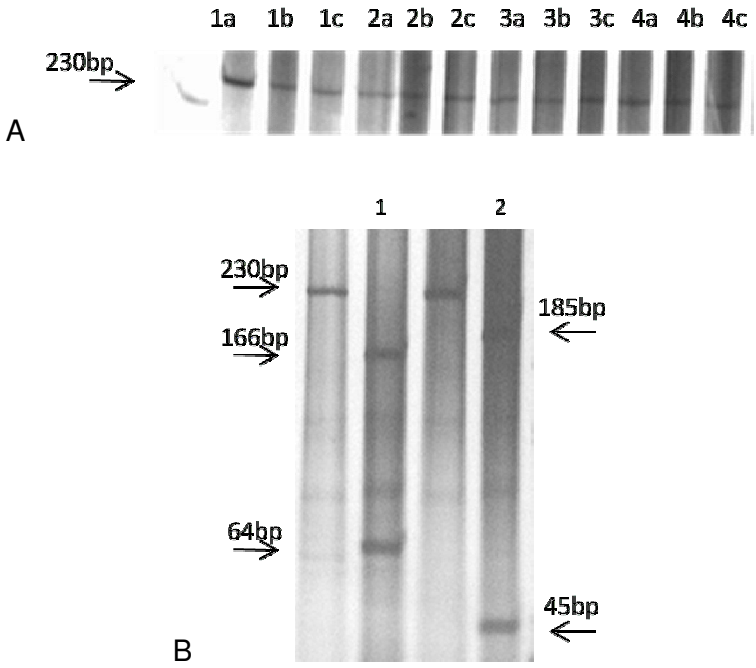

Figure 2. **A**. Methylation accessed by *HpaII* and *HaeIII* restriction enzymes and PCR amplification of genomic DNA from microdissected human Dental Papilla:1 -embryo 10 weeks; 2 -embryo 12 weeks; 3 -embryo 11 weeks; 4 -embryo 4 months old. Enzymatic reactions and controls (no enzymes) a: 3dwater; b:*HpaII*; c:*HaeIII*. Ladder 100bp (Promega™) **B**. Control reactions performed with *HaeIII* (1) and *HpaII* (2) and using PCR products of DNA extracted from buccal epithelial cells.

Discussion

PAX9 is a transcription factor that is expressed in dental mesenchyme at initiation, bud, cap and bell stages of odontogenesis (Vainio et al. 1993, Peters et al. 1998). Protein products of this gene serve as transcription factors that are responsible for the crosstalk between epithelial and mesenchymal tissues and are essential for the establishment of the odontogenic potential of the mesenchyme (Jernvall and Thesleff 2000). The expression of PAX9 in the mesenchyme appears to be a marker for the sites of tooth formation as it occurs before any morphological manifestation of this process (Neubuser et al. 1997). Pax9-deficient mice were shown to lack teeth and pharyngeal pouches derivates and have severe craniofacial anomalies (Peters et al. 1998).

In this study, PAX9 methylation pattern was examined by analysis of its promoter sequence in human embryos paraffin-embedded microdissections through restriction enzyme methylation-sensitive. In this analysis, restriction enzyme cleavage is blocked when the DNA recognition sequence is methylated, so amplification products are only detected when digestion is inhibited by methylation. A major limitation of this technique is the incomplete digestion of DNA samples due to insufficient exposure to the restriction endonuclease, which may result in false-positives. We have taken preventive measures to avoid such drawbacks, the use of two restriction enzymes and the use of extended (overnight) reaction times. Comparative analysis showed that the *HpaII* site located at -157 is located at a poorly conserved area while the GGCC sequence cut by *HaeIII* is located at position -139 is conserved only among primates (http://genome.ucsc.edu/).

We believe that the present protocol has two main improvements over previous manual microdissection procedures. The staining directly on waxed section preserves the material from air exposure and prevents DNA degradation. Methylene blue is routinely used for staining of semi-thin sections of tissues embedded in hydrophobic resins

(Gevondyan and Avtandilov 1982), and here we shown that it can also be used to stain tissue sections embedded in paraffin. Although the methylene blue staining is limited only to the surface of the section it is sufficient to distinguish the tissues and organs present in the material been studied. Additionaly, the microdissection in waxed sections avoids the fragmentation of the material. In our experience, fragmentation, and in some cases powdering, is frequently observed in sections of paraffin embedded material that were dewaxed with xylene. The scrapping of waxed sections usually produces a single mass containing paraffin and the organic material that that adheres to the needle and is easily collected.

The protocol used for DNA extraction is based on salting out the proteins with 8M ammonium acetate solution. Ammonium acetate precipitation of proteins has been used for DNA purification of seeds (Krishna and Jawali 1997), bacteria, protozoarium, and white blood cells (Ikuta et al. 1992), buccal swabs (Walker et al. 1999) and formalin-fixed paraffin-embedded tissue sections (Santos et al. 2008). This reagent has also been used in commercially available Kits (Feigelson et al. 2001, Garcia-Closas et al. 2001, King et al. 2002). Besides simple and cost effective the procedure described here avoids the use of toxic organic solvents as phenol and chloroform that are commonly used in the extraction of DNA.

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Capítulo 2

Artigo em vias de submissão

Title: Transcriptional activity analysis of human PAX9 gene under Retinoic Acid and Dexamethasone regulation *in vitro*

Running Title: Transcriptional activity of PAX9 gene

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Abstract

Objective: PAX9 is a key regulator during tooth development and plays an essential role in the patterning of murine and human dentition. The objectives of this study were to gain new insights into the transcriptional activity within the promoter region and transcripts of human PAX9 gene *in vitro*.

Methods: Pax9 encoding mRNA from Rat embryonic cell culture and from Mouse odontoblast cell–like culture (MDPC-23) were amplified by reverse transcriptase semi-quantitative PCR. Transcriptional activities of PAX9 gene promoter constructs were assessed by dual luciferase assay in transfected Rat embryonic cell culture. Cell cultures were submitted to selective regulation by Retinoic Acid (RA) and Dexamethasone (Dex) drugs.

Results: Reverse Transcriptase PCR analysis revealed higher transcriptional levels for Dex treated cell group comparatively to RA treated group. *ApaI*-digested pGL3Basic-PAX9 promoter construct was not activated by RA.

Conclusions: Dex and RA steroid hormone receptors may bind directly to DNA sequences of murine PAX9 gene promoter. In addition, we suggest that a 513bp region within PAX9 promoter may contain binding sites for RA receptors. The observed transcriptional activity may give rise to new perspectives on PAX9 expression.

Key words: PAX9, transfection, semi-quantitative PCR, human.

Introduction

PAX genes encode transcription factors with nine members in mammals, characterized by a DNA-binding domain called "paired domain"[1]. In mouse embryos, Pax9 is an early marker of tooth development, appearing in odontogenic mesenchyme before ectodermal thickening and prior to the expression of other tooth signaling genes. Besides, PAX9 is expressed in dental papilla at cap stage and is required for the mesenchymal expression of Bmp4, Msx1 and Lef1, suggesting that its function is essential to establish the inductive capacity of this tissue[2]. In humans, mutations in PAX9 are associated with unique phenotypes of familial tooth agenesis that mainly involve posterior teeth [3]. Mouse molar teeth is frequently used as a model system to study the molecular regulation in vertebrate odontogenesis[4,5]. It has been demonstrated that in Pax9-deficient mouse embryos, odontogenesis is arrested at the bud stage. These animals also exhibit secondary cleft palate and many other craniofacial abnormalities [6].

All-*trans* retinoic acid (ATRA) is found in cells during embryological development of many organs. Retinoids, particularly ATRA, are also potent regulators of cell proliferation and do so, in part, by promoting cell differentiation[7,8,9]. Retinoids regulate gene transcription by binding to and activating nuclear transcription factors. Dexamethasone is the steroid analogue to ATRA. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of transcripts. A common question is the quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions. While *in vivo* animal models provide valuable insights to overall understanding of transcription factors genes, cell culture-based models are useful for dissecting the various molecular signalling pathways under different conditions. In typical transfection assays, eukaryotic vectors for

expression of transcription factors are introduced into the cell along with a promoter construct driving expression of a reporter gene.

The aim of this study was to investigate human PAX9 promoter constructs as well as Pax9 transcripts behavior under Retinoic Acid (RA) and Dexamethasone (Dex) regulation.

Materials and Methods

Cells and reagents- Mouse odontoblast-like cells – MDPC-23 were the generous gift from Dr.Alexandre Zaia (Endodontics Department, Piracicaba Dental School, SP, Brazil). After acquiring Animal Ethics Committee approval, embryonic cell lines were collected from Wistar Rats at 13 day-old. RA and Dex were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions for RA and Dex were prepared in absolute ethanol at 1mM and the final ethanol concentration in all cultures did not exceed 0.1% vol/vol. The cells were plated in the presence of 1μM, 0.1μM and 1nM concentrations of each drug. Experiments were performed in duplicate. Unless otherwise indicated cells were cultured in standard medium containing either vehicle or Dex/RA.

RT-PCR- Rat embryonic cells and Mouse odontoblast-like cells-MDPC-23 were plated and established in p35 tissue culture plates and grown in DMEM containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37^0C in the presence of 5% CO_2. Cells were incubated with either vehicle or Dex/RA at 1μM, 0.1μM and 1nM concentrations in duplicate for each cell line for 24h. Cells were homogenized and RNA were isolated with TRIzol™, as per manufacturer's instructions (Invitrogen) and purified samples quantitated at OD260 using a spectrophotometer. The isolated mRNA was converted into cDNA using Superscript III following manufacturer's instructions (Invitrogen). As with any PCR reaction, initial optimization of the thermal-cycling parameters was carried out. In two independent experiments, PCR amplifications were performed using gene-specific primers for Pax9/β-actin genes. Primer sequences and optimal PCR annealing temperatures (at) are listed in Table. To ensure that no false positive PCR fragments would be generated from pseudogenes in contaminating genomic

DNA, primer sequences were designed to span intron regions. Polymerase chain reactions were performed on a TC-512 PCR machine (Techne Incorporated Burlington, NJ, USA) using ~1 µg of cDNA, 5 pmoles of each oligonuclotides primers, GoTaq® Green Master (Promega) in a 25 µl volume. The PCR program initially started with a 94°C denaturation for 4 min, followed by 5 to 40 cycles of 94°C/1 min, at°C /1 min, 72°C/1 min and 10 sec with a 72°C for final extension for all set of RT-PCR primers. β-Actin gene amplifications were performed in separated tubes. The gels were stained with ethidium bromide [10 µg/ml] and photographed on top of a 280 nm UV light box. The gel images were digitally captured with a CCD camera and analyzed with the NIH Image J program[10] .

Construction of Mammalian Expression Plasmids pGL3-Basic-PAX9 *and pGL3-Basic*-PAX9 *obtained from ApaI restriction digest* — The PAX9 promoter region between positions – 1,106 and +92 was amplified by PCR (Table) and subcloned into *SacI-HindIII* sites of TOPO TA vector (Invitrogen) to generate high copies for cloning into pGL3Basic vector. The following mammalian expression plasmids were constructed for use in transfection assays: *pGL3-Basic*-PAX9 *promoter, pGL3-Basic*-PAX9 *promoter* obtained from *ApaI* restriction digest - pGL3 Basic-PAX9 promoter lacking 513bp with the firefly luciferase gene as the reporter. The pRL-TK vector (Promega) contains the reporter *Renilla* luciferase gene which was used to correct for variations in transfection efficiency.

Transient Transfection—Rat embryonic cells for primary culture were obtained from Wistar rats at 13 day-old following a previously described primary culture protocol [11]. The day of detection of a vaginal plug was noted as the day 0. At day 13, pregnant female was killed by cervical dislocation and had the uterus asseptically removed out into a 25ml screw-capped tube containing 20ml Phosphate-Buffered Saline (PBS) plus

penicillin/streptomycin. Embryos was dissected out the uteri in a flow hood area and transferred to a fresh dish of sterile PBS. Disaggregation was achieved by physical disruption cutting the tissue into pieces. Dissected embryos were transferred to a new p100 dish containing DMEM, 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37^0C in the presence of 5% CO_2. After culture establishment cells were transferred to 24-well tissue culture plate (Corning Glass) and incubated with either vehicle or Dex/RA at 1µM, 0.1µM and 1nM concentrations. Transient transfections were performed when 90-95% of confluence was reached through lipofection by using Lipofectamine 2000™ reagent (Invitrogen) in the presence of Reduced Serum Medium according to the manufacturer's instructions. The total amount of 0.8 µg of total plasmid (0.65 µg of the appropriate expression plasmid and 0.15µg of the pRL-TK vector that was always co-transfected for normalization (Promega™)) was used. Transfected cells were incubated at 37°C in the presence of 5% CO_2 for 24h.

Luciferase Assay— Twenty-four hours after transfection, cell extracts were collected and firefly and *Renilla* luciferase activities were measured (dual luciferase reporter assay; Promega™). The cells were lysed and reacted with the luciferase assay substrate for 1 hour. 100 µl of each sample were quantitated using a 96-well microplate reading luminometer (Veritas™- Turner Biosystems). The firefly luciferase activity measured by relative luciferase units in each sample was normalized to *Renilla* luciferase absorbance to correct for variations in transfection efficiency. Experiments were performed in duplicate.

Table. PCR primers used for cloning and RT–PCR

Name	Sequence (5'-3')	Product size (bp)
PAX9 human F (a)	CTCGAGGTGTAGACCGCAGC	1198
PAX9 human R (a)	ACCGACCCGCACCAATCACCATGCAGCTT(*HindIII*)	
PAX9 human F (b)	AGTTTCTGTCTGGGAGTGCG	336[a]
PAX9 human R (b)	GCTTGTAGGTCCGGATGTGT	
Primer actin-b R (mouse)	GGTGTAAAACGCAGCTCAGTA	197[b]
Primer actin-b F (mouse)	CTCCTAGCACCATGAAGATCA	
Pax9 Rat F	GAGTTCCATCAGCCGGATTC	391[c]
Pax9 Rat R	CAAGGCTCCCTTCTCCAATC	
Primer actin-b F (rat)	TGACATCCGTAAAGACCTCT	210[d]
Primer actin-b R (rat)	AGATGTGATCAGCAAGCAG	

Restriction site introduced for cloning purposes is underlined.

[a]at= 57^{0}C, 29 amplification cycles.

[b]at= 55^{0}C, 19 amplification cycles.

[c]at= 56^{0}C, 29 amplification cycles.

[d]at= 56^{0}C, 30 amplification cycles.

Results

Dex stimulated Pax9 transcripts in Primary Rat embryonic cells and MDPC-23 cells, whilst RA had an inhibitory effect in rat embryonic cell line (Figure 1). pGL3Basic-PAX9 promoter (*ApaI* digest) construct was not activated by RA. Conversely, pGL3Basic-PAX9 promoter was greatly influenced under the same treatment (Figure 2).

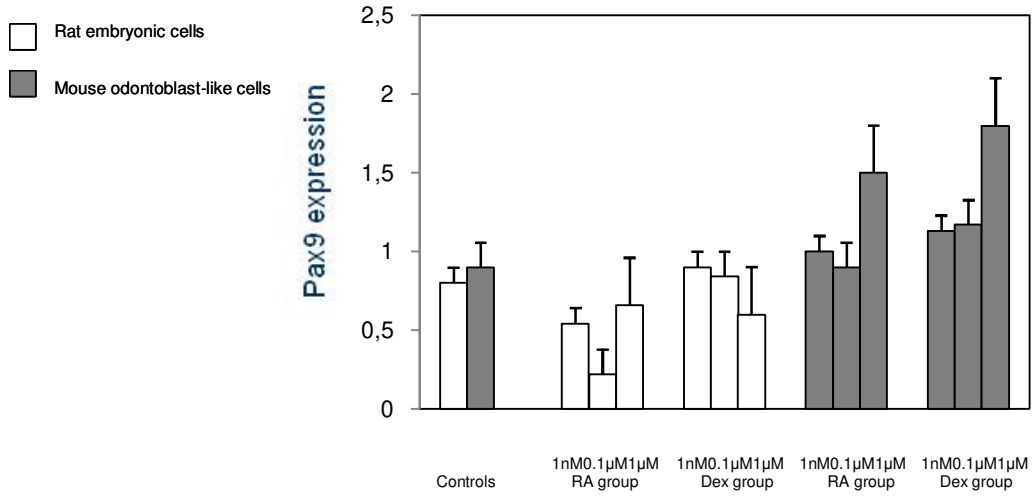

Figure 1 .Dexamethasone increases Pax9 expression in rat (white bars) and MDPC-23 (gray bars) cells as detected by semi quantitative PCR. Gene transcript quantity was measured by using as standard β-actin signal as the denominator as described in the materials and methods section. Error bars represent standard error. Controls: ethanol alone.

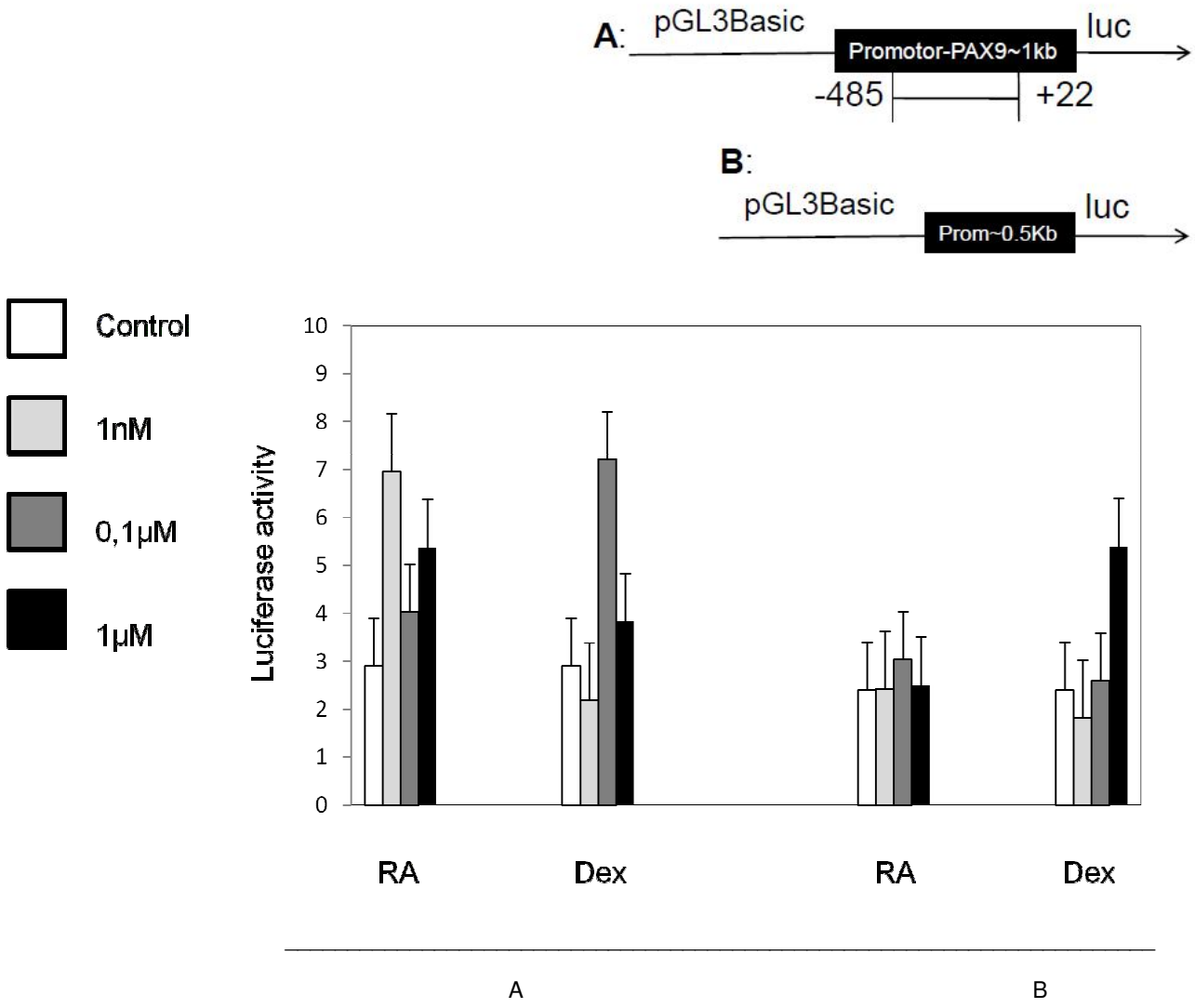

Figure 2. Luciferase activities of pGL3Basic-PAX9 promoter (A) and pGL3Basic-PAX9 promoter (*ApaI* digest) (B) reporter constructs for transfection assays performed in Rat Embryonic cell line. RA, Retinoic Acid, Dex, Dexamethasone, respectively. pGL3 Basic-PAX9 promoter (*ApaI* digest) construct was not activated by RA. Error bars represent standard error. Controls: ethanol alone.

Discussion

In our experiments semi-quantitative RT-PCR was performed to measure gene expression of Pax9 *in vitro* by using Mouse odontoblast cell-like- MDPC-23 and Rat Embryonic cell in culture under selective regulation of RA and Dex. In transcriptional studies of collagen type II gene, Dex and RA inhibited the collagen type II promoter-enhancer-mediated chloramphenical acetyltransferase (CAT) activity[12]. In the present study, greater concentrations of RA activated pGL3Basic-PAX9 promoter in comparison with ethanol alone group. Instead, RA did not altered pGL3 Basic-PAX9 promoter (*ApaI digest*) behavior suggesting that a 513bp deletion or -644 to -137 of this construct may contain binding sites for RA receptors. Also, PAX9 is required for secondary palate development and certain teratogens such as retinoic acid have been identified as inducers of a cleft secondary palate[13,14]. Dex increased luciferase relative unit levels for both constructs with the maximum concentration 1μM. As detected by semiquantitative PCR cells, Dex treated cells group presented an overall increasing of PAX9 transcripts comparatively to AR treated cells group. The same result was achieved in Pigment epithelial-derived factor (PEDF) assays which demonstrated greater than a four fold increase in PEDF expression after treatment with 1 μM dexamethasone for 24 h in transfected cells[15].

Both dexamethasone and retinoic acid directly bind to PAX9 gene promoter and upregulate Pax9 transcriptional activity, although the former had a greater effect in similar concentrations. A 513bp region (-644 to -137) within PAX9 promoter may harbor binding sites for dexamethasone and retinoic acid. Taken together, our results provide new molecular insights into the PAX9 promoter transcriptional activities *in vivo* .

Acknowledgments

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Conclusão

Os resultados obtidos nesta pesquisa mostram que o promotor a região que corresponde a 513 pares de bases (-644 até -137) na região promotora do PAX9, contém sítios de ligação para receptores do Ácido Retinóico. Mostram também que o gene Pax9 presente nas células de camundongo, em cultura, foi mais responsivo à ação das drogas, Ácido Retinóico e Dexametasona. Por fim, o sítio metilado encontrado na região promotora fornece um dado novo para o entendimento da função do promotor no processo normal de diferenciação celular.

A análise do padrão de metilação de células da papila dentária, mostrou que as sequências analisadas no promotor do gene PAX9 apresentavam-se metiladas, sugerindo que a metilação nestas sequências não interfere com a atividade transcricional.

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APÊNDICE: **Artigo publicado no periódico Pathology Ressearch and Practice em 21 de Abril de 2008. Classificação Qualis A Internacional. ISSN 0344-0338**

Full Title: Extraction of genomic DNA from paraffin embedded tissue sections of human fetuses fixed and stored in formalin for long periods

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Abstract

The advent of PCR technology has increased the interest in fetal specimens housed in anatomy museums, as they may represent a unique source of genetic material for the study of uncommon or rare pathological conditions such as congenital malformations, neoplastic processes and parasitic as well as other infectious diseases. The aim of this study is to evaluate the quality of genomic DNA extracted from paraffin-embedded tissue sections of human fetuses that have been maintained in formalin for several years. Fetal tissues were paraffin embedded and tissue sections were submitted to ethanol/xylene dewaxing, followed by DNA extraction with ammonium acetate. DNA fragments were amplified from DNA extracted from formalin-fixed tissue sections but not from Bouin-fixed tissues (average yield of 13.7 µg/ml from ten umbilical cord sections of 10 µm; A_{260}:A_{280} =1.55). The addition of bovine serum albumim increased the yield of PCR amplification. Genomic DNA can be reliably amplified from paraffin-embedded human fetal tissues that had been fixed in formalin during 19 years and used for microdissection studies. This simple, cost-effective and non-laborious method should facilitate the molecular analysis of a large number of specimens fixed for long periods of time.

Key words: Paraffin-embedded; Formalin-fixed; DNA extraction; microdissection; museum specimens

Introduction

The advent of PCR technology has increased the interest in fetal/neonatal specimens housed in anatomy museums, as they may represent a unique source of genetic material for the study of uncommon or rare pathological conditions that may include congenital malformations, neoplastic processes and parasitic as well as other infectious diseases [1]. Obtaining DNA from museum specimens is not always possible, since they are usually maintained in preservative liquids that were not meant for preserving DNA. Despite of the difficulties several papers have already described successful DNA extraction from tissues maintained for long periods in formalin and ethanol [2-4].

The PCR amplification of microdissected formalin fixed and paraffin embedded tissue samples allows the molecular analysis of pure groups of cells. This technique has been mostly used in the quantitation of differential gene expression between normal and abnormal tissues. This procedure, however, may also be used for the analysis of DNA. Detection of mutations and changes in DNA methylation pattern in tumors [5, 6], detection of infectious agents on a specific tissue, separation of chorionic villi from endometrial mucosa for paternity testing [7], and isolation of sperm in postcoital slides [8] are among the examples where the analysis of DNA extracted from of microdissected cells where required. Although, laser capture microdissection has been increasingly of value, manual microdissection techniques are still used [9, 10].

Obtaining good quality polymerase chain reaction (PCR) products from DNA extracted from fixed, paraffin wax embedded tissue is not always an easy task because, in general, this material is scarce, and often contains remnants of substances that inhibit the amplification reaction, such as formalin [11] or inhibit proteinase K used in the extraction

procedure, such as xylene [10]. Additionally, fetal tissues frequently present some degree of autolysis [12].

In this study we describe a simple and inexpensive protocol to obtain genomic DNA from paraffin slides of fetal specimens that have been maintained in formalin during 19 years.

Materials and methods

DNA was extracted from 10 umbilical cords and 4 maxillas derived from human fetuses, with ages ranging from 4 to 8 months. Specimens were obtained from the Museum of Anatomy of the Faculty of Dentistry of Piracicaba – State University of Campinas, São Paulo, Brazil. The specimens had been fixed in formalin (12 specimens) or Bouin's (2 specimens) solution during 19 years (since 1988). Umbilical cords were dehydrated with ethanol and diaphanized in xylene, before embedded in paraffin wax. The maxillas were decalcified in 5% EDTA for 7 days (EDTA solution was changed daily) before ethanol dehydration.

Procedure

Five 10 µm slices of paraffin wax embedded tissue sections were collected in 1.5 ml micro-tube and 1 ml of pre-heated xylene was added to each micro-tube and kept for 10 minutes at 65°C. The tubes were then centrifuged at 13620g for 5 minutes and the supernatant was discarded. This procedure was repeated 5 times to assure that no paraffin residues remained. The pellet was washed in a descending series of ethanol (absolute, 95%, 75%). Every change was preceded by homogenization and centrifugation at 13620g for 5 min. The pellet was diluted in 50 µl Mili-Q water and washed in 1 ml of TNE [17 mM Tris/HCl (pH 8.0), 50 mM NaCl and 7 mM EDTA]. The tubes were centrifuged

at 17000 g for 5 min. A volume of 1.3 ml of lysis solution [10mM Tris-HCl (pH 8.0), 0.5% SDS, 5mM EDTA] and 10 µl of proteinase K (Sigma Chemical Co., St.Louis, MO, USA)(20mg/ml) was added in pellet and vortexed for 5 seconds, followed by an overnight incubation at 55°C. After incubation, 500 µl of a solution containing 8 M of ammonium acetate and 1 mM EDTA was added, followed by centrifugation at 17000 *g* for 10 min. Nine hundred µl of supernatant were carefully poured into two clean micro-tubes containing 540 µl of isopropanol (2-propanol). The solutions were mixed by gently inverting the tubes 20 times and centrifuging at 17000 *g* for 5 min. The supernatant was poured out, and 1 ml of 70% ethanol was added in pellet and centrifuged at 17000 *g* for 5 min. The pellet was dried at 50°C for 15 min. The DNA was re-suspended in 30 µl of TE buffer [10 mM Tris-HCl (pH 7.8) and 1 mM EDTA].

Tissue sections of fetal maxillas were placed on glass slides and stained with hematoxylin. Before dewaxing dental papillae were microdissected with a sterile 22-gauge needle under a microscope.

The amplification of fragments of 170 bp from human MMP20 and 362 bp from human PAX9 genes was carried out using PCR containing 5 µl of DNA (mean of 100 ng/ml), in a volume of 50 µl in a reaction mixture containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 4 mM of $MgCl_2$, deoxyribonucleotides (200uM each), 1 µM of each primers, 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and 0.5 µg/µl bovine serum albumim. Two pairs of primers were used (Table 1). Amplification was carried out in a *Perkin-Elmer GeneAmp 2400 thermal cycler* with 35 amplification cycles at annealing temperatures of 58°C. Amplification products were visualized by electrophoresis on vertical 10% poliacrylamide gels in 1 X TBE (89 mM Tris-Borate, 89 mM boric acid, 2mM EDTA), followed by silver staining [13].

Results

The 170 and 362 bp fragments were amplified from DNA extracted from formalin fixed tissue sections but not from Bouin fixed section (Fig 1). The extractions produced DNA suitable for the PCR (average 13.7 µg/ml from ten umbilical cord sections of 10 µm; A_{260}:A_{280} =1.55,). The addition of bovine serum albumim increased the yield of PCR amplification with the MMP20 primers. The amplifications with the PAX9 gene primers were only positive when bovine serum albumim was used.

Table 1. Primers sequences. F=forward, R=reverse

Fragment size	Primer sequences (5'-3')
170 bp	F: AAGTGCAAACGTGCACTGTC
	R: GGTTTTCTAGGGCAGAGGAG
362 bp	F: GGCATGGACTGAAGTGAGGTA
	R: CTCTTTCAGGCTAGCTCCCC

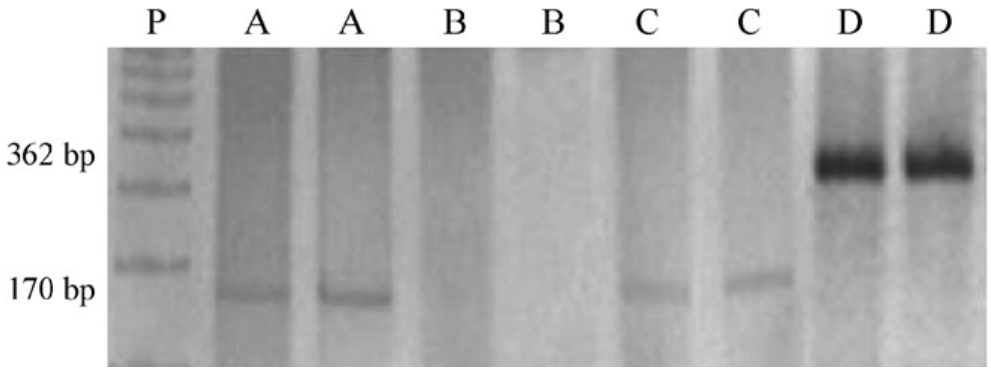

Fig. 1. Polyacrylamide gel electrophoresis showing PCR products of DNA extracted from 8 distinct samples: P- Molecular marker standard (100 bp ladder), A- buccal epithelial cells, B- Bouin's solution fixed tissues, C- formalin fixed tissues (fragment of 170 bp), D- microdissection of dental papilla from two formalin fixed specimens (fragments of 362 bp).

Discussion

The procedures for DNA extraction described in this paper avoid the use of organic solvents. This is achieved by salting out the cellular proteins with 8 M ammonium acetate solution. Ammonium acetate precipitation of proteins has been used for DNA purification of seeds [14], bacteria, protozoarium, and white blood cells [15], buccal swabs [16] and formalin-fixed paraffin-embedded tissue sections [17]. This reagent has also been used in commercially available Kits [18-20].

Over the years, different fixatives have been tested for molecular studies on histological sections. Success or failure of DNA/RNA extraction from archival tissue mainly depends on the fixing procedure. Ethanol was reported to be a good fixative for PCR analysis because it does not cause extensive cross-linking. Ethanol, however, is not commonly used in museum specimens, since it causes tissue dehydration and a consequent shrinkage of the specimens [21]. Bouin's solution contains picric acid, which causes DNA depurination. Neither DNA nor RNA could successfully be extracted from

highly cross-linking fixative such as glutaraldehyde, or modified formalins containing picric acid [22]. In contrast, Gloghini et al., (2004) [23] showed that high-quality RNA can be extracted from Bouin-fixed and paraffin embedded tissues. However, the short time of fixation (4 to 5 hours) can explain this discrepance. Rogers et al., (1990) [24] reported that PCR amplification decreases with fixing time. Our results provide an advance in this area by showing that DNA can be reliably amplified from paraffin embedded fetal tissues that had been maintained in formalin during 19 years. This simple, cost-effective and non-laborious method should facilitate the molecular analysis of a large number of specimens fixed for long periods of time in retrospective studies.

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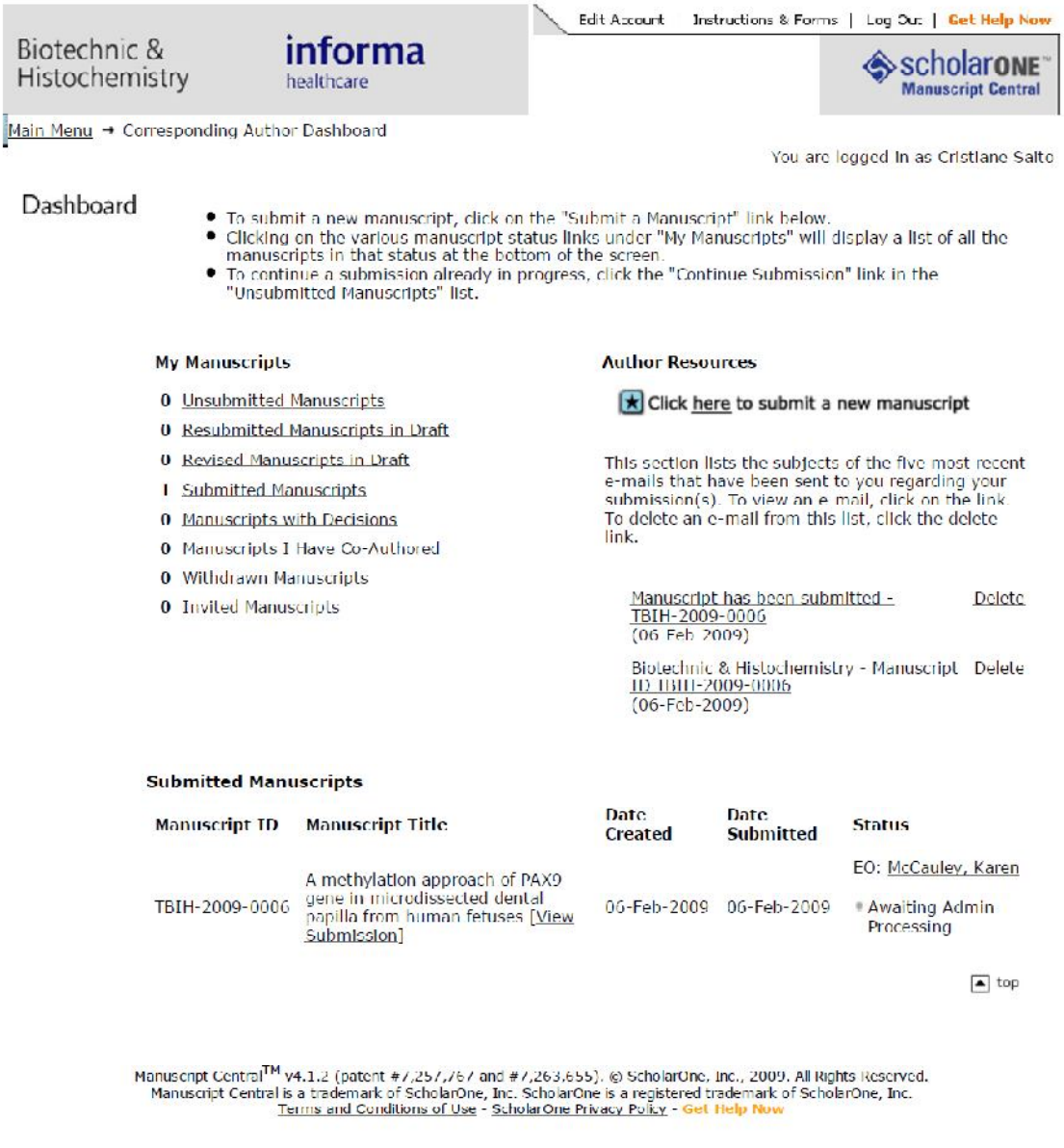
ANEXO 1: Documento comprovante de submissão do artigo inserido no capítulo 1



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ANEXO 2: Certificação do Comitê de Ética

COMITÊ DE ÉTICA EM PESQUISA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Defeitos na formação do órgão dental"**, protocolo nº 217/2004, dos pesquisadores **SERGIO ROBERTO PERES LINE, CRISTIANE PEREIRA BORGES SAITO e FÁBIO JOSÉ BIANCHI**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 16/02/2005.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project **"Defects in the formation of tooth organ"**, register number 217/2004, of **SERGIO ROBERTO PERES LINE, CRISTIANE PEREIRA BORGES SAITO and FÁBIO JOSÉ BIANCHI**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 16/02/2005.

Fernanda Klein Marcondes
p/ **Cinthia Pereira Machado Tabchoury**
Secretária
CEP/FOP/UNICAMP

Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

ANEXO 3: Declaração de não infração dos dispositivos da Lei n.º9.610/98

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Piracicaba, 03 de março de 2009.

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