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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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ANÁLISE DO EFEITO DA DEXAMETASONA, ÁCIDO RETINÓICO E ERGOCALCIFEROL NA ATIVIDADE TRANSCRICIONAL DA REGIÃO PROMOTORA DO GENE *PAX9* HUMANO

Tese apresentada à Faculdade de Odontologia, da Universidade Estadual de Campinas, para Obtenção do Título de Doutor em Biologia Buco-Dental, Área de Histologia e Embriologia.

Orientador: Prof. Dr. Sérgio Roberto Peres Line

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RESUMO

O gene *PAX9*, pertencente à família Pax, é amplamente expresso em vários tecidos craniofaciais durante o desenvolvimento. Sabe-se que mutações neste gene em humanos causam fenótipo de oligodontia, afetando os dentes molares e segundos pré-molares. Grande variedade de agentes fisiológicos e farmacológicos externos podem ter impacto relevante na regulação da atividade transcricional de genes modulando fatores de transcrição. A presente tese focaliza o estudo da região 5´do gene *PAX9* humano e tem como objetivo analisar a influência da dexametasona, ácido retinóico e ergocalciferol (vitamina D2) na atividade transcricional de sua região promotora, utilizando construções em vetor plasmideano que dirige a transcrição do gene da luciferase de vagalume (*Photinus pyralis*, pGL3 basic vector). Para ensaios de transcrição, foram amplificados através de ensaios com transcriptase reversa, transcritos do gene *PAX9* de células mamárias de adenocarcinoma MCF-7 e células odontoblastóides de camundongo MDPC23. Estes transcritos foram quantificados através de PCR quantitativo. Fragmentos da região promotora do gene *PAX9* humano de 1198pb (−1106 - +92), 843pb (−751- +92) e 691bp (−1106 - +92 com deleção de 507pb nos sítios -645 e -138) foram recombinados com vetor de expressão pGL3Basic e denominados *PAX9*-pGL3B1, *PAX9*-pGL3B2 e *PAX9*-pGL3B3, respectivamente. As contruções foram transfectadas em cultura de células mamárias de adenocarcinoma MCF-7 e células odontoblastóides de camundongo MDPC23. Todas as placas de cultura foram submetidas à ação de três drogas: dexametasona (DEX), ácido retinóico (RE) e ergocalciferol (VITD2). Após lise das células, os níveis relativos de expressão da proteína luciferase foram analisados com o uso do kit Dual-Glo Luciferase em luminômetro. Os resultados referentes às células mamárias de adenocarcinoma MCF-7 mostraram que: 1) Altas concentrações de ácido retinóico aumentaram a síntese de RNA mensageiro transcrito. 2) Fragmentos do promotor *PAX9* de 1198pb (*PAX9*-pGL3B1) e 843pb (*PAX9*-pGL3B2) foram ativados na presença de ácido retinóico mas suas transcrições desestimuladas na presença da dexametasona e ergocalciferol. 3) A atividade da luciferase na construção *PAX9*-pGL3B2 foi mais fraca que outras duas construções, indicando que a sequência -1106 and -751 ou 355pb era importante para a atividade transcricional. 4) Fragmento do promotor clivado nos sítios -645 e -138 com deleção de 507pb (*PAX9*-pGL3B3) foi ativado negativamente somente na presença do ergocaciferol,

enquanto que com a dexametasona e ácido retinóico o mesmo não foi afetado. Quanto às células odontoblastóides de camundongo MDPC23, os resultados mostraram que: 1) Todas as concentrações de ergocalciferol influenciaram positivamente a síntese de RNA mensageiro transcrito. 2) A atividade promotora das construções *PAX9*-pGL3B1 e *PAX9*-pGL3B2 foi aumentada com baixa concentração de dexametasona e ergocaciferol enquanto que alta concentração diminuiu esta atividade. 3) Na construção *PAX9*-pGL3B3, todas concentrações de ergocaciferol influenciaram a transcrição do promotor negativamente, enquanto que com a dexametasona e ácido retinóico, a mesma não foi afetada. Concluímos que as drogas dexametasona, ácido retinóico e ergocalciferol podem modular a expressão do gene *PAX9.* A região de 507pb deletada do promotor do gene *PAX9* humano pode conter sítios de ligação para receptores do ácido retinóico e dexametasona.

Palavras Chaves: Região promotora do gene *PAX9*, atividade de transfecção, ensaios de luciferase, pGL3 Basic Vector.

ABSTRACT

PAX9, member of the family homeobox, has important functions in embryogenesis and it is widely expressed in various craniofacial tissues during development. *PAX9* mutations in human families cause autosomal dominant oligodontia, characterized by the absence of permanent molars and pre-molars. A great variety of physiological or pharmacological environmental factors may have impact on downstream signaling cascades and transcriptional regulation of gene modulating transcription factors. This work focused on the analysis on the 5'-flanking region of the *PAX9* gene studying the influence of retinoic acid, dexamethasone and vitamin D on the expression of *PAX9* by expression constructs that carry the reporter gene luciferase (*Photinus pyralis*, pGL3 basic vector). In the present study, we have PCR amplified cDNAs encoding mouse *Pax9* from Mouse Odontoblast Cell-Like-23 (MDPC23) and *PAX9* from Human breast adenocarcinoma (MCF-7) and quantified by Quantitative PCR. We examined the transcriptional activity of human *PAX9* promoter from constructions: 1) *PAX9*-pGL3B1 construct clone *PAX9* gene promoter 1198bp from −1106 upstream to +92 downstream of translation start site (ATG). 2) *PAX9*-pGL3B2 construct clone *PAX9* gene promoter 843bp from −751 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG). 3) *PAX9*-pGLB3 construct clone *PAX9* gene promoter 691bp from −1106 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG) using deletion of 507bp in restriction sites (-645 and -138) of ApaI enzyme. These constructions were transfected into Mouse Odontoblast Cell-Like-23 (MDPC23) and *PAX9* from Human breast adenocarcinoma (MCF-7). Cell cultures were all submitted to selective regulation of tree drugs: dexamethasone (DEX), retinoic acid (RE) and ergocalciferol (VITD2). Relative luciferase expression units were obtained by dual luciferase assay kit. The results in Human breast adenocarcinoma (MCF-7) showed that retinoic acid and dexamethasone influenced negatively the expression of *PAX9* promoter. *PAX9*-pGL3B1 and *PAX9*-pGL3B2 promoter was inhibited under the treatment of dexamethasone and ergocalciferol. Retinoic acid and dexamethasone did not altered *PAX9*-pGL3B3 (−1106 to +92, 507bp deleted with ApaI digest) behavior. Luciferase activity in plasmid *PAX9*-pGL3B2 was always weaker than the other two constructions indicating that sequence present between -1106 and -751 or 355bp

were important for the transcriptional activity of *PAX9* promoter. The results in Mouse Odontoblast Cell-Like-23 (MDPC23) showed that it *PAX9*-pGL3B1 and *PAX9*-pGL3B2 promoter activity was increased by the treatment of lower concentration of dexamethasone and ergocalciferol, whereas higher concentration of the same drugs decreased this activity. The effect of the retinoic acid in the luciferase activity of *PAX9*-pGL3B1 has the same pattern but for the *PAX9*-pGL3B2, all concentrations increased the promoter activity. For the *PAX9*-pGL3B3 construction, concentrations of ergocalciferol had a statistically significance decreasing the activity of the promoter and no effect of the activity was observed in the dexamethasone and retinoic acid treatment. In conclusion, dexamethasone, retinoic acid and ergocalciferol may bind to *PAX9* gene promoter and up or down-regulate *PAX9* transcriptional activity. A 507bp region (-645 and -138) within *PAX9* promoter may harbor biding sites for dexamethasone and retinoic acid since none of concentrations of these reagents influenced changes in promoter activity.

Key words: *PAX9* promoter gene, transcriptional activity, luciferase assays, pGL3 Basic Vector.

SUMÁRIO

INTRODUÇÃO

Os genes da família homeobox são conhecidos por desempenhar papéis importantes nas interações indutivas dos tecidos, amplamente expresso em vários tecidos craniofaciais durante o desenvolvimento funcionando como fatores de transcrição durante a organogênese dos vertebrados (Vainio S. et al. 1993). O gene *PAX9*, o nono membro pertencente à família de fatores de transcrição *Pax*, participa como regulador da embriogênese e desenvolvimento dos vertebrados sendo amplamente expresso em vários tecidos craniofaciais durante o desenvolvimento e funciona como iniciadores da diferenciação celular e mantendo a pluripotência de células em desenvolvimento (Gruss P. et al. 1992). Acredita-se que o *Pax9* desempenhe também uma função essencial no desenvolvimento do germe dental, sendo expresso nas fases de iniciação, botão, capuz, e estágio de sino da odontogênese (Thesleff, 2003, Kapadia et al., 2007 Neubüser A. 1995, Peters et al. 1998, Peters et al. 1999).

Mutações no gene *Pax9* foram reportadas em famílias com um padrão autossômico dominante de agenesia dental afetando principalmente segundos pré-molares e terceiros molares (Vastardis et al 1996, Van den Boogaard 2000). Outras mutações no *PAX9* também foram associadas com a agenesia dental em humanos, afetando principalmente segundos pré-molares e todos os molares. Também nestes casos as alterações eram transmitidas como um padrão autossômico dominante (Stockton et al. 2000, Nieminen et al. 2001; Frasier-Bowers et al. 2002; Das et al. 2002; Das et al. 2003; Mostowska et al. 2003, Jumlongras et al. 2004). Polimorfismos na região 5´ do gene *PAX9* parecem estar associados com a hipodontia em humanos (Peres et al. 2005). É interessante notar que enquanto a agenesia dental foi a principal (na maioria dos casos a única) alteração observada nas famílias com mutação nos genes *PAX9*, a mutação deste gene em camundongo foi acompanhada por múltiplas alterações craniofaciais incluindo agenesia de todos os dentes (Satokata e Maas 1994, Peters et al. 1998). Apesar de serem produzidos em vários tecidos durante a embriogênese a mutação dos dois alelos dos genes *Pax9* afeta principalmente o desenvolvimento craniofacial.

Acredita-se que a expressão proteica é principalmente regulada durante a transcrição (Veitia et al. 2006, Tomilin et al. 2008, Celniker et al. 2007). Neste processo, a

região promotora possui um papel fundamental mudando a expressão gênica devido seu potencial de incorporar sinais endócrinos e ambientais (Vlahopoulos S et al. 2004). Grande variedade de agentes fisiológicos e farmacológicos externos podem ter um impacto relevante na regulação da atividade transcricional de genes modulando variada classe de fatores de transcrição. Drogas podem se ligar diretamente ou indiretamente modular classe de receptors nucleares de fatores de transcrição através de cascatas de sinalização como, por exemplo, o tamoxifen utilizado no tratamento de cancer de mama e vários outros tipos de anti-inflamatórios e hormônios esteróides (Overington et al., 2006).

Pouco se conhece sobre a atividade transcricional do gene *PAX9* humano. Para elucidarmos nosso conhecimento sobre sua regulação, o presente trabalho focaliza o estudo da região 5´do gene *PAX9* humano e tem como objetivo analisar a influência da dexametasona, ácido retinóico e ergocalciferol (vitamina D2) na atividade transcricional de sua região promotora, utilizando construções em vetor plasmideano que dirige a transcrição do gene da luciferase de vagalume (*Photinus pyralis*, pGL3 basic vector).

Transcritos do gene *PAX9* de células mamárias de adenocarcinoma MCF-7 (Human breast adenocarcinoma) e células odontoblastóides de camundongo MDPC23 (Mouse Odontoblast Cell-Like-23) foram amplificados através de ensaios com transcriptase reversa nos ensaios de transcrição. Para a análise da atividade transcricional da região promotora do gene *PAX9*, a região promotora inteira ou fragmentos menores obtidos com deleção através de restrição com enzima ApaI, foram recombinadas ao vetor de expressão pGL3Basic. Sob influência de drogas que notoriamente influenciam na transcrição de alguns genes, dexametasona, ácido retinóico e ergocalciferol (vitamina D2), células mamárias de adenocarcinoma MCF-7 (Human breast adenocarcinoma) e células odontoblastóides de camundongo MDPC23 (Mouse Odontoblast Cell-Like-23) sofreram transfecção com os recombinantes citados acima, com finalidade de avaliar o comportamento transcricional destes frente à ação das drogas.

A análise da expressão de transcritos de cDNA provenientes da cultura de células mamárias de adenocarcinoma MCF-7 (Human breast adenocarcinoma) e células odontoblastóides de camundongo MDPC23 (Mouse Odontoblast Cell-Like-23) através de reação de PCR Quantitativo forneceu dados complementares sobre o padrão de expressão gênica do PAX9 sob a ação da dexametasona, ácido retinóico e ergocalciferol. O estudo

realizado visa contribuir para o melhor entendimento da atividade transcricional do gene *PAX9* humano.

CAPÍTULO 1

Em vias de submissão ao periódico *Cell Biochemistry and Function*

Full Title: Transcriptional Activity Analysis of Promoter Region of Human *PAX9* Gene under Retinoic Acid, Dexamethasone and Ergocalciferol treatment in MCF-7.

Running Title: Transcriptional activity of *PAX9* gene.

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ABSTRACT

PAX9 is a member of the family homeobox of transcription factors and performs important functions in development and organogenesis. Mutations in *PAX9* coding sequences have been implicated in autosomal dominant oligodontia affecting predominantly permanent molars and second premolars. Previous studies have shown that *PAX9* is required for secondary palate development and certain teratogens have been identified as inducers of a tooth and craniofacial malformations. This work focused on the analysis on the 5'-flanking region of the *PAX9* gene studying the influence of retinoic acid, dexamethasone and vitamin D on the expression of *PAX9* by expression constructs that carry the reporter gene luciferase. As results, retinoic acid and dexamethasone showed progressive decrease of *PAX9* expression. *PAX9*-pGL3B1 and *PAX9*-pGL3B2 promoter was inhibited under the treatment of dexamethasone and ergocalciferol. Retinoic acid and dexamethasone did not altered *PAX9*-pGL3B3 (−1106 to +92, 507bp deleted with ApaI digest) behavior. Luciferase activity in plasmid *PAX9*-pGL3B2 was always weaker than the other two constructions indicating that sequences present between -1106 and -751 were important for the transcriptional activity of *PAX9* promoter.

Keywords: *PAX9* promoter gene, transcriptional activity, pGL3 Basic Vector, Luciferase Assays, Human breast adenocarcinoma MCF-7 cells.

INTRODUCTION

PAX9 (14q12-q13) is ninth member of highly conserved Pax homeobox family of transcription factors that play an active role in vertebrate development (Stapleton et al., 1993; Wallin et al., 1993). *PAX9* expression displays a spatially and temporally restricted pattern in various embryonic tissues including the somites, the distal limb buds, nasal mesenchyme, the mandibulary and maxillary components of the first branchial arch and the tongue mesenchyme, suggesting a critical role in morphogenesis (Neubüser et al. 1997). Targeted inactivation in mouse embryo has shown that *Pax9* is required for the proper development of pharyngeal pouch derivatives, the craniofacial skeleton, palate, digits and teeth, where it is expressed in the initiation, bud, cap, bell stages of odontogenesis (Thesleff, 2003, Kapadia et al., 2007, Neubüser A. 1995, Peters et al. 1998, Peters et al. 1999). *PAX9* mutations in human families cause autosomal dominant oligodontia, characterized by the absence of permanent molars and pre-molars (Goldenberg et al. 2000, Stockton et al. 2000, Nieminen et al. 2001). It is interesting to note that ablation of both alleles in knockout mice is accompanied by many craniofacial alterations (Satokata and Maas 1994, Peters et al. 1998). Dental agenesis was the main (in most cases the only) change observed in families with mutations in *PAX9* gene.

It is believed that protein expression is mainly regulated during transcription (Veitia et al. 2006, Tomilin et al. 2008, Celniker et al. 2007). In this process, the promoter region has a key role in changing gene expression due to its potential to incorporate endocrine and environmental signals (Vlahopoulos S et al. 2004). A great variety of physiological or pharmacological environmental factors may have impact on downstream signaling cascades and transcriptional regulation of gene since they may bind to promoter region. Drugs may directly target and indirectly modulate the nuclear receptor class of transcription factors through signaling cascades such as tamoxifen for the treatment of breast cancer and various types of anti-inflammatory and anabolic steroids (Overington et al., 2006).

Little is known about transcriptional regulation of human *PAX9* gene. In order to broaden our knowledge on *PAX9* expression, the aim of this work was to determine whether dexamethasone (DEX), retinoic acid (RE) and ergocalciferol (VITD2) treatment may change the expression of *PAX9* and to evaluate if these drugs can interfere with the transcriptional activity of this gene.

MATERIALS AND METHODS

DNA extraction and amplification

Genomic DNA from healthy patients was extracted from samples of epithelial oral cells collected by mouthwash with dextrose 3% and purified using 8M ammonium acetate and 1mM EDTA (Aidar & Line 2007). Sequences between positions -1106 to +92 and -751 a +92 were amplified by PCR using primers *PAX9* SacI-HindIII and *PAX9* KpnI-HindIII, respectively, designed with Primer3 version 0.2 (Massachusetts Institute of

Technology, Boston, MA) (Table 1). PCR reactions were performed in a total volume of 25 µl containing 20 ng/2 µl genomic DNA, 10 pg of each primer, 2.5 µl MgCl2 (25 mM), 2.0 µl dNTP mixture (2.5 mM each), 2.5 µl of 10× reaction buffer and 0.2–0.4 unit of thermostable DNA polymerase. DNA was denaturated at 95°C for 2 min, followed by 35 cycles of denaturation of 30s at 95°C, annealing for 45s at 60–64°C, extension of 45–60s at 72°C, and final extension of 10 min at 72°C on GeneAmp 2400 PCR Thermal Cycler (ABI corporations). Aliquots (5µl) of the PCR product were electrophoresed on 1% agarose gels and visualized under UV illumination after staining with ethidium bromide.

Generation of promoter constructs

The PCR products of 1198 bp and 843 bp containing the 5'-flanking region of *PAX9* gene was generated from human genomic DNA obtained from oral epithelial cells of a healthy individual. The PCR products were cloned in the luciferase reporter plasmid using the vector pGL3 Basic. Luciferase reporter recombinant *PAX9*-pGL3B1 and *PAX9*-pGL3B2 were generated by inserting the promoter fragment into SacI-HindIII and KpnI-HindIII sites of pGL3-Basic vector, respectively. The luciferase reporter recombinant *PAX9*-pGLB3 was generated by deleting 507bp from the 1198bp promoter fragment already cloned in the vector pGL3-basic using ApaI restriction enzyme. Constructs were confirmed by DNA sequencing, restriction enzymes digest with SacI, KpnI, HindIII combined and PCR. The pRL-TK vector (Promega) containing the reporter *Renilla* luciferase gene was used as a control to correct variations in transfection efficiency. The constructs are shown in Figure 1.

Fragment	**Forward primer (5´ - 3´)**	**Reverse primer (5´- 3´)**	**Product Size**
***PAX9* SacI-HindIII**	CTCGAGGTGTA***G******AGCTC***GCAGC	***AAGCTT***CTAGGTGATTGGTGCGGGTCGGT	1198bp
***PAX9* KpnI-HindIII**	TCTGGATTGTCAG***GTACC***AGTAACAG	***AAGCTT***CTAGGTGATTGGTGCGGGTCGGT	843bp

Table 1. Primers used to amplify regions PAX9 gene promoter. Restriction sites of enzymes SacI, KpnI and Hind III in italic.

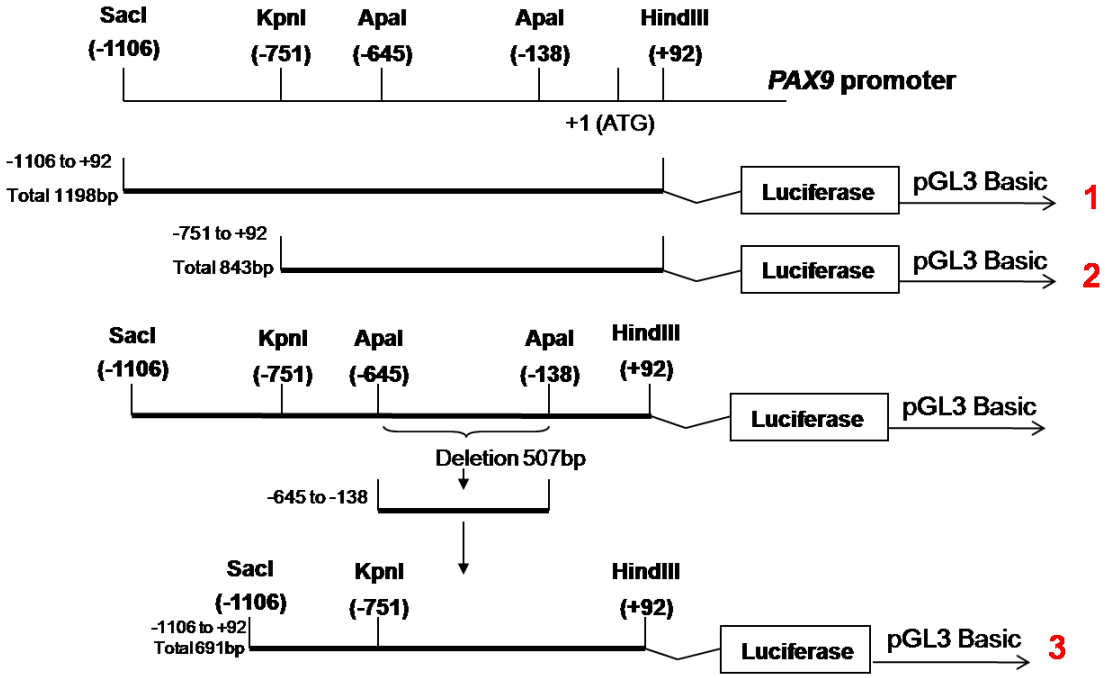

Figure 1. Schematic diagram of promoter constructs. 1) *PAX9*-pGL3B1 construct clone PAX9 gene promoter 1198bp from −1106 upstream to +92 downstream of translation start site (ATG). 2) *PAX9*-pGL3B2 construct clone PAX9 gene promoter 843bp from −751 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG). 3) *PAX9*-pGLB3 construct clone PAX9 gene promoter 691bp from −1106 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG) using deletion of 507bp in restriction sites (-645 and -138) of ApaI enzyme.

DNA sequencing

In order to confirm cloning assays, samples of constructions were subjected to automatic sequencing in 20 µl containing 1 µl of DNA, 3 µM of sense primer and 8 µl of Big Dye Terminator Ready v.2.0 (Applied Biosystems, Foster city, CA, USA). The cycle sequencing product was then run through an ABI 3739 automated sequencer (Perkin Elmer, Applied Biosystems, USA) according to the manufacturer´s protocol. The screening of the sequence was performed by comparing the output nucleotide sequence with published sequence of the *PAX9* gene (GenBank database) using Clustawl and naked eye check by Bioedit sequence alignment Editor Software /version 7.0.5.3. The potential cis-acting factor biding sites were predicted by the MatIsnpector software (www.genomatix.de).

Culture cells and drug treatments

Human breast adenocarcinoma MCF-7 cells were generously provided by Dr. Zoltan Tokes, University of Southern California (Los Angeles, CA) and cultured according

to the suppliers protocol at 37°C humidified atmosphere, 5% CO_2 in DMEM (Invitrogen, Carlsbad, CA) media supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 1% GlutaMax (Invitrogen, Carlsbad, CA). Unless otherwise stated, cells were plated at 500,000 cells/10 cm plate. The medium was replaced every 2–3 days. The mycoplasma test was performed using MycoAlert® Mycoplasma Detection Kit (Lonza).

Chemicals

Dexamethasone (9α-Fluoro-16α methylprednisolone - DEX), All-*trans*-Retinoic acid - RE (Tretinoin) and Ergocalciferol (Irradiated ergosterol/Vitamin D_2 - VITD2) (St. Louis, MO, USA) was diluted in ethanol at final concentration of 10mM as stock solution. Approximately 5 x 10^4 to 1 x 10^5 MCF-7 cells was maintained in 12-well culture plates and exposed in triplicate to reagents at concentrations of 0.1 μg/ml, 1μg/ml, or 10μg/ml, to ethanol 100% (vehicle control added to untreated samples) 48 hrs prior to mRNA extraction, according to conditions previously described (Antonova L. and Mueller C. 2008).

Reverse transcription and Real-time PCR

Total RNA was harvested from cells and purified using the Aurum Total RNA mini kit (BioRad), following the manufacturer's recommendation. cDNA was synthesized from 3μg total RNA using the High capacity archive cDNA kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed on the 7900 Fast RealTime PCR machine (Applied Biosystems, Carlsbad, CA) and TaqMan Assay kits (Applied Biosystems, Carlsbad, CA) that include primers and FAM-labeled probe sets specifically targeting human *PAX9* gene and TATA box binding protein gene. Real-time PCR was conducted using standardized conditions with a 60°C annealing temperature for 30s. All primers used were validated realtime PCR primers purchased from Applied Biosystems (Carlsbad, CA).

Transient transfections and Dual luciferase assay system

MCF-7 cells (3x10^5cells/well) were plated in 12-well plates (Becton Dickinson, San Jose, CA, USA) and incubated for 3 days. On the third day (cells reached 80%-90% of confluence), the culture medium was aspirated, and the cell monolayer was washed with pre-warmed sterile PBS and trypsinazed with 1x Trypsin-EDTA (0.05% trypsin/0.02% EDTA) (Invitrogen, Carlsbad, CA). Cells were tranfected by eletroporation using Electro Square Porator ECM830 (BTX, CA) according to the manufacturer's instructions (240V with a single 25-ms pulse) with 4000ng of pGL3-basic promoter construct *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3 encoding a firefly luciferase gene (pGL3-basic vector) and co-transfected with 300ng of pRL-TK (Promega, USA) encoding the *Renilla* luciferase gene. Cells were kept under triplicate treatment of concentrations of 10μg/ml, 1μg/ml and 0μg/ml for each reagent (Dexamethasone, Retinoic acid and Ergocalciferol) for 48 hours. Then cells were lysed with the passive lysis buffer (Promega, Madison, WI) and assayed in triplicate for luciferase activity on a 1420 Multilabel Counter Victor^3 V plate reader (Perkin-Elmer, Turku, Finland) using the Dual-Luciferase Assay System (Promega)

according to the manufacturer's instructions. Promoter activities were expressed as a ratio of firefly luciferase to *Renilla* luciferase luminescence in each well.

Trypan blue assays

Cell viability was determined by trypan blue assays testing whether dexamethasone, retinoic acid and ergocalciferol challenged cells. Trypan blue assays exclusion test challenged cells were performed as previously described by Lissy 2000 and Devireddy 2001. In brief, cells were harvested by brief trypsinization 24 h after the addition of Dexamethasone, Retinoic Acid and Ergocalciferol (Sigma). Both floating and attached cells were assayed. At least 150 cells per treatment were counted after being stained with trypan blue at a final concentration of 0.2%. Assays were performed in duplicate in tree independent experiments.

Statistical analysis

With respect to Real-time PCR *PAX9* gene expression analysis, an inter-group analysis was performed using nonparametric Kruskal–Wallis test at the level of 5%, followed by Dunn's test when differences were detected. Sample means and standard deviations were calculated for the luciferase assays which were performed in triplicate. Individual experiments were carried out at least three times and a representative experiment is shown. Statistical analysis calculations for luciferase assay results were performed using the two-sample Student's t-test function assuming equal variances. The values of $P \leq 0.05$ were regarded as significant.

RESULTS

PAX9 mRNA expression in MCF-7 under DEX, RE and VITD2 treatment

In order to assess the effect of various drugs on *PAX9* expression in MCF-7 cells, cells were treated with various concentrations of dexamethasone, retinoic acid and ergocalciferol for 48 hours. Dexamethasone did not affect *PAX9* mRNA synthesis in MCF-7 cells. In the same culture conditions, 0.1μg/ml, 0.1μg/ml 10μg/ml of retinoic acid increased *PAX9* mRNA expression in 3, 5 and 7%, respectively when compared with untreated cells ($p \leq 0.001$) (Figure 2).

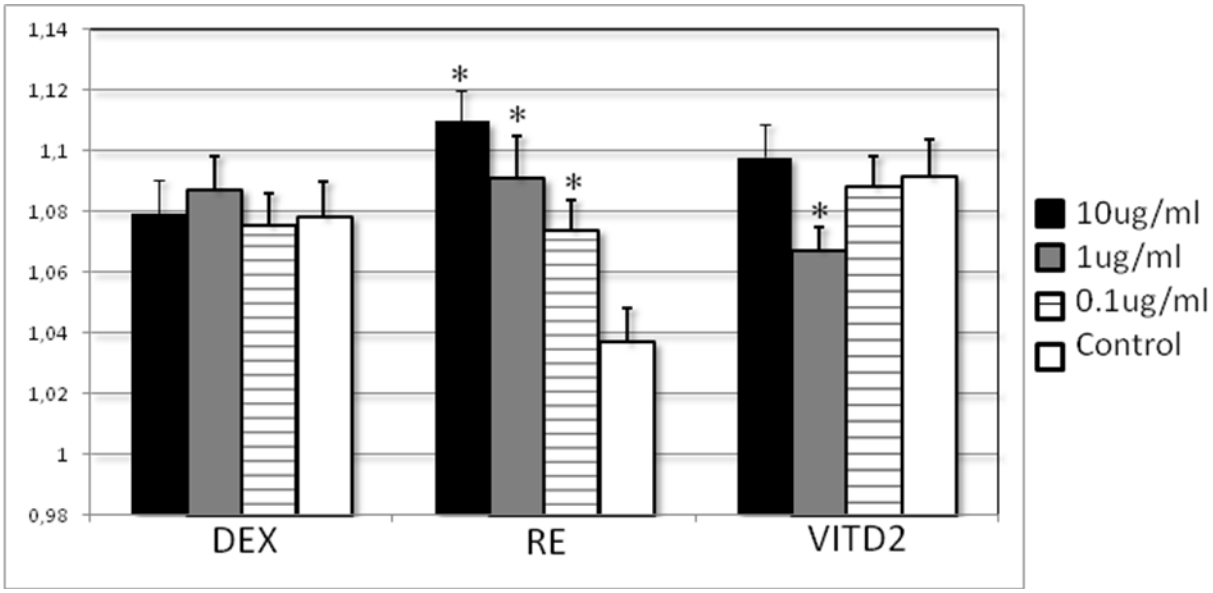

Figure 2. The influence of various concentrations of Dexamethasone (DEX), Retinoic Acid (RE) and Ergocalciferol (VITD2) in MCF-7 breast cancer cells. Exposure time 48hrs. Control=100% ethanol. Data presented as mean values ± SD (n=3). *p≤0.001.

PAX9-pGL3B1, PAX9-pGL3B2 and PAX9-pGL3B3 construct confirmation

In total, we combined tree specific sequences of the promoter region of human *PAX9* gene with the pGL3-basic vector encoding a firefly luciferase gene (*PAX9*-pGL3B1 - from −1106 to +92, *PAX9*-pGL3B2 - from −751 to +92 and *PAX9*-pGLB3 - from −1106 to +92 with deletion of 507bp), which were confirmed by sequencing, restriction enzyme cutting and PCR (Figure 3A and 3B).

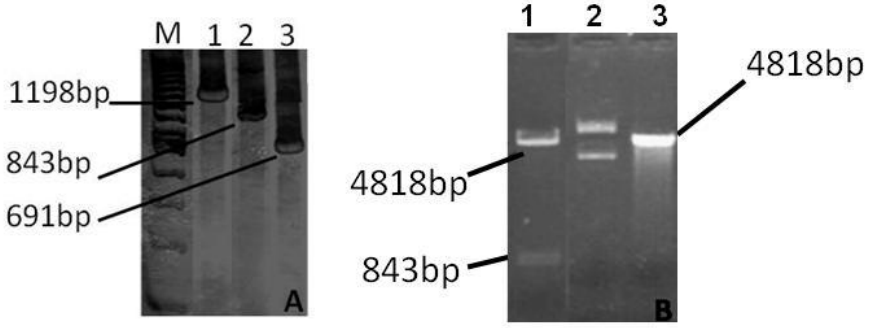

Figure 3. *PAX9*-pGL3B1 and *PAX9*-pGL3B2 construct confirmation by restriction enzymes digest and PCR - gel electrophoresis. A) M: Marker 100bp; A1, A2 and A3: PCR product of *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3 constructs respectively (primers on table 1). B) 3B) pGL3-Basic; 2B) pGL3-Basic single cut with KpnI; 1B) Recombinant *PAX9*-pGL3B2 construct confirmed by cut with KpnI and HindIII.

Transcriptional activity of PAX9 promoter in MCF-7 under DEX, RE and VITD2 treatment

To examine the transcriptional activity of the *PAX9* gene promoter, 1198bp fragment (-1106 to +92), 843bp (-751 to +92) and 691bp (deletion of 507bp from region -1106 to +92) was placed in the upstream of the firefly luciferase gene in the pGL3-Basic vector. The resultant plasmid *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3 was transiently transfected into the MCF-7 cells that were treated with various concentrations of dexamethasone - DEX, retinoic acid – RE and ergocalciferol – VITD2 for 48 hours. Then, the firefly luciferase activity obtained was compared with those from the pRL-TK-control plasmid. Treatment with 10μg/ml of dexamethasone decreased luciferase activity to 47% and 82% in *PAX9*-pGL3B1 and *PAX9*-pGL3B2, respectively ($p \leq 0.003$, figure 4A).

The same effect of downregulation in luciferase activity was observed with ergocalciferol treatment of *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3. There was a 28% decrease with 1μg/ml and 37% with 10μg/ml for *PAX9*-pGL3B1 ($p \leq 0.002$). For plasmid *PAX9*-pGL3B2, there was a 29% decrease in luciferase activity with 1μg/ml, and 65% with 10μg/ml ($p \leq 0.002$) (figure 4C). For plasmid *PAX9*-pGL3B3, the decrease in luciferase activity for concentrations of 10μg/ml and 1μg/ml were 34% and 26%, respectively.

Conversely, 1μg/ml of Retinoic acid increased luciferase activity in 73% for *PAX9*-pGL3B1 and 267% for *PAX9*-pGL3B2 construction ($p \leq 0.002$) (figure 4B).

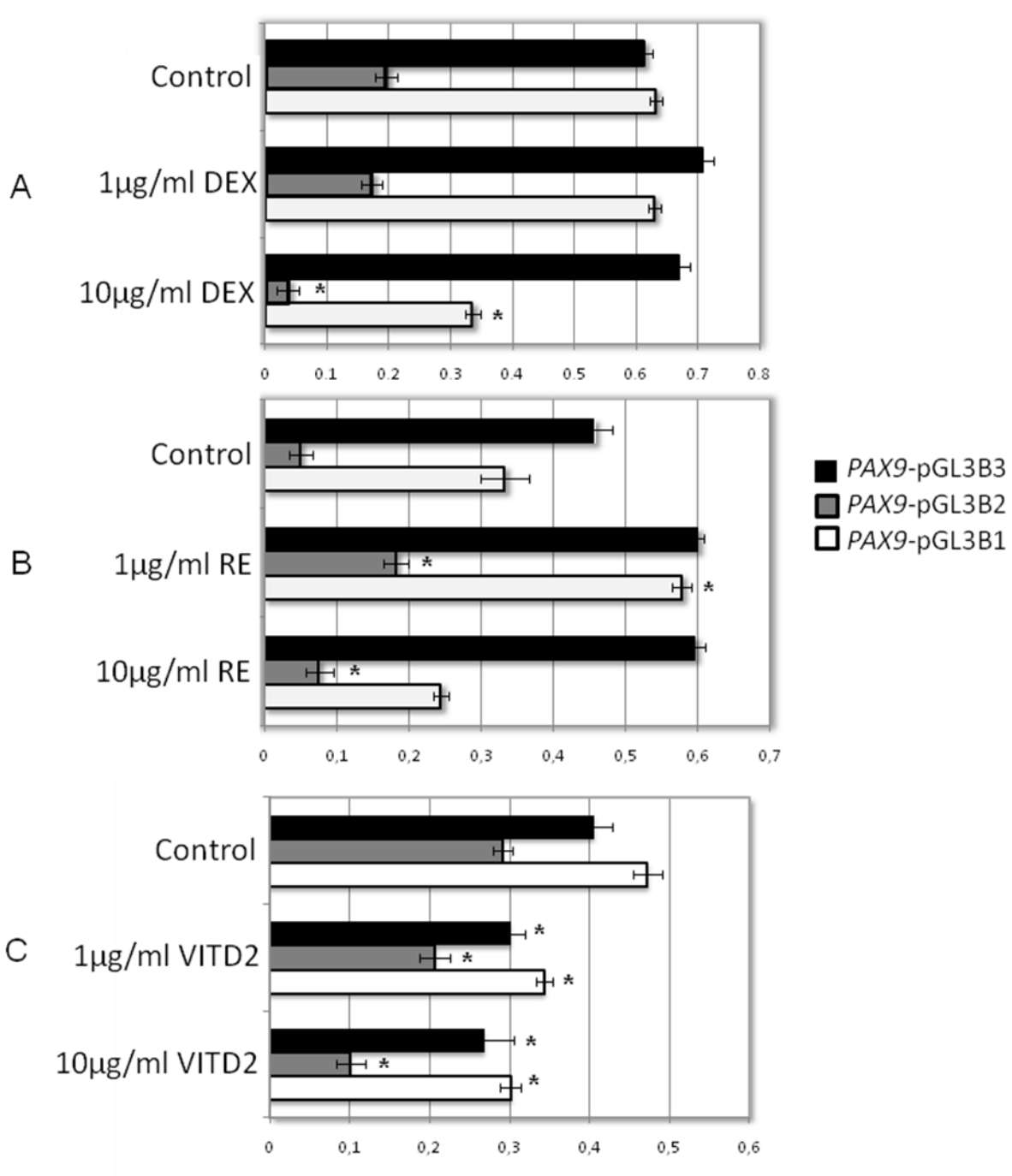

Figure 4. Functional analysis of *PAX9* gene promoter by the Dual luciferase assay in MCF-7 cells. Luciferase activity in MCF-7 cells transfected with *PAX9*-pGL3B1, *PAX9*-pGL3B2, *PAX9*-pGL3B3 and pRL-TK as a control under Dexamethasone (DEX) (4A), Retinoic Acid (RE) (4B) and Ergocalciferol (VITD2) (4C) treatment.

DISCUSSION

We have shown in time and concentration dependent manner that retinoic acid increases expression activity of *PAX9* gene in MCF-7 cancer cells on RT-PCR experiment. It is known that retinoic acid receptors act as transcription factors regulating gene expression/protein production (Balmer and Blomhoff 2002). Besides, more than 532 genes have been put forward as regulatory targets of retinoic acid (Blalock and Gifford 1977). It is also has been reported that *PAX9* is required for secondary palate development and certain teratogens such as retinoic acid have been identified as inducers of cleft secondary palate (Sulik et al. 1988, Gorlin et al. 1990, Peters et al. 1998). In early developmental stages retinoic acid receptors act directly in the regulatory regions and transcription of homeobox genes targets controlling anterior/posterior patterning (Holland LZ. 2007). Our study are in agreement with previous works confirming that genes containing homeobox domains are among the various types of genes regulated directly through the classical Retinoic acid pathway (Balmer and Blomhoff 2002), thereby activating *PAX9* gene transcription directly. However, no significant changes in *PAX9* expression were found in RT-PCR analysis with dexamethasone and ergocalciferol treatment.

We found with dual luciferase promoter function assay that lower concentration of retinoic acid activates *PAX9*-pGL3B1 (−1106 to +92) and *PAX9*-pGL3B2 (−751 to +92) luciferase if compared with ethanol group alone. It is interesting to observe that the influence of retinoic acid was different when comparing RT-PCR and Luciferase assays. Retinoic acid increased the expression of *PAX9* mRNA, while the same concentration decreased expression in the luciferase assays. This may be explained because the transcriptional activity of the *PAX9* gene may be influenced by other sequences distant from transcription start site. In fact, cis-regulatory sequences of *Pax9* gene were identified in intronic sequences of the neighboring gene *Slc25a21* (Santagati et al 2003).

Different from *PAX9*-pGL3B1 and *PAX9*-pGL3B2 constructions, retinoic acid and dexamethasone did not alter luciferase activity in *PAX9*-pGL3B3 (−1106 to +92, 507bp deleted with ApaI digest). This result suggests that 507bp deletion or -645 to -138 of this construction may contain biding sites for retinoic acid and dexamethasone receptors or other transcription factors, whose expression is modulated by these drugs. The analysis of the DNA biding sites on MatInspector software (http://www.genomatix.de/cgi-bin/eldorado/main.pl) revealed that the region of 507bp deleted in the construction *PAX9*-pGL3B3 has around 85 transcriptional biding motifs that may be sites for signaling cascade proteins initially activated by the presence of extracellular retinoic acid. This region of 507bp is located within various core binding sequences, such as, zinc binding proteins, GATA binding factors, homeodomain transcription factors and zinc fingers kruppel-like transcription factors. Although there is no specific study on the expression of these *PAX9* promoter factors on development, the Kruppel-like transcription factor, for instance, was shown to be expressed by the mesenchymal cells of first arch at E11.5 (Garrett-Sinha 1996). The same analysis on MatInspector software revealed the presence of one biding site for glucocorticoid responsive and relative elements (core sequence: GTGC) within the 507bp deleted sequence. DNA sequences from human, dog, mouse, rat and chimpanzee were obtained using the Blat Search Genome (http://genome.ucsc.edu/cgi-bin) and aligned with the software Clustalw (www.ebi.ac.uk/clustal). The sequence TGTGC (containing the glucocorticoid responsive element) is conserved among these species, suggesting that it has an active role in the transcriptional regulation of *PAX9* gene (Figure 5).

Luciferase activity in plasmid *PAX9*-pGL3B2 was always much weaker than the other two constructions used, indicating that sequences present between -1106 and -751 or 355bp were important for the transcriptional activity of *PAX9* promoter. It was showed in previous studies that polymorphisms in the promoter region from -1106 to -751 of *PAX9* gene may have an influence on the transcriptional activity of this gene and are associated with hypodontia in humans. T-912C polymorphism in the 5' region of the gene *PAX9* appears to be associated with hypodontia in humans (Peres et al. 2005).

Concerning the ergocalciferol treatment, a significant reduction (−50%; $p \leq 0.001$) of luciferase expression compared to the control was observed in all the constructions, especially when cells were treated with 10μg/ml. Vitamin D plays an important role in tissue mineralization and calcium/phosphate homeostasis during skeletal development (Wentz et al 1958, Berdal et al. 1995). Nutritional vitamin D deficiency could cause morphogenic abnormalities, matrix disorganization and hypomineralization during tooth formation (Zhang X et al. 2007, Papagerakis et al. 1999, Davideau JL et al. 1996). In this study, we characterized the transcripitional activity of specific regions of the *PAX9* promoter gene and we demonstrated that ergocalciferol can modulate the transcriptional inhibits the promoter activity of *PAX9* gene.

In summary, dexamethasone, retinoic acid and ergocalciferol can regulate the transcriptional activity of human *PAX9* gene. It is worth to mention that the receptors for these drugs may also modulate the transcription of other genes which eventually repress or stimulate *PAX9* activity. In this study, we observed that deletions of 507 bp (*PAX9*-pGL3B3) and 355 bp (*PAX9*-pGL3B2) have profound effect on the *PAX9* gene transcription. It will be interesting to examine whether other modifications add further layer of complexity to the regulation of transcriptional activity on PAX9 promoter.

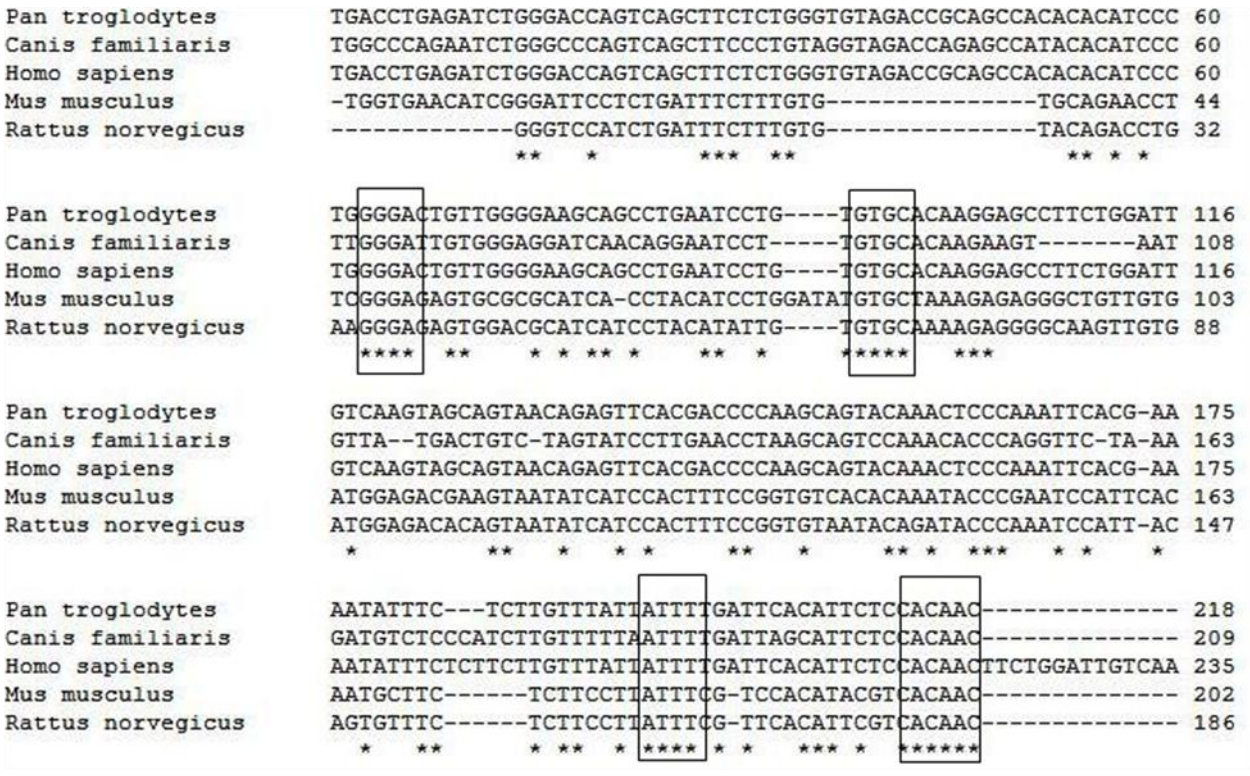

Figure 5. *PAX9* 5´ flanking sequences of chimpanzee, dog, mouse, and rat aligned with human sequences. DNA sequences were obtained using the Blat Search Genome (http://genome.ucsc.edu/cgi-bin) and aligned with the software Clustalw (www.ebi.ac.uk/clustalw). Conserved consensus sequences are underlined by asterisk (*). Four regions with 4 consecutive conserved bases are marked inside squares: binding sites to GGGA, ATTT, CAAC and GTGC transcription factors. Biding site for glucocorticoid responsive and relative elements (GTGC) is shown to be conserved among species.

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CAPÍTULO 2

Em vias de submissão ao periódico *Archives of Oral Biology*

Full Title: Transcriptional Activity Analysis of Promoter Region of Human *PAX9* Gene under Retinoic Acid, Dexamethasone and Ergocalciferol treatment in MDPC23.

Running Title: Transcriptional activity of *PAX9* gene.

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ABSTRACT

Pax genes play important roles in mammalian development and organogenesis. *Pax9*, a member of this transcription factor family, is expressed in somites, pharyngeal pouches, mesenchyme involved in craniofacial, tooth, and limb development, as well as other sites during mouse embryogenesis. The aim of this work was to determine changes in *PAX9* expression under dexamethasone (DEX), retinoic acid (RE) and ergocalciferol (VITD2) treatment and observe their influence in the transcriptional activity of this gene. Our results have showed that *PAX9*-pGL3B1 and *PAX9*-pGL3B2 promoter activity was increased by the treatment of 1μg/ml of dexamethasone and ergocalciferol; 10μg/ml of the same reagents decreased this activity. Retinoic acid and dexamethasone did not altered *PAX9*-pGL3B3 (−1106 to +92, 507bp deleted with ApaI digest) behavior. This result suggests that 507bp deletion or sequence between -645 to -138 is important for the transcriptional activity of *PAX9* gene. In conclusion, we suggest that 507bp region within *PAX9* promoter in *PAX9*-pGL3B3 may contain biding sites that are responsive for retinoic acid and dexamethasone since none of concentrations of this reagent influenced change in promoter activity.

Keywords: *PAX9* promoter gene, Transcriptional activity, pGL3 Basic Vector, Luciferase Assays, Mouse odontoblast like cell line MDPC-23.

INTRODUCTION

Pax9 is a member of a transcription factor family that is characterized by a common motif, the DNA-binding paired domain encoded by the paired box; a conserved DNA region originally identified in *Drosophila* (Bopp et al. 1986, Baumgartner et al. 1987). The *Pax9* gene is present in all vertebrates analyzed so far, including zebrafish, chick, mouse, and man (Stapleton et al. 1993, Neubüser et al. 1995, Nornes et al. 1996) and during mouse development is widely expressed in neural crest-derived mesenchyme involved in craniofacial and tooth development (Deutsch et al. 1988, Timmons et al. 1994, Neubüser et al. 1995, 1997). Spontaneous as well as targeted mutations in *Pax9* gene revealed that it performs essential functions during mammalian embryonic development since it is observed various craniofacial malformations in the skeleton, palate, digits and teeth (Thesleff, 2003, Kapadia et al., 2007 Neubüser A. 1995, Peters et al. 1998, Peters et al. 1999, Satokata I., 1994). *PAX9* mutations in human families cause autosomal dominant oligodontia, characterized by the absence of permanent molars and pre-molars (Goldenberg et al. 2000, Stockton et al. 2000, Nieminen et al. 2001).

Furthermore, *PAX9* is required for secondary palate development and certain teratogens such as retinoic acid have been identified as inducers of a cleft secondary palate (Sulik et al. 1988, Gorlin et al. 1990). It is believed that retinoic acid functions determining position along embryonic anterior/posterior axis in chordates. It acts through homeobox genes, which ultimately control anterior/posterior patterning in early developmental stages binding to retinoic acid response elements in the regulatory regions of direct homeobox genes targets, thereby activating gene transcription directly (Holland LZ, 2007). Retinoic acid has effects on the fundamental process of morphogenesis and on the growth and differentiation of normal and transformed cells.

Other hormones and vitamins can influence embryologic development. Vitamin D has been reported to play an important role in tooth mineralization (Wentz et al. 1958, Berdal et al. 1995, Zhang X et al. 2007, Papagerakis et al. 1999, Davideau JL et al. 1996). Corticosteroids are involved in a wide range of physiologic systems and glucocorticoid hormones also control gene transcription by activating glucocorticoid receptor response elements in the regulatory region (Wu W et al. 2005) and may influence the expression of genes related with odontogenesis.

Considering that a great variety of physiological or pharmacological environmental stimulus may have impact on transcriptional regulation of genes, this study focused on the analysis of the 5' region of the *PAX9* gene that may be mediated by the effect of dexamethasone, retinoic acid and ergocalciferol on *PAX9* promoter expression in Mouse odontoblast like cell line MDPC-23.

MATERIALS AND METHODS

DNA extraction and amplification

Human genomic DNA from healthy patients was extracted from samples of epithelial oral cells collected by mouthwash with dextrose 3% and purified using 8M ammonium acetate and 1mM EDTA (Aidar & Line 2007). Promoter regions between positions -1106 to +92 and -751 to +92 were amplified by PCR using primers *PAX9* SacI-HindIII and *PAX9* KpnI-HindIII, respectively, designed with Primer3 version 0.2

(Massachusetts Institute of Technology, Boston, MA) (Table 1). PCR reactions were performed in a total volume of 25 µl containing 20 ng/2 µl genomic DNA, 10 pg of each primer, 2.5 µl MgCl2 (25 mM), 2.0 µl dNTP mixture (2.5 mM each), 2.5 µl of 10× reaction buffer and 0.2–0.4 unit of thermostable DNA polymerase. DNA was denaturated at 95°C for 2 min, followed by 35 cycles of denaturation of 30s at 95°C, annealing for 45s at 60–64°C, extension of 45–60s at 72°C, and final extension of 10 min at 72°C on GeneAmp 2400 PCR Thermal Cycler (ABI corporations). Aliquots (5µl) of the PCR product were electrophoresed on 1% agarose gels and visualized under UV illumination after staining with ethidium bromide.

Generation of promoter constructs

The PCR products of *PAX9* promoter fragment of 1198bp and 843bp upstream in the 5'-flanking region of *PAX9* gene was generated from genomic DNA of epithelial oral cells from healthy patients. The PCR products were cloned in the luciferase reporter plasmid using the vector pGL3 Basic. Luciferase reporter recombinant *PAX9*-pGL3B1 and *PAX9*-pGL3B2 were generated by inserting the promoter fragment into SacI-HindIII and KpnI-HindIII sites of pGL3-Basic vector, respectively. The luciferase reporter recombinant *PAX9*-pGLB3 was generated by deleting 507bp from the 1198bp promoter fragment already cloned in the vector pGL3-basic using ApaI restriction enzyme. Constructs were confirmed by sequencing, cutting with SacI, KpnI, HindIII combined and PCR. The pRL-TK vector (Promega) contains the reporter *Renilla* luciferase gene was used as a control to correct variations in transfection efficiency. The constructs are shown in Figure 1.

Fragment	**Forward primer (5´ - 3´)**	**Reverse primer (5´- 3´)**	**Product Size**
***PAX9* SacI-HindIII**	CTCGAGGTGTA***AGCTC***GCAGC	***AAGCTT***CTAGGTGATTGGTGCGGGTCGGT	1198bp
***PAX9* KpnI-HindIII**	TCTGGATTGTCA***GGTACC***AGTAACAG	***AAGCTT***CTAGGTGATTGGTGCGGGTCGGT	843bp
Mouse 18S rRNA	GAGGTAGTGACGAAAAATAACAAT	TTGCCCTCCAATGGATCCT	99bp
Pax9 mouse	CATCCGACCTTGTGACATCA	GGTCACTACGACTCCTATAA	336bp

Table 1. Primers used to amplify regions *PAX9* gene promoter for cloning (restriction sites of enzymes SacI, KpnI and Hind III in italic) and Primers used for *Pax9* and 18S RT-PCR amplification.

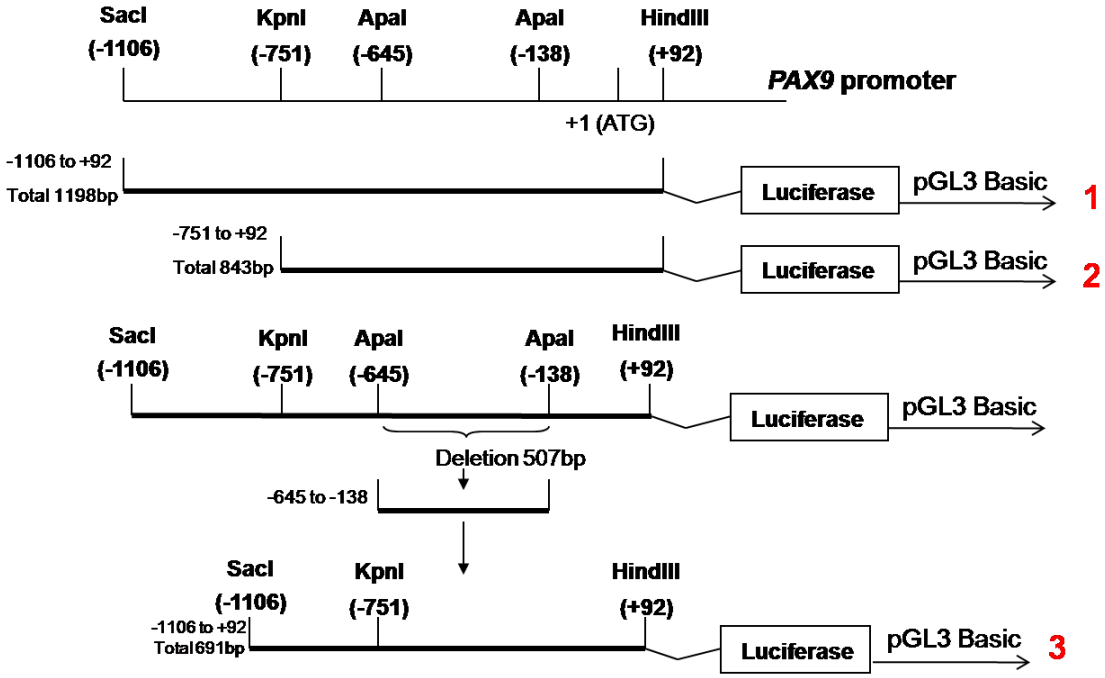

Figure 1. Schematic diagram of promoter constructs. 1) *PAX9*-pGL3B1 construct clone *PAX9* gene promoter 1198bp from −1106 upstream to +92 downstream of translation start site (ATG). 2) *PAX9*-pGL3B2 construct clone *PAX9* gene promoter 843bp from −751 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG). 3) *PAX9*-pGLB3 construct clone *PAX9* gene promoter 691bp from −1106 upstream of translation start site (ATG) to +92 downstream of translation start site (ATG) using deletion of 507bp in restriction sites (-645 and -138) of ApaI enzyme.

DNA sequencing

In order to confirm cloning assays, samples of constructions were subjected to automatic sequencing in 20 µl containing 1 µl of DNA, 3 µM of sense primer and 8 µl of Big Dye Terminator Ready v.2.0 (Applied Biosystems, Foster city, CA, USA). The cycle sequencing product was then run through an ABI 3739 automated sequencer (Perkin Elmer, Applied Biosystems, USA) according to the manufacturer´s protocol. The screening of the sequence was performed by comparing the output nucleotide sequence with published sequence of the *PAX9* gene (GenBank database) using Clustawl and naked eye check by Bioedit sequence alignment Editor Software/version 7.0.5.3. The potential biding sites were selected by the promoter transcription factor biding predict software (www.genomatix.de;www.gene-regulation.com).

Culture cells and reagent treatments

Mouse odontoblast like cell line MDPC-23 were generously provided by Dr. Zoltan Tokes, University of Southern California (Los Angeles, CA) and cultured according to the

suppliers protocol at 37°C humidified atmosphere, 5% CO_2 in DMEM (Invitrogen, Carlsbad, CA) media supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Unless otherwise stated, cells were plated at 500,000 cells/10 cm plate. The medium was replaced every 2–3 days. The mycoplasma test was done using MycoAlert® Mycoplasma Detection Kit (Lonza).

Chemicals

Dexamethasone (9α-Fluoro-16α methylprednisolone - DEX), All-*trans*-Retinoic acid - RE (tretinoin) and Ergocalciferol (irradiated ergosterol/vitamin D_2 - VITD2) (St. Louis, MO, USA) were diluted in absolute ethanol at final concentration of 10mM as stock solution. Approximately 5 x 10^4 to 1 x 10^5 MDPC23 cells was maintained in 12-well culture plates and exposed in triplicate to reagents at concentrations of 0.1 μg/ml, 1μg/ml, or 10μg/ml, to ethanol 100% (vehicle control added to untreated samples) 48 hrs prior to mRNA extraction, according to conditions previously described (Antonova L. and Mueller C. 2008).

Reverse transcription and Real-time PCR

Total RNA was harvest from cells and purified using the Aurum Total RNA mini kit (BioRad Hercules, CA), following the manufacturer's recommendation. RNA was quantified and the quality tested by photometric measurement. We only used highly purified RNA (A260/A280>1.95). cDNA was synthesized from 3μg total RNA using the kit High capacity archive cDNA (Applied Biosystems, Carlsbad, CA). The selected specific and optimal sequences of primers 18S rRNA and *Pax9* mouse, shown in Table 1, were chosen to prevent amplification of genomic DNA. Real time PCRs in triplicate were carried out in a final volume of 25μl, containing 1 μM of primers, 1x Syber Green supermix (Bio-Rad), and variable amounts of RT products. Thermal cycling was peformed in DNA Engine Opticon real-time PCR detection system (PTC-200 DNA Engine Cycler and CFD-3200 Opticon Detector) from BioRad. Data were treated with the accompanying software analysis with melting curve. The program profile used for *Pax9* mouse amplification was: 95˚C for 3 min and 50 cycles of denaturation for 20 seconds at 95˚C, and annealing for 15 seconds at 58˚C and extension for 30 seconds at 72˚C. The program profile used for 18S rRNA was 94˚C for 3 min followed by 30 cycles of denaturation, annealing and extension for 30 sec each at 94˚C, 67.5˚C and 72˚C, respectively.

Transient transfections and Dual luciferase assay system

MDPC23 cells (3 x 10^5 cells/well) were plated in 12-well plates and incubated for 3 days. On the third day (cells reached 80%-90% of confluence), the culture medium was aspirated, and the cell monolayer was washed with pre-warmed sterile PBS and trypsinazed with 1x Trypsin-EDTA (0.05% trypsin EDTAo4Na) (Invitrogen, Carlsbad, CA). Cells were tranfected by eletroporation using Electro Square Porator ECM830 (BTX, CA) according to the manufacturer's instructions (240V with a single 25-ms pulse) with 4000ng of pGL3-basic promoter constructs *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3 encoding a

firefly luciferase gene (pGL3-basic vector) and co-transfected with 300ng of pRL-TK (Promega, USA) encoding the *Renilla* luciferase gene. The Cells were kept under triplicate treatment of concentrations of 10μg/ml, 1μg/ml and 0μg/ml for each reagent (Dexamethasone, Retinoic acid and Ergocalciferol) for 48 hours. Then cells were lysed with the passive lysis buffer (Promega, Madison, WI) and assayed in triplicate for luciferase activity on a 1420 Multilabel Counter Victor^3 V plate reader (Perkin-Elmer, Turku, Finland) using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions. Promoter activities were expressed as a ratio of firefly luciferase to *Renilla* luciferase luminescence in each well.

Trypan blue assays

Cell viability was determined by trypan blue assays testing whether dexamethasone, retinoic acid and ergocalciferol challenged cells. Trypan blue assays were performed as previously described by Lissy 2000 and Devireddy 2001. In brief, cells were harvested by brief trypsinization 24 h after the addition of dexamethasone, retinoic acid and ergocalciferol (Sigma). Both floating and attached cells were assayed. At least 150 cells per treatment were counted after being stained with trypan blue at a final concentration of 0.2%. Assays were performed in duplicate in tree independent experiments.

Statistical analysis

With respect to Real-time PCR *Pax9* gene expression analysis, an inter-group analysis was performed using nonparametric Kruskal–Wallis test at the level of 5%, followed by Dunn's test when differences were detected. Sample means and standard deviations were calculated for the luciferase assays which were performed in triplicate. Individual experiments were carried out at least three times and a representative experiment is shown. Statistical analysis calculations for luciferase assay results were performed using the two-sample Student's t-test function assuming equal variances. The values of $p \leq 0.05$ were regarded as significant.

RESULTS

Pax9 mRNA expression in MDPC23 under DEX, RE and VITD2 treatment

In order to assess the effect of various drugs on *Pax9* mRNA expression in MDPC23 cells, cells were treated with various concentrations of dexamethasone, retinoic acid and ergocalciferol for 48 hours. Figure 2 shows that 1ug/ml of dexamethasone and retinoic acid decreased expression of the *Pax9* gene in MDPC23 cell in 12% and 7%, respectively (*$p<0.001$). Ergocalciferol treatment promoted a 47% to 51% increase the mRNA expression of *Pax9*.

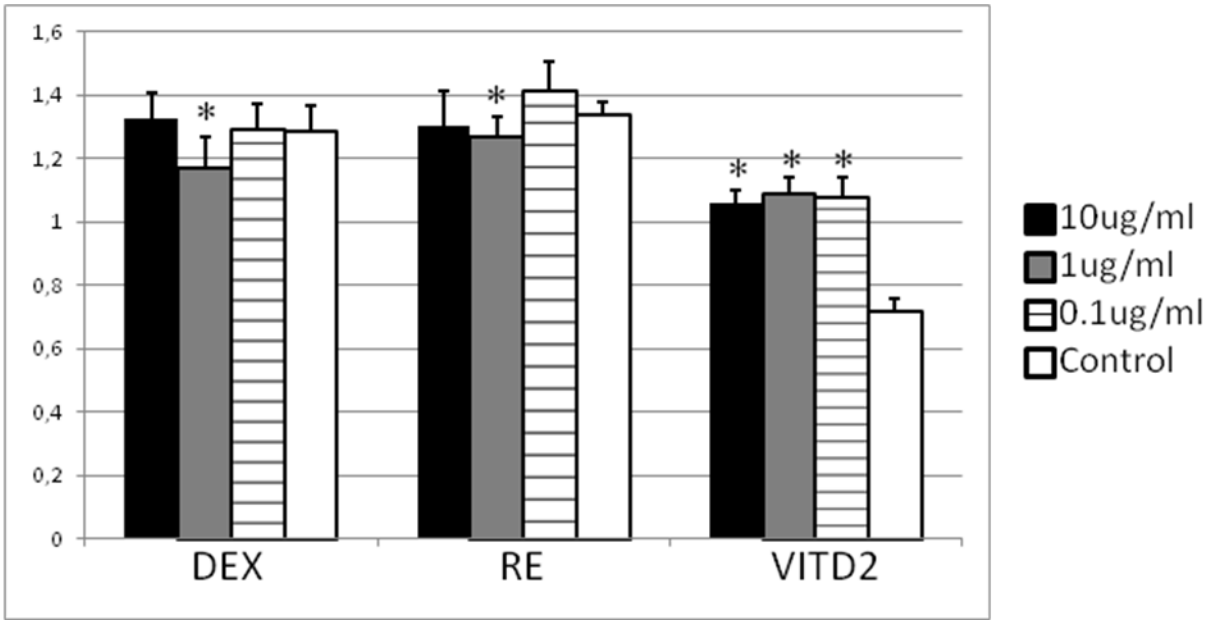

Figure 2. The influence of various concentrations of Dexamethasone, Retinoic Acid and Ergocalciferol in the expression of Pax9 mRNA expression in MDPC23 cells. Exposure time 48 hrs. Control=100% ethanol. Data presented as mean values ± SD (n=4). *p≤0.001.

PAX9-pGL3B1, PAX9-pGL3B2 and PAX9-pGL3B3 construct confirmation

In total, we combined tree specific sequences of the promoter region of human *PAX9* gene with the pGL3-basic vector encoding a firefly luciferase gene (*PAX9*-pGL3B1-from −1106 to +92, *PAX9*-pGL3B2-from −751 to +92 and *PAX9*-pGLB3-from −1106 to +92 with deletion of 507bp), which were confirmed by sequencing, restriction enzyme cutting and PCR (data not shown).

Transcriptional activity of PAX9 promoter in MDPC23 under reagent treatment

To examine the transcriptional activity of the *PAX9* gene promoter, 1198bp (-1106 to +92), 843bp (-751 to +92) and 691bp (deletion of 507bp from region -1106 to +92) fragments were placed in the upstream of the firefly luciferase gene in the pGL3-Basic vector. The resultant plasmid *PAX9*-pGL3B1, *PAX9*-pGL3B2 and *PAX9*-pGL3B3 were transiently transfected into the MDPC23 cells that were treated with various concentrations of dexamethasone - DEX, retinoic acid – RE and ergocalciferol – VITD2 for 48 hours. Then, the firefly luciferase activity obtained was compared with those from the pRL-TK-control plasmid. In figure 3A and 3C, *PAX9*-pGL3B1 and *PAX9*-pGL3B2 lucifarase activity was increased by the treatment of 1μg/ml of dexamethasone and ergocalciferol in 16 to 19%; 10μg/ml of the same reagents decreased this activity in 23 to 35% (p≤0.001). Under retinoic acid treatment, the luciferase activity of *PAX9*-pGL3B1 was increased with 1μg/ml in 65% and decreased with 10μg/ml in 23%, but for the *PAX9*-pGL3B2, concentrations of 1μg/ml and 10μg/ml increased the promoter activity in 98% and 39%,

respectively (Figure 3B). For the *PAX9*-pGL3B3 construction, 1µg/ml and 10µg/ml of ergocalciferol promoted decrease in the luciferase activity in 65% ($p<0.005$) while dexamethasone and retinoic acid treatment did not alter luciferase activity (Figure 3C).

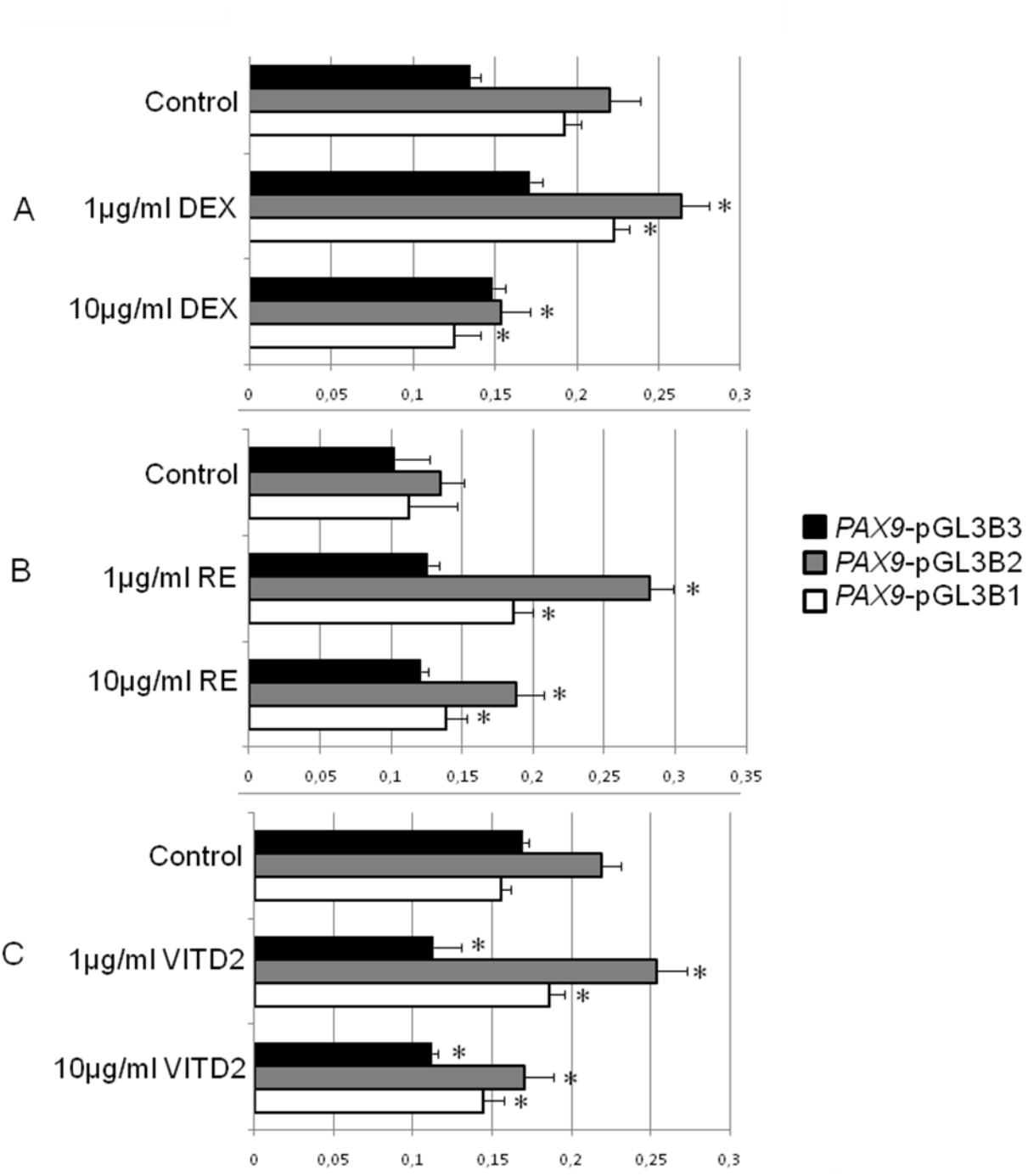

Figure 3. Functional analysis of *PAX9* gene promoter by the Dual luciferase assay in MDPC23 cells. Luciferase activity in MDPC23 cells transfected with *PAX9*-pGL3B1,

PAX9-pGL3B2, *PAX9*-pGL3B3 and pRL-TK as a control under Dexamethasone (DEX) (3A), Retinoic Acid (RE) (3B) and Ergocalciferol (VITD2) (3C) treatment.

DISCUSSION

In this study, the results obtained with the RT-PCR experiments showed that ergocalciferol promoted a significant concentration-dependent increase in the expression of *Pax9* in concentrations of 10μg/ml, 1μg/ml and 0.1μg/ml in mouse odontoblast like cell line MDPC-23. Vitamin D plays an essential role in a variety of biological events such as calcium homeostasis, bone formation-metabolism and cellular differentiation (Bouillon et al.1995; Walters, 1992; Chen & DeLuca, 1995). Hormonal form of vitamin D acts as a ligand for the vitamin D receptor (VDR), and VDR–vitamin D complex activates the target gene expression at the trancriptional level (Freedman, 1999, Demay et al., 2006). Data obtained in this study shows that *Pax9* expression in odontoblast like cell line was responsive to vitamin D2 treatment. It is known that cells devoted to enamel and dentin formation at the bell stage of odontogenesis have express Vitamin D receptor presented in both dental epithelial and mesenchymal tissues (Mark et al., 1995) and matrix proteins of mineralized tissues, such as osteocalcin, is responsive to vitamin D (Demay et al., 1990; Ozono et al., 1990; Breen et al., 1994). Previous studies in animal model suggest that specific dysplasia of rachitic enamel (decrease of intraprismatic enamel) is secondary to vitamin D dysregulation of amelogenin expression (Papagerakis et al., 1999).

In the luciferase assays, we observed that luciferase activity was increased by lower concentrations (10ug/ml) of dexamethasone, ergocalciferol and acid retinoic in constructs *PAX9*-pGL3B1 (−1106 to +92) and *PAX9*-pGL3B2 (−751 to +92) whereas higher concentration (10ug/ml) inhibits promoter activity of these constructions. It is interesting to observe that there is a pattern followed all constructions since all greatly responded with 1ug/ml of reagents but inhibited with 10ug/ml. Different from *PAX9*-pGL3B1 and *PAX9*-pGL3B2 constructions, retinoic acid and dexamethasone did not altered *PAX9*-pGL3B3 (−1106 to +92, 507bp deleted with ApaI digest) behavior. This result suggests that 507bp deletion or -645 to -138 of this construct contains biding sites for retinoic acid and dexamethasone receptors. The analysis of the DNA biding sites on MatInspector software (http://www.genomatix.de/cgi-bin/eldorado/main.pl) revealed that the region of 507bp removed from the construction *PAX9*-pGL3B3 is located within various core binding sequences, such as, Kruppel-like transcription factors. This transcription factor was shown to be expressed by the mesenchymal cells of first arch at E11.5 and thereby, may have an important role in development (Garrett-Sinha et al. 1996). The same analysis on MatInspector software demonstrated that there are biding sites for glucocorticoid responsive and relative elements in this 507bp deleted sequence, explaining why the promoter activity in this construction was also not altered with dexamethasone treatment. Moreover, DNA sequences were obtained using the Blat Search Genome (http://genome.ucsc.edu/cgi-bin) and aligned with the software Clustalw (www.ebi.ac.uk/clustal) showed that this 507bp region was well conserved in evolution, in chimpanzee, dog, rat and mouse sequences; suggesting that this region may have an important role on the transcriptional regulation of *PAX9* gene.

RT-PCR and Luciferase assays results were different from each other. All concentrations of ergocalciferol increased the expression of the gene in the RT-PCR and

the same concentrations decreased this expression of the *PAX9*-pGL3B3 promoter in the luciferase assays. Ergocalciferol appears to act at the transcriptional level of the *PAX9* to reducing expression and activity of the promoter in pGL3 vector. In culture cells the same gene expression may have influence of other environmental factors.

In conclusion, dexamethasone, retinoic acid and ergocalciferol may affect the transcriptional activity of transcription factor and also interact with other genes modulating the transcriptional activity of *PAX9* gene promoter. We observed that 507bp deleted in *PAX9*-pGL3B3 and 355bp deleted in *PAX9*-pGL3B2 may contain biding sites for dexamethasone, retinoic acid and ergocalciferol and may have effect on the *PAX9* gene function.

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CONCLUSÃO

Verificamos com este estudo que drogas como a dexametasona, ácido retinóico e vitamina D podem atuar como moduladores da expressão gênica do gene *PAX9* tanto em células humanas quanto de camundongo. E ainda, os resultados mostram que a região promotora do gene *PAX9* humano estudada entre -1106 a +92 possui receptores de ligação que podem ser influenciados pelas drogas usadas neste estudo. Mostrou-se também que as sequências deletadas do promotor -1106 a -751 e -645 a -138 nas construções *PAX9*-pGL3B2 e *PAX9*-pGL3B3, respectivamente, são de importante relevância na regulação da atividade transcricional deste gene. Verificamos que a região correspondente a 507 pb deletada do promotor na construção *PAX9*-pGL3B3 contém sítios de ligação para proteínas que são influenciadas por estas drogas. Estes resultados observados com o ensaio da luciferase e o PCR quantitativo vem a acrescentar novo entendimento da expressão do gene *PAX9 in vivo*.

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APÊNDICE: Artigo submetido no periódico *Journal of Applied Oral Science.*

Full Title: Transcriptional analysis of the human PAX9 promoter

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Abstract

Pax9 belongs to a transcriptional factor genes family named Pax family. This gene is expressed in embryonic tissues like somites, pharyngeal pouch endoderm, distal limb buds and neural crest-derived mesenchyme. Some polymorphisms in the upstream promoter region of the human Pax9 have been associated with human non-syndromic forms of autosomal dominant tooth agenesis. In the present study, we verified the in vitro expression of this gene and the influence of two promoter sequences in Pax9 gene, in embryo tissues obtained from digits, face and midbrain and hindbrain regions. These fragments were cloned into reporter plasmid and were transfected into the different cells cultures. Our semi-quantitative RT-PCR results showed that in vitro E13.5 limb bud and CNS cells express Pax9, but not in derived facial region cells. Moreover, the luciferase assay showed that this protein activity of the constructed vector by themselves were weaker than pgl3-Basic alone, suggesting that they are not sufficient to drive Pax9 gene transcription.

Key words: Pax9. Transcriptional analysis. Promoter region. pGL3.

Introduction

The gene PAX9 belongs to a family composed of nine genes that encode transcription factor proteins, the Pax proteins, characterized by the presence of the paired domain, and a highly conserved DNA binding region of about 128 amino acids [1, 2]. These factors play important roles in embryonic patterning and organogenesis being expressed in various embryonic tissues like somites, pharyngeal pouch endoderm, distal limb buds and neural crest-derived mesenchyme that develop into craniofacial structures, like teeth, expressed in the mesenchyme of developing tooth germs, especially at the bud and cap [8, 11,] and are essential during later stages of tooth development [2, 5, 9, 12]. It is known that they are under the control of Fgf/Bmp signaling [4].

Pax9 protein products are essential for the establishment of the odontogenic potential of the mesenchyme, where the expression seems to be a marker for the sites of tooth formation due to its occurrence before any morphological manifestation of this process [9]. Analysis of mouse embryos showed that the Pax9 is an early marker of tooth development, appearing at E10 stage in the mesenchyme, before the ectodermal thickening and prior to the expression of other tooth signaling genes. High levels of Pax9 expression are subsequently maintained throughout the initiation (E11.5), bud, and cap stages and down regulated at the bell stage (E16)[11].

The initial description of the tooth agenesis caused by the mutation in Pax9 was made by Stockton et al. [14], since then, many other autosomal dominant mutations have been identified throughout the entire gene, been the majority of mutations is located in the paired domain, the DNA-binding domain of PAX9. Polymorphisms in the upstream promoter region of the human PAX9 have been associated with variable forms of oligodontia [10]. To date, it is known that heterozygous deletion of whole PAX9 gene is associated with a severe form of non-syndromic tooth agenesis that involves all the primary molars and some posterior permanent teeth (premolars and molars) [3, 5, 6, 14]. However, the precise mechanisms for the development of tooth agenesis remain unclear [17].

The aim of the present study is to verify the influence of promoter sequences in the transcription of PAX9 gene. Two fragments of the promoter region were cloned into reporter plasmid containing luciferase gene and transfected into three different rat embryo tissues: digits, face and midbrain and hindbrain regions.

Material and Methods

Construction of Expression Plasmids - The PAX9 promoter region between positions – 1209 and +92 was amplified from human DNA by PCR and subcloned into SacI-HindIII restriction sites of TOPO TA vector (Invitrogen) to generate high copies for cloning into pGL3-Basic vector. The second plasmid was constructed by the pGL3-Basic-PAX9 promoter digested with ApaI restriction enzyme, resulting on an insert with 590 bp (pGL3-Basic-PAX9-ApaI). In both, the +92 base was oriented to luciferase gene (fig. 1).

Maternal and Fetal Surgical Manipulation - The day of sperm found is E0, and at day 13.5 pregnant females was anesthetized with Ketamine (100mg/ml) and after the surgery, it was killed by cervical dislocation and the uterus was aseptically removed out into a 25ml screw-capped tube containing 20ml Phosphate-Buffered Saline (PBS) and 1% antibiotic solution (Anti/Anti Invitrogen). Embryos were dissected out the uteri in a flow hood area and transferred to a fresh dish of sterile PBS. The embryos digits, face and midbrain and hindbrain regions (these cells will be referred as CNS) were placed in 35mm cell culture dishes. Experiments were performed in duplicate. The cells were grown in

Dulbecco's Modified Eagle's Medium DMEM – High glucose (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic solution (Anti/Anti - Invitrogen) and incubated at 37 ° C in the presence of 5% CO_2. Ethical approval was granted by the Ethics Committee of the Piracicaba Dental School.

mRNA analysis - After 48 hours of culture, cells were homogenized and total RNA were isolated with TRIzol™, following manufacturer's instructions (Invitrogen). The cDNA was synthesized from 1μg of total RNA using Superscript III Reverse Transcriptase following manufacturer's instructions (Invitrogen). DNase I digestion of RNA was performed prior to the reverse transcriptase reaction. In two independent experiments, PCR amplifications were performed using gene-specific primers for Pax9 and β-actin genes. Pax9 primer sequences were: Pax9Rat 5' (GAGTTCCATCAGCCGGATTC) and Pax9Rat 3' (CAAGGCTCCCTTCTCCAATC). Polymerase chain reactions were performed on a TC-512 PCR machine (Techne Incorporated Burlington, NJ, USA) using 5μL 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 40 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 minutes for all set of RT-PCR primers. β-actin gene amplifications were performed in separated tubes. PCR program initially started with a 94°C denaturation for 4 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 minutes. Amplifications were verified in 2% agarose gels 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.

Transient transfection - 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. 0.8 μg of pGL3 (Promega™) was used for a co-transfection with 0.08 μg of the pRc-CMV vector (kindly provided by Dr. Kleber Franchini - Unicamp) for luciferase analysis normalization. Transfected cells were incubated at 37°C in the presence of 5% CO_2.

Luciferase analysis - Forty eight hours after the transfection, cell extracts were collected and firefly and Renilla luciferase activities were measured (dual glo luciferase assay system; Promega™). Briefly, 75μL of remain DMEM serum free medium were mixtured with 75uL of Dual-Glo™ Luciferase Reagent and incubated for 1 min. The lysate were measured for firefly luciferase activity in 96-well microplate reading luminometer (Veritas™ - Turner Biosystems). Each sample was normalized to Renilla luciferase absorbance to correct for variations in transfection efficiency using 75μL of Stop & Glo® Reagent added to the same well and incubated for 10 min after reading. Experiments were performed in duplicate.

Results

mRNA analysis - In our experiments, a semi-quantitative assay RT-PCR was performed to measure the in vitro expression of the gene Pax9, using E13.5 rat embryonic cell in culture. The results indicated that the Pax9 gene is not expressed in face at this day, while in digits and CNS this gene is expressed (figure 2). The presence of amplicons for the

amplification of ß-actin gene validates our approach, indicating that the gene is repressed in face tissues of the evaluated embryos.

Luciferase analysis – The experiments revealed that both transfected constructions pGL3/Pax9 plasmids were not able to highly express the Luc protein. However, values of expression were found in decreasing the protein synthesis in digits and CNS transfected cells, and inhibited it in face transfected cells (figure 3).

Discussion

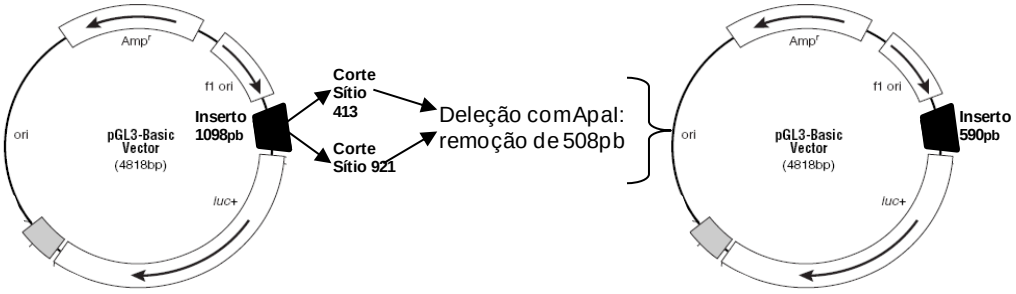
In this study, Pax9 transcriptional regulatory pattern was examined by analysis of its promoter sequence in rat embryos. In our experiments semi-quantitative RT-PCR was performed to detect gene expression of Pax9 in vitro by using rat embryonic cell. We also analyzed the capacity of Pax9 promoter region to drive the transcriptional activity by transfecting pGL3 basic plasmids vectors containing inserts of Pax9 promoter region in rat embryos primary cell culture of digits, face and CNS.

Experiments showed that Pax9 gene were expressed in day E13.5 in limb bud and CNS cells, but surprisingly was not expressed in cells derived from facial region. Kriangkrai et al [7] showed that Pax9 was expressed on facial region of rats at stages E13 and E14. One possible explanation for this discrepancy is that the transcription of PAX9 was inhibited when facial cells were cultured. In fact, one thing that is evidenced in studies that analyze the expression of PAX9 in mouse and rat embryonic tissues is that the expression of this gene changes rapidly during ontogeny [7, 9]. Therefore, it is conceivable PAX9 transcription may be influenced subtle changes in cell environment. In the present study two polymorphisms in the PAX9 promoter located within the region, shown to be associated with third molar agenesis in humans, were analyzed [15]. Moreover, two conserved non-coding regions with enhancer activities for expression of Pax9 were already identified, where one of these sequences contained a consensus Gli-binding motif [13]. In addition, those authors also identified highly conserved sequences in the 5' regions of Pax9 gene that were located about 2kb distant from the transcription initiation site and in the third intron of this gene.

Conclusions

The results of the present study showed that the luciferase activity of pGL3-Pax9 constructs were weaker than pGL3-Basic vector alone, suggesting that PAX9 5' flanking sequences alone are not sufficient to drive Pax9 gene transcription and that this region might contain inhibitory cis-acting sequences. These results also indicate that enhancer sequences located in 5' sequences distant from the transcription origin or in intronic regions are necessary for Pax9 transcription.

Legends



pGL3B+ Inserto de 1098pb

pGL3B+ Inserto 590pb após deleção e ligação

ApaI G-GGCC'C - 2 Cortes: local 413 e 921 – Coesivo
C'CCGG-C

Figure 1: Construct Pgl3Pax9/ApaI (-1106 to + 92) was digested with ApaI and re-ligated resulting in a plasmid containing the 590bp (by Ramenzoni, L.L.).

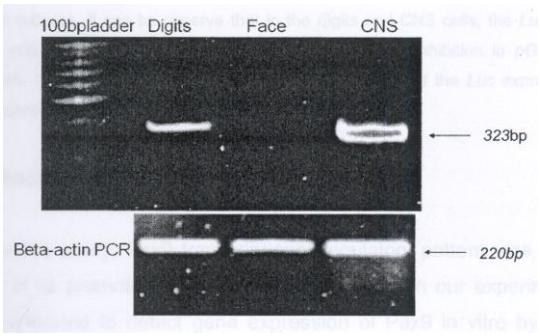


Figure 2: Pax9 expression in rat embryos cells were detected by semi-quantitative PCR in 2% agarose with ethidium bromide.

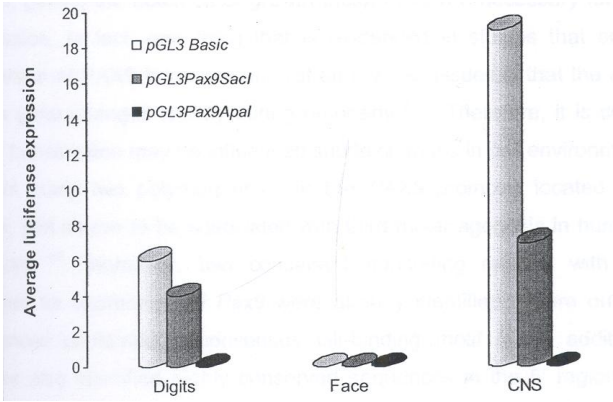


Figure 3: Luciferase expression and inhibition evidences in rat embryo 13.5 days of digits, face and CNS cells cultures. It can be observed that in the digits and CNS cells, the Luc was down expressed in pGL3Pax9SacI transfected cells, and its complete inhibition in

pGL3Pax9ApaI transfected cells. In face cells, there was the complete inhibition of the Luc expression of all plasmids transfected cells.

Acknowledgments

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COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

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, FÁBIO JOSÉ BIANCHI e LIZA LIMA RAMENZONI**, 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.

Piracicaba, 26 de setembro de 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, FÁBIO JOSÉ BIANCHI and LIZA LIMA RAMENZONI**, 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.

Piracicaba, SP, Brazil, Setember 26 2005

Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 1

ANEXO 2

[JAOS] Article Submission Advise — Terça-feira, 3 de Março de 2009 8:04

De: "Carlos Ferreira dos Santos" <appscielo@bireme.org>

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Article: "JAOS-1021 - Transcriptional analysis of the human PAX9 promoter"

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