

Evaluation of the Impact of Alveolar Bone Resorption on the Root Formation of Molars in Transgenic Mice with RANK Over-expression

Evaluación del Impacto de la Resorción Ósea Alveolar sobre la Formación Radicular de Molares en Ratones Transgénicos con Sobreexpresión de RANK

Berdal, Ariane^{*}; Betancur, John Jairo^{**}; Morales, María Carolina^{***}; Pimienta, Carolina^{***} & Castañeda, Beatriz^{****,*****}

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ABSTRACT: Alterations in the balance of the osteoblastic-osteoclastic activity (osteopetrosis, osteolysis) have an impact on the dental development. The over activation of the osteoclastogenesis pathway RANK/RANKL/OPG in RANK^{Tg} mice produces an acceleration of tooth eruption and root elongation, suggesting this pathway could control the speed of tooth formation. Evaluate the effect of osteoclastic hyperactivity on the root formation of molars in RANK^{Tg} mice. Histologic study both descriptive and comparative of the impact of hyper-resorption of the alveolar bone in Hertwig's epithelial root sheath (HERS) of molars in both RANK^{Tg} mice and controls. This is done through the immuno-detection of matrical, epithelial and cellular proliferation proteins with histological, histoenzymology and immunohistochemical techniques. Osteoclastic hiperactivity in alveolar bone does not alter the root structure and integrity of molars in RANK^{Tg} mice; the acceleration in root formation does not alter the HERS integrity. An area of cellular hyper-proliferation in the apical follicular tissue of HERS was found, which could regulate root growth in response to osteoclastic activity. The overexpressed RANK produces an inhibition of amelogenin expression at 5 days of age, suggesting an indirect regulation of these cells by RANK/RANKL. Exploring other molecular factors expressed in HERS, and the related engram, would make possible the use of new therapies for the control of osseous and inflammatory pathologies during root formation.

KEY WORDS: bone resorption, root development, epithelial cells.

INTRODUCTION

The teeth and alveolar bone form both a structural and functional unit. During growth, even a minute disturbance in one of these two elements will have repercussions on the other. Therefore, in agenesis cases, the alveolar bone is not formed (Orestes-Cardoso *et al.*, 2001) and in reciprocity, in alterations of the alveolar bone modeling, like osteopetrosis (Del Fattore *et al.*, 2007; de Vernejoul, 2008) or osteolysis (Osterberg *et al.*, 1988; Mitchell *et al.*, 1990; Dickson *et al.*, 1991; Hughes *et al.*, 1994; Hughes *et al.*, 2000; Olsen *et al.*, 1999; Palenzuela *et al.*, 2002; Font, 2002),

the tooth development is affected with a severity proportional to the degree of the osseous alteration (Helfrich, 2005).

The studies which describe the effects of osteopetrosis (Kornat *et al.*, 2001; Chalhoub *et al.*, 2003; Bjorvatn *et al.*, 1979; Friede *et al.*, 1985; Bénichou *et al.*, 2000; Gelb *et al.*, 1996) and osteolysis (Osterberg *et al.*; Mitchell *et al.*; Dickson *et al.*; Hughes *et al.*, 1994, 2000; Olsen *et al.*; Palenzuela *et al.*; Font) in the craneofacial complex have suggested that

^{*} Ph.D in Sciences Biologiques, Université Paris 7 Denis Diderot, Director of the Laboratory of Molecular Oral Pathophysiology, Centre de Recherche des Cordeliers, Institut National de la Santé et de la Recherche Médicale, Paris, France.

^{**} Specialist in Orthodontics, associate Professor, School of Dentistry, University of Antioquia, Medellín, Colombia.

^{***} Specialist in Orthodontics, School of Dentistry, University of Antioquia, Medellín, Colombia.

^{****} Ph.D in Sciences Biologiques, Associate Professor, School of Dentistry, University of Antioquia, Medellín, Colombia.

^{*****} Research Associate at the Laboratory of Molecular Oral Pathophysiology, Centre de Recherche des Cordeliers, Institut National de la Santé et de la Recherche Médicale, Paris, France.

osteoclastic activity of the alveolar bone will have an impact on the tooth development and specifically on the root formation (Helfrich), but the molecular mechanisms responsible for the interactions between dental and bone cells are not understood.

The receptor activator of nuclear factor kB ligand (RANKL), the receptor activator of nuclear factor kB, (RANK), and osteoprotegerin (OPG) constitute the main pathway of osteoclastogenesis. RANKL (TNFSF11), synthesized by stromal cells, is an activator of the osteoclastic differentiation through its binding to the RANK (TNFRS11A) receptor which is present in the surface of the osteoclast precursor cells (Theill *et al.*, 2002). OPG on its own, is a soluble protein whose structure is similar to RANK, which allows it to bind to RANKL and block its inductor effect on osteoclastogenesis (Lacey *et al.*, 1998). These three proteins are implicated in numerous developmental phenomena, like the maturing and activation of osteoclasts, the communication between dendritic cells and T lymphocytes, the organogenesis of lymphatic ganglia, the development of mammary glands and pilous follicle, tooth eruption, and the survival of endothelial cells of blood vessels (Theill *et al.*).

Some studies have demonstrated the direct implication of the RANKL/RANK/OPG triad in early dental morphogenesis (Pispa *et al.*, 1999; Lauriikkala *et al.*, 2001; Ohazama *et al.*, 2003a, 2003b, 2004), in physiological root resorption (Fukushima *et al.*, 2003), in alveolar bone modeling during growth (Heinrich *et al.*, 2005), and in tooth eruption (Wise *et al.*, 2002).

In other circumstances such as orthodontic movement, changes in the RANK, RANKL and OPG expression in the dental supporting tissues (Oshiro *et al.*, 2002) have been demonstrated, but its effect on tooth development, and specifically on root formation, has not yet been reported.

A recent study pertaining to the characterization of the dentoalveolar phenotype in the transgenic mouse with overexpressed RANK (Castaneda *et al.*, 2010) showed that in these mice, which present a hyper-resorption of the alveolar bone, tooth eruption and root growth were more precocious than in the control mice or wild type without presenting great morphological or functional modifications. These results suggest the activity of the osteoclasts during alveolar bone modeling could exert some control over the root formation.

The purpose of this study was to evaluate the effect of the osteoclastic hyperactivity of alveolar bone on the root formation of molars using a transgenic mouse model of RANK over-expression in osteoclast precursors (Castaneda *et al.*; Duheron *et al.*, 2011), having Hertwig's epithelial root sheath (HERSRANK) as the reference structure.

MATERIAL AND METHOD

A descriptive and comparative analysis was done of the impact of alveolar bone hyper-resorption on root integrity and on the cells of Hertwig's epithelial root sheath (HERS) between type CD1 mice with overexpressed RANK and control mice (wild type).

Frontal sections were made every 8 μ at the level of the first mandibular molar in a total of 173 mice: 71 RANK^{Tg} and 102 controls or wild type, classified in 5 subgroups according to age: 3–5 days, 7–10 days, 11–14 days, 15–20 days, and 21–28 days (Table I).

The histological stains used to evaluate the structure and integrity of the mandibular alveolar bone, periodontal ligament, and the root were Masson Trichrome stain and Collagen 1a1 immunostaining. TRAP histoenzymology was used to identify clastic cells (osteoclasts and odontoclasts). The cell proliferation markers PCNA and P21, were used to associate the areas activated during root growth with the areas of resorption hyperactivity in the alveolar bone. Keratin 14 was used to trace the epithelial identity of root cells and to facilitate their quantification. Amelogenin was used as a marker of the transformation of enamel epithelium to root epithelium. Finally, the immunolocalization of the RANK, RANKL and OPG factors allowed the evaluation of the effect of the overactivation of the RANK pathway in the epithelial cells of HERS.

Animal Model. The RANK^{Tg} mice of CD1 type used in this study (Fig. 1) (Castaneda *et al.*; Duheron *et al.*) are crossbred, reproduced, and sacrificed by people certified for animal experimentation following the protocols validated by the management of veterinarian services (Ministry of Agriculture of France). The postnatal age was determined starting at the day of birth (12 h: day 1). The genotype of these mice is determined by PCR (Polymerase Chain Reaction) with 100 ng of genomic DNA extracted from the section of the last 5 millimeters of each animal's tail (Fig. 1).



Fig. 1. RANK^{Tg} Mouse A. Construction of the de RANK^{Tg} sequence under the MRP8 human promoter. B. Genotyping of the control mice (wild type) and RANK^{Tg}, the sign corresponds to the RANK inserted sequence (both mice at 8 days).

For the RANK transgene, the following two sequences were used:

5'FlagRANK : ATGGACTACAAAGACGATGACGAC
3'RANK : TGCCAGGATCCACCGCCACCA

The amplification conditions were: 95 °C (10 s), 64 °C (30 s) and 72 °C (1 min) for 35 cycles. After the amplification by PCR, the tube contents are run on a 2% agarose gel. RANK^{Tg} produces a 320 pb band (Fig.1).

A minimum of 5 mice were analyzed per age group, between control mice or wild type and RANK^{Tg} mice (Table I). The age groups were defined according to the molecular activity curves reported during the eruption process and root elongation process (Wise *et al.*; Castaneda *et al.*) For all evaluations, control mice and transgenic mice of the same cohort were compared.

Histology. The heads of the mice are demineralized for 4 weeks at 4 °C in an agitator with PBS (*Phosphate Buffer Solution*) pH 7.4 containing 10% of ethylenediaminetetraacetic (EDTA). This decalcifying solution is changed every week. After demineralization, all samples are washed in PBS 1X at 4 °C, dehydrated in ethanol solutions at increasing concentrations (70-85-95-100%), then they are coated in paraffin through successive immersions at 65 °C in toluene, toluene/paraffin, and pure paraffin. The samples are then

decreasing ethanol solutions at 100, 90, 80, 70, 50, 30% for 2 min in each solution, continuing with a rehydration in tap water and then in distilled water (2

Table I. Number of animals evaluated by age group.

Age group	WT	RANK +	Total
3–5 days	29	20	49
7–10 days	21	15	36
11–14 days	24	13	37
15–20 days	11	8	19
21–28 days	17	15	32
Total			173

inserted in paraffin molds adapted to room temperature. Serial frontal sections of 8 µm in thickness are done with a microtome (RM 2145 Leica, France).

The sections are collected in slides coated with poly-L-Lisine (100 mg/ml) (Sigma-Aldrich St. Louis, USA), and used for Masson's Trichrome, immunohistochemistry and immunohistochemical stains. After Masson's Trichrome stain, the section is mounted between the slide and coverslip with Eukitt (K, Freiburg, Germany), observed and photographed with a photonic microscope (DMRB Leica, France).

Masson's Trichrome Stain. The sections are deparaffinized in Safesolv (Labonord, Z.I de Templemars, France) for 5 min and immersed in

min). Then, the slides are stained with Hematoxiline (10 min), Fuchsin-ponceau (5 min), Orange G phosphotungsten (5 min), and Luminous green (2 min). Finally they are mounted with Eukitt resin (K, Freiburg, Germany).

Tartrate-resistant Acid Phosphatase (TRAP) Histoenzymology. The sections are deparaffinized by immersing them in room temperature toluene for 5 minutes, rehydrated through immersion in decreasing ethanol solutions (100, 95, 80, 60, 30%), rinsed with sterile water (5 min), before the PFA (Paraformaldehyde) 4% fixation (5 min) at room temperature. The slides are then immersed in Tartrate-resistant acid phosphatase (TRAP) solution which contains 60 mg of Naphtol AS-TR-phosphate, 300 µl of N-N dimetilformamide, 60 mg of acetate buffer PH 5.2, 1.38 g of sodium tartrate, and 60 mg of FastRedTRsalt, at room temperature and in a dark and humid chamber until a red marker is obtained. The slides are rinsed with sterile water at room temperature for 5 min and then they are mounted with Aquamount (BDH Laboratories, France).

Immunohistochemistry. The sections are deparaffinized in toluene (5 min), rehydrated by immersing them in decreasing grades of ethanol solutions (100, 95, y 70%, 5 min each), followed by immersion in water, and then in PBS 1X.

For peroxidase conjugated antibodies (Table II), the endogenous peroxidases are inactivated by treating them with H₂O₂ (hydrogen peroxide). The slides are rinsed three times, each for 3 min, in PBS 1X; the tissue is saturated with 10% goat serum at room temperature for 60 min (buffer). The sections are incubated with the primary antibody in the concentration indicated by the manufacturer (Table II) at 4 degrees centigrade for one night, leaving one section in a slide as a negative control by incubating it in a buffer instead of in the primary antibody. The sections go again through 3 rinses PBS 1X and all sections are incubated with the secondary antibody indicated by the manufacturer (Table II). After rinsing with PBS 1X, the reaction to the enzymatic activity HRP (Horseradishperoxidase) is done using the

Table II. Inputs used in immunohistochemical protocol.

Primary antibody	Type	Code M®	Dilution	Secondary antibody type	Code M®	Dilution	Description
RANK	Anti-human RANK/ TNFRSF11A	R&D AF683	1/20	Anti-goatIgG	Jakson 805-065-180	1/400	Biotin-SP-conjugated, Pure Bovine; bovine/streptavidin Horseradish Peroxidase Vector
RANKL	Anti-human TRANCE/TNFSF 11/RANKL	R&D AF626	1/20	Anti-goatIgG	Jakson 805-065-180	1/400	Biotin-SP-conjugated, Pure Bovine; bovine/streptavidin Horseradish Peroxidase Vector
OPG	Anti-human Osteoprotegerin/ TNFRSF11B	R&D AF805	1/10	Anti-goatIgG	Jakson 805-065-180	1/400	Biotin-SP-conjugated, Pure Bovine; bovine/streptavidin Horseradish Peroxidase Vector
COLLAGEN I	Rabbit Policlonal	Abcam ab292	1/100	GoatAnti-Rabbitpoliclonal	Dako P 0448	1/200	Peroxidase conjugated
PCNA	Rabbitpoliclonal	Abcam ab2426	1/500	GoatAnti-Rabbitpoliclonal	Dako P 0448	1/500	Peroxidase conjugated
P21	Rabbitpoliclonal	SC- 397	1/50	GoatAnti-Rabbitpoliclonal	Dako P 0448	1/200	Peroxidase conjugated
KERATINA 14	Rabbitpoliclonalke ratina 14	Covance AF 64	1/1000	Biotinylated anti-rabbitIgG	Vector BA-1100	7/1000	Streptavidin-AP conjugated, ROCHE sistema NBT/BCIP
AMELOGENIN	Anti-amelogenin Rabbitpoliclonal	Kamia PC-062	1/1000	Fluorophorlabeled Donkey anti-rabbit	Invitrogen A 21206	1/500	Fluorescence

NovaRED kit (Vector, Laboratories, Burlingame, USA) and DAB (DAKO) kit for 15 min. The sections are then dehydrated and mounted with Eukitt (K, Freigburg, Germany). For the RANK/RANKL/OPG antibodies the Biotine-Streptavidine peroxidase system was used (Vector).

For Collagen I α 1 and the Keratin, the section undergo an unmasking of the antigens in a citrate buffer before incubating them in the buffer (time according to manufacturer).

To compare the number of epithelial cells present in the Hertwig's epithelial root sheath (HERS) and in the epithelial cell rests of Malassez (ERM) between the molars of RANK^{Tg} mice and the controls, a quantification of cells marked with Keratin 14 was done for each molar and averaged according to the number of sections; the statistical analysis was done with the t-test GraphPadPrism program. The sections from two age groups were compared: the start of root formation in control mice between 5 and 7 days of age and in transgenic mice between 3 and 5 days of age, and root elongation between 14 and 21 days of age, according to the growth spurts reported by a previous study (Castaneda *et al.*).

Immunofluorescence. Tissues mounted in Freeze Gel (Labonord, Z.I. de Templemars, France) were used for cryostat sections. The decalcified heads of mice were immersed for 24 h in a 4% PFA bath, then in PBS1X, and then the tissues were saturated in 15% sucrose and 30% PBS1X. The slides with the sections were air dried, then the sections were saturated in 1% PBS-BSA for 30 min to block nonspecific sites. The slides are incubated with the primary antibody (Amelogenin: Kamia Biomedical Company, Seattle, USA) diluted in PBS at room temperature for an hour. After rinsing three times with PBS for 5 min each, the sections are incubated with the secondary antibody at room temperature for an hour, rinsed and incubated for 10 min with DAPI (4,6-Diamidino-2-phenylindole dihydrochloride). After rinsing with PBS, the slides are mounted with cover slips and a fluorescence mounting medium Fluoprep (BioMérieux, Marcy l'Etoile, France).

RESULTS

With the purpose of analyzing the impact of alveolar bone modeling over the root growth in molars, the structure and integrity of the mandibular alveolar

bone of RANK^{Tg} mice was initially evaluated at 5, 7, 11, 14, 15, 17 and 21 days of age using the expressed Collagen Ia1 compared to the control mice of the same ages. The use of this marker allowed an initial evaluation of the integrity of the root matrix, specifically the root dentin. The alveolar bone of RANK^{Tg} mice shows a more trabecular and less dense structure plus a decrease in its size compared to 5 day old control mice (Fig. 2A and B). There is an absence of alveolar bone between the dental nerve and HERS in the molars of RANK^{Tg} mice whereas it is present in control mice (arrows in Fig. 2C). This result coincides with an increased osteoclastic activity in the bone adjacent to the growing molar in RANK^{Tg} mice, evident through the TRAP marker (Fig. 2A). The Hertwig's epithelial root sheath, HERS, presents evident alterations during root growth, keeping its integrity in mice with over-expressed RANK (Fig. 2B).

In a more advanced age (14 days) it was observed that the fibers of the periodontal ligament in the RANK^{Tg} molars present a disorganized array and the insertion is not well defined compared to the control molars (Fig. 3B). At the apical level, the tissue is also not well defined as compared with the control molars (Figs. 3A-B).

Additionally, the bone surface adjacent to the periodontal ligament in 11 to 14 days old RANK^{Tg} mice shows an increase in positive TRAP cells when compared to control mice which have none of these cells in the dentin (arrows Fig. 3C and D). There are no observed evident changes in the expressed collagen profile of both bone and dental structures (Figs. 3E and F).

The cellular proliferation was evaluated using PCNA (Proliferating Cell Nuclear Antigen) immunostaining to enable the association of accelerated growth of the molar root with the alveolar bone modeling in RANK^{Tg} (Castaneda *et al.*, 2010) mice. The use of the stain showed an evidence of increase cellular proliferation in the peridental tissues of RANK^{Tg} mice with respect to the control animals (Fig. 4). At 17 days of age, the stain is more evident at the apical level (arrow Fig. 4D).

At 5 days of age, a strong proliferation of the pulp tissue in the cusps was found (Fig 4 A and C), so an evaluation with P21, a marker for enamel nodules (Matalova *et al.*, 2012), was used. A strong expression in the apical tissue adjacent to HERS in 5 days old RANK^{Tg} mice (Fig. 5 B and D) in comparison

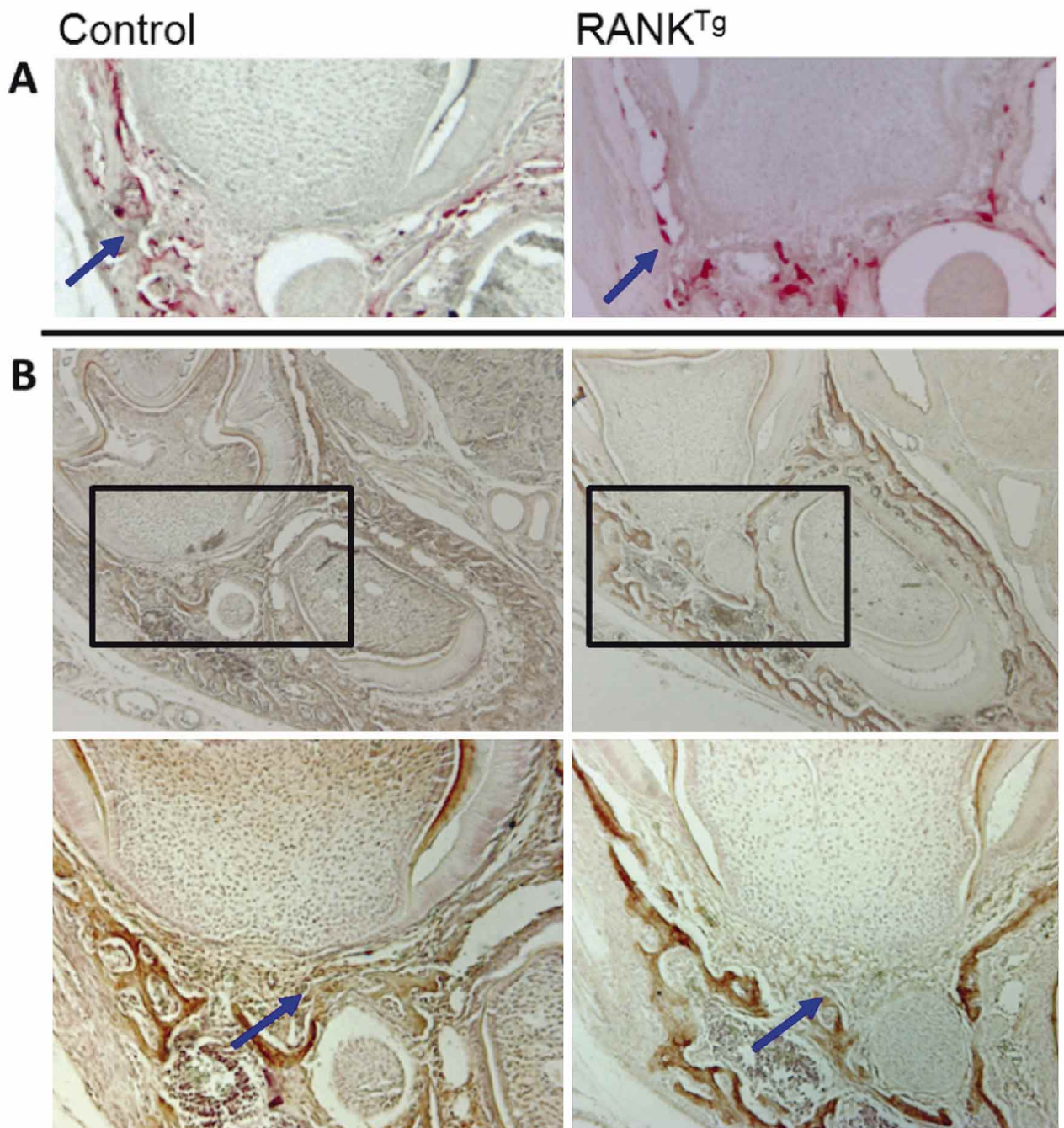


Fig. 2. Comparative histologic analysis of the periodontal unit between 5 day old control mice (Left) and RANK^{Tg} mice (Right). A. TRAP Histoenzymology: The arrows point to the positive TRAP cells in the alveolar bone. B. and C. Collagen I δ 1 Immunohistochemistry: the arrows in C point to the absence of alveolar bone between HERS and the dental nerve of RANK^{Tg} molars, compared to control molars. Scale A: 10X/0.30; B: 5X/0.12; C: 20X/0.50.

to no such evidence in control mice of equal age (Fig 5 A and C) was observed. This is not evident in 14 days old mice.

To evaluate the effect of hyperactivity of the

apical tissues on the proliferation of epithelial root cells, the number of epithelial cells of HERS and of the epithelial cell rests of Malassez were quantitatively compared, using Keratin 14 marker to identify them. The results show that the profile of Keratin present

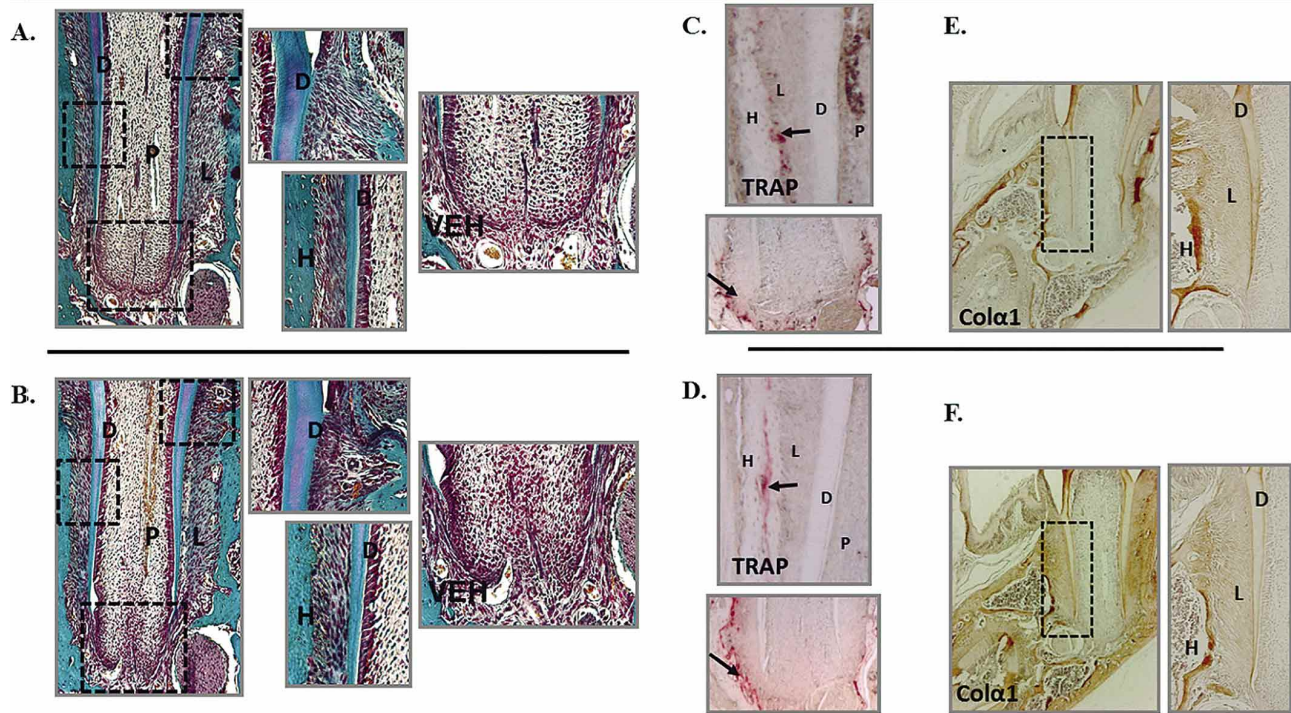


Fig. 3. Comparative histologic analysis of the phenotype of the periodontal unit of 14 day old molars, between control mice and RANK^{Tg} mice. A. Masson's Trichrome Stain. Scale: 10X/0.30h control mouse. B. Masson's Trichrome Stain. Scale: 10X/0.30h RANK^{Tg} mice. C. TRAP Histoenzymology. Scale: 10X/0.30h (upper) and 20X/0.50h (lower). Control mouse. D. TRAP Histoenzymology. Scale 10X/0.30h (upper) and 20X/0.50h (lower). RANK^{Tg} mice. E. Collagen Iα1 Immunohistochemistry in control mice, scale 20X/0.50h. F. Collagen Iα1 Immunohistochemistry in RANK^{Tg} mice, scale 20X/0.50h. H:bone, D:dentin, L: ligament, P: pulp.

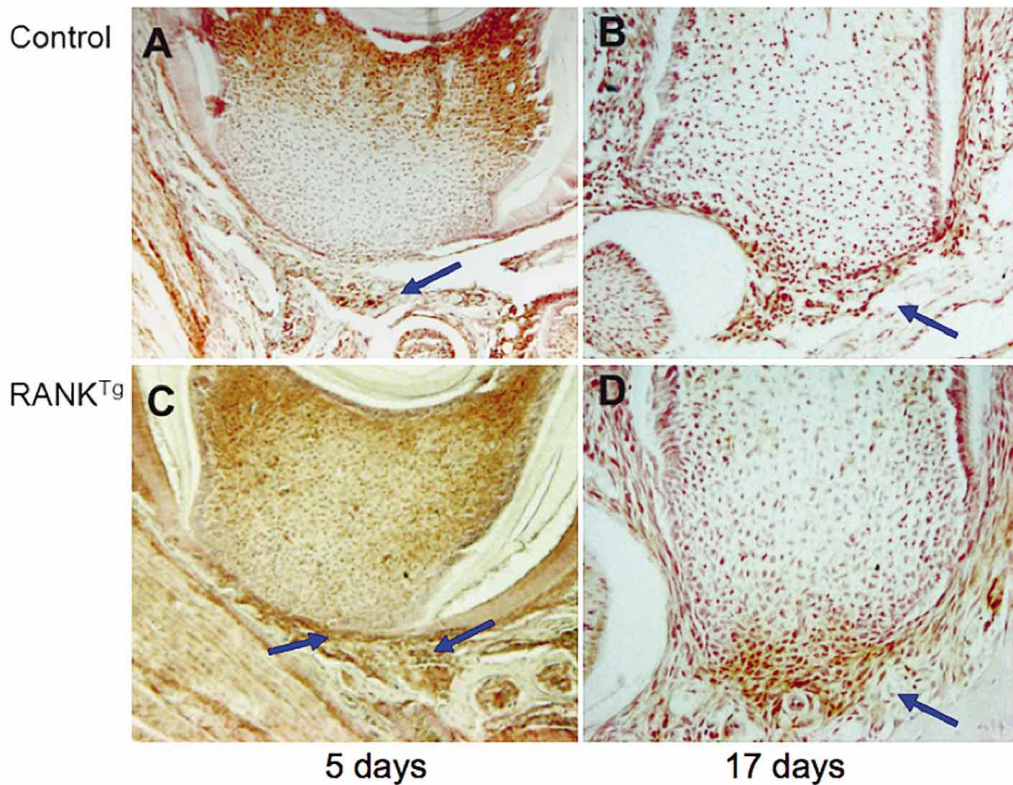


Fig. 4. Cellular proliferation immunohistochemistry with PCNA immunostaining. First mandibular molar of control mice (A and B) and RANK^{Tg} mice (C and D) at 5 and 17 days of age. The arrows show the difference in cellular proliferation in the region adjacent to HERS. Scale: A and C: 10X/0.30; B and D: 20X/0.50

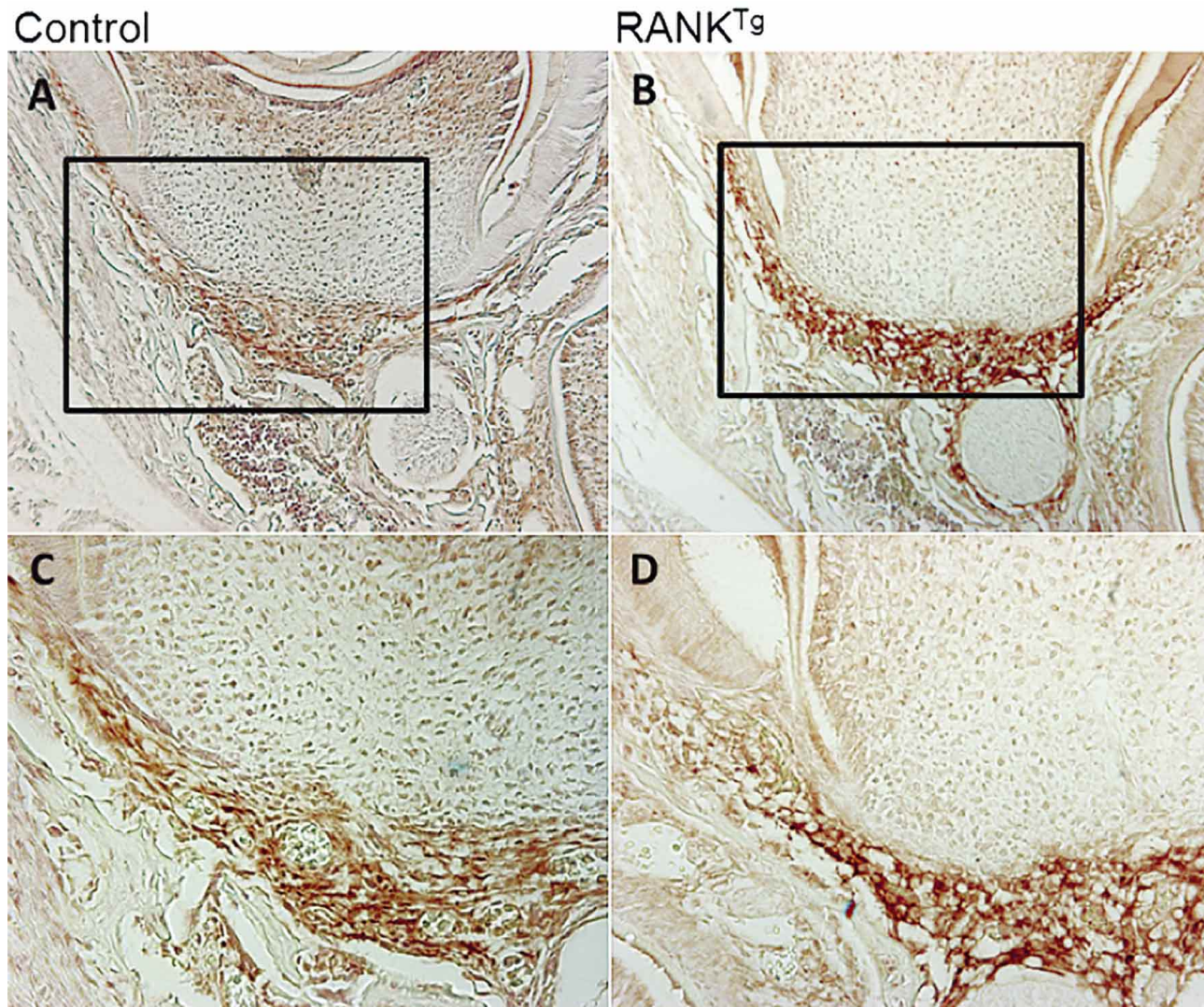


Fig. 5. Comparative analysis of cellular proliferation with immunohistochemistry of expressed P21 in the mandibular molar at 5 days of age. A. Control molar, scale 10X/0.30 B. RANK^{Tg} molar, scale 10X/0.30. C and D. Amplified rectangle in A and B shows the Hertwig's Epithelial Root Sheath region. Scale 20X/0.50.

no alterations in the RANK^{Tg} molars when compared to the controls (Figs. 6 A and B). The expression of Keratin 14 is specific to epithelial cells of HERS at the studied ages and for epithelial cell rests of Malassez starting at 14 days of age (arrows Fig. 6). But, the number of marked cells in HERS at 3-7 days of age is greater in the molars of RANK^{Tg} mice than in control molars (Fig. 6C). In the 14 to 21 day age group, the number of epithelial cells in the root sheath shows no significant difference between molars of transgenic and control mice (Fig. 6C, graph 14 - 21 days of age). The number of ERM (Epithelial Cell Rests of Malassez) in this age group is greater in RANK^{Tg} molars than in the molars of the control group.

The start of root formation was determined with the amelogenin marker (Fig. 7), keeping in mind that its expression implies the transformation of enamel cells into root cells (Aioub *et al.*, 2007). The stain was present in the epithelial sheath of control molars of 5 days of age while at the same age, the stain was absent in RANK^{Tg} molars.

The expression of RANK, RANKL and OPG proteins was evaluated through immunohistochemistry with the intent of identifying if the stimulation of the cell proliferation in HERS is a direct activation of the over-expressed RANK. RANK was found in pulp cells, in preodontoblasts (arrows Fig. 8b), and in the cells

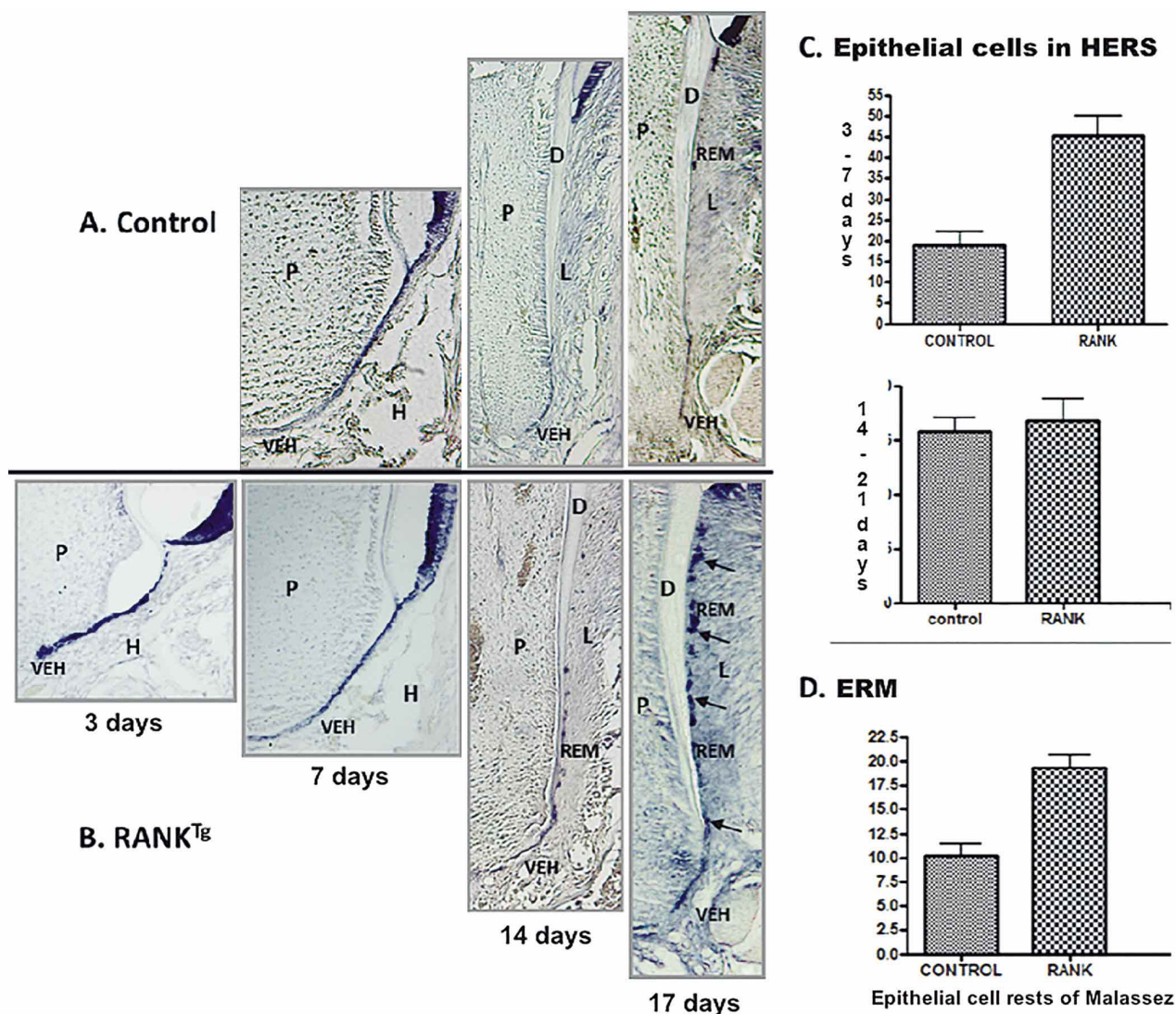


Fig. 6. Immunohistochemistry of the expressed Keratin 14 during root formation. Staining of the epithelial cells of the root. Scale A-F: 10X/0.30. A. Control mice at 7, 14 y 17 days of age and B: RANK^{Tg} mice at 3, 7, 14 and 17 days of age. C. Comparison of the number of epithelial cells in HERS in control molars between 3-7 days of age and RANK^{Tg} molars. Statistical analysis and standard deviation calculation using t-test: GraphPadPrism. D. Comparison of the number of epithelial cell rests of Malassez (ERM) at 14-21 days of age between control molars and RANK^{Tg} molars. Statistical analysis and standard deviation calculation using t-test: GraphPadPrism.

of the apical follicular tissue (star Fig. 8a and arrow Fig. 8g). RANKL, was found to be expressed in the pulp cells adjacent to HERS (arrow Fig. 8d) and in the apical follicular tissue and alveolar bone (stars Fig. 8c and 8d). Finally, the expressed OPG was equally found in the pulp cells adjacent to HERS (Fig. 8f), in the apical follicular tissue and alveolar bone (stars Fig. 8e and 8f), although less strong than the RANKL expression for RANK^{Tg} molars (Fig. 8f vs 8d), but similar to control molars (Figs. 8h and 8i).

DISCUSSION

Some studies on osteopetrotic mice models (de Vernejoul; Helfrich; Aïoub *et al.*, 2007) and in RANK^{Tg} mice with hyper-resorption of the alveolar bone (Oshiro *et al.*) have shown the impact of osteoclastic activity on tooth development (Helfrich).

The first phenotypic study of the RANK^{Tg} mouse

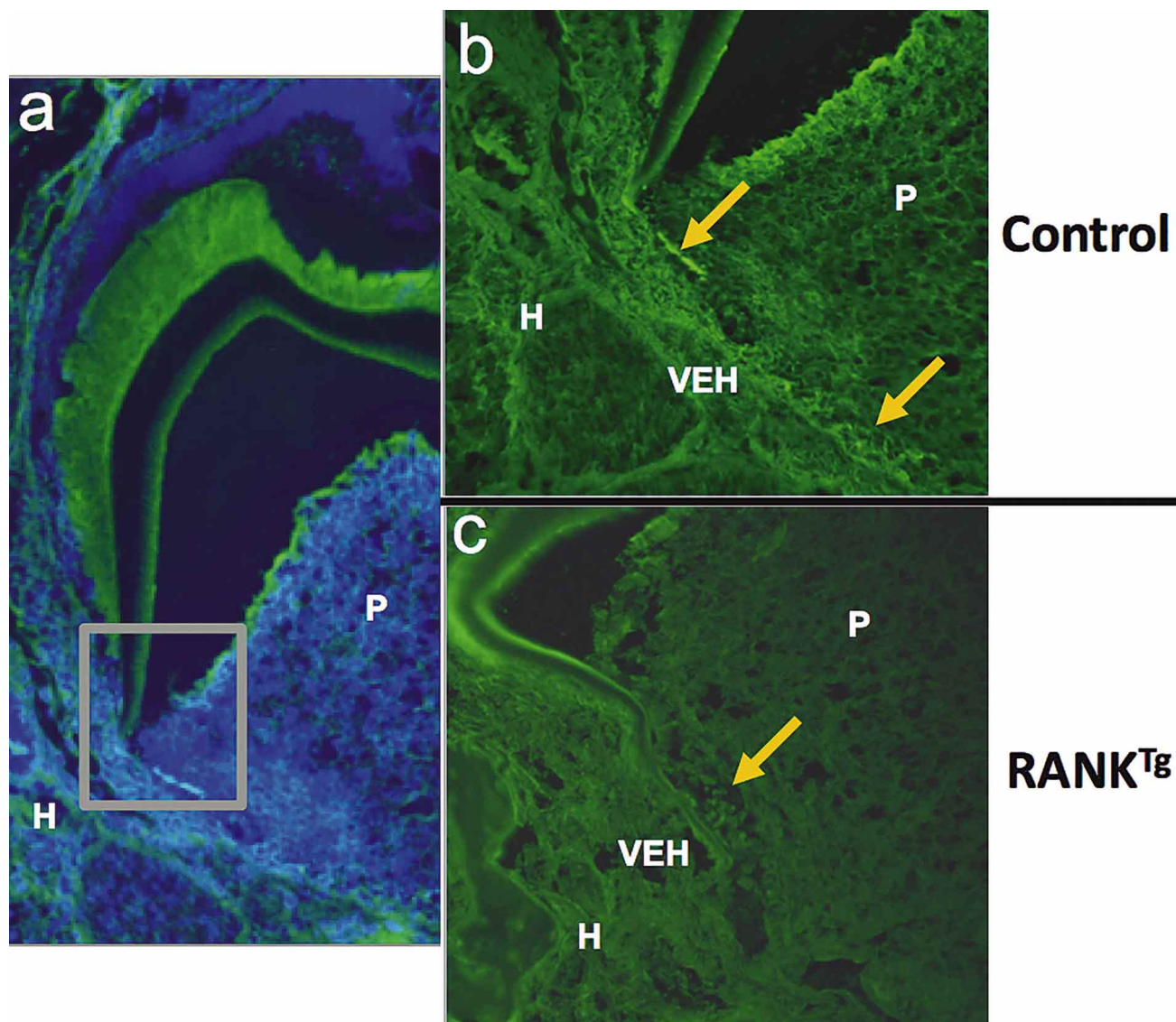


Fig. 7. Amelogenin Immunofluorescence. Comparison between the molars of control mice (B) y $RANK^{Tg}$ mice (C) at 5 days of age. The arrows on B, point to the presence of positive signaling for amelogenin in control animals, while the arrow on C points to the absence of the signal. Scale A-F: 10X/0.30.

made evident the implication of alveolar bone resorption activity on root growth and on the periodontal homeostasis in the adult (Castaneda *et al.*). The results of this study generated hypotheses about the effect of osteoclastic activity on the control of root growth. It was considered that a more detailed analysis of the signaling pathways involved in the osteoclastic cells-epithelial cells interactions could provide elements of the characterization of the alveolar bone-root growth relationship. Up until now, all studies have been focused on the processes which intervene in tooth eruption (Castaneda *et al.*), which suppose that the same

signaling pathways were involved on both the crown level and root level. This research is a preliminary descriptive study of this relationship.

To comply with the objective to analyze the effect of osteoclastic hyperactivity of the alveolar bone on root growth, the response of Hertwig's epithelial root sheath (HERS) cells of the $RANK^{Tg}$ mouse to osteolysis of the peri-radicular tissues was evaluated. This study is a preliminary evaluation of the interaction between the main RANK/RANKL/OPG osteoclastogenesis pathway and the HERS epithelial cells.

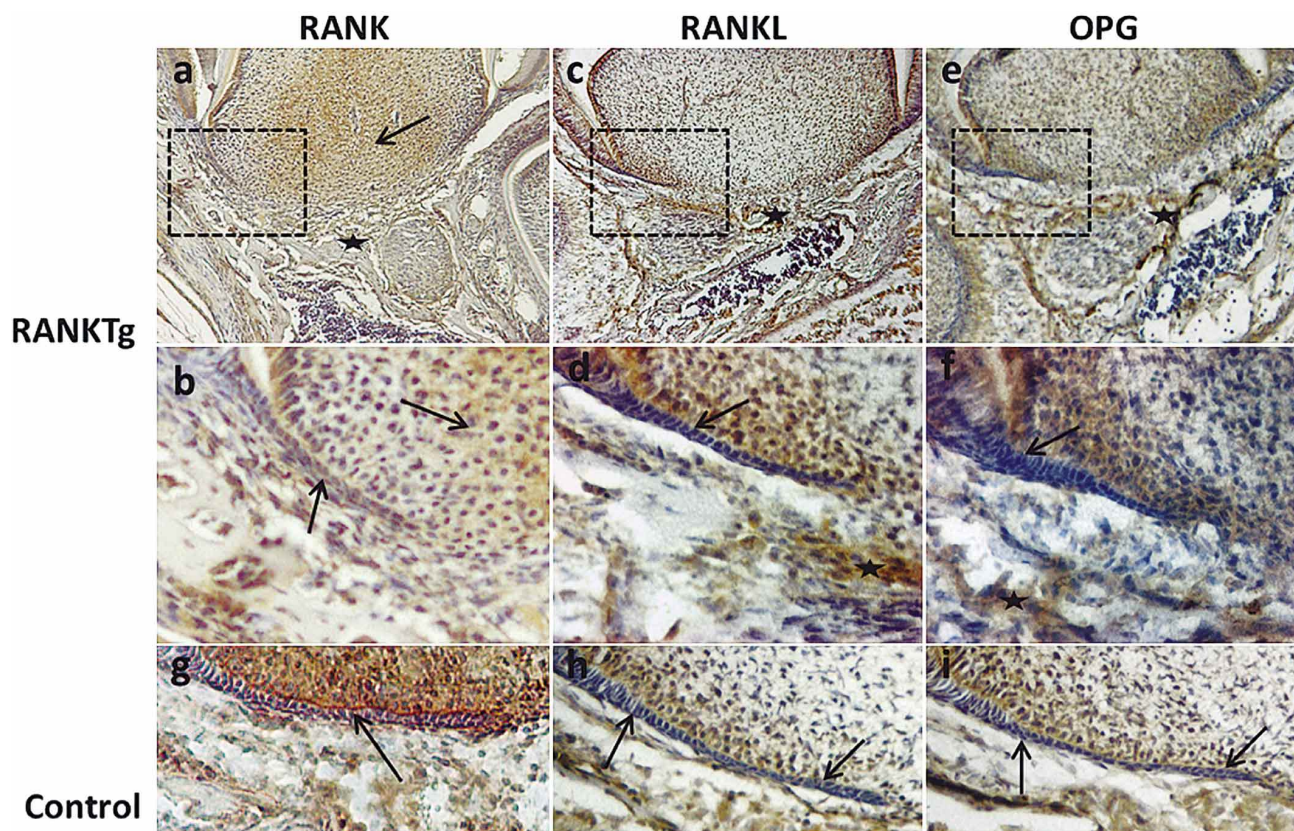


Fig. 8. Immunolocalization of RANK, RANKL and OPG factors in Hertwig's epithelial root sheath. a. RANK in RANK^{Tg} mice. b. amplification. c. RANKL in RANK^{Tg} mice. d. amplification. e. OPG in RANK^{Tg} mice. f. amplification. g. immunostaining of RANK in the HERS of control molars. h. immunostaining of RANKL in the HERS of control molars. i. immunostaining of OPG in the HERS of control molars. The stars in a, c and e show the expression in the apical mesenchymal tissue, while the arrows in b, d, f, g, h, and i, show the absence of expression in the epithelial cells of Hertwig's Epithelial Root Sheath. Scale a, c, e: 10X/0.30; b, d, f: 20X/0.70; g, h, i: 40X/0.70.

The expression of Collagen 1 δ 1, shows macro changes in the bone matrix of the RANK^{Tg} mouse when compared with the control mouse. At 5 days of age, the mandibular alveolar bone is much more trabecular in RANK^{Tg} mice, as opposed to more compact in control mice. This is explained by an increase in osteoclastic differentiation and a more active bone resorption. The absence of alveolar bone between the dental nerve and the apical region of RANK^{Tg} molars at 5 days of age suggests that the acceleration of growth and root elongation in these molars does not allow bone deposit in this area contrary to what was seen in the control molars. Although these changes occurred in the bone matrix, there was no evidence of alterations in the dentin structure of the developing molars. The absence of positive TRAP cells in the root surface which is just starting to form indicates that the resorption activity affects solely the periradicular bone matrix without affecting the dental cells. The integrity of Hertwig's epithelial root sheath is maintained without

significant changes despite the peridental osteoclastic hyperactivity.

This result coincides with the reports of other studies which have shown that osteoclastic hyperactivity, like the one generated by orthodontic movement, does not induce in itself the resorption of dentin in developing roots (Mavragani *et al.*, 2002).

The preliminary results of the adult molar evaluation (unpublished data), showed some areas of dentin resorption without associated positive TRAP cells. These cells were present in the adjacent alveolar bone and some in the periodontal ligament. The observed situation is compatible with the dentoalveolar phenotype described in studies concerning root resorption due to orthodontic forces applied to adult teeth (Low *et al.*, 2005). The effect of an overactivated RANK pathway on the periodontal homeostasis and root integrity of the adult should be evaluated. Other

markers of macrophages (for example, cathepsin K) would have to be used to confirm the presence of odontoclasts.

Hertwig's epithelial root sheath (HERS) determines the root morphology and growth. Recent studies have established the implication of epithelial mesenchymal interactions with respect to SHH/Msx2 (SHH: Sonic hedgehog homolog) and IGF/BMP4 pathways, in its formation. In fact, the expressions of these factors have been shown in HERS cells and epithelial cell rests of Malassez (Andeasen *et al.*, 1990; Grzesik & Narayanan, 2002; Yamashiro *et al.*, 2003; Yamamoto *et al.*, 2004; Fujiwara *et al.*; 2005; Nakatomi *et al.*, 2006; Khan *et al.*, 2007; Tummers *et al.*, 2007; Hosoya *et al.*, 2008). By analogy with the epithelial enamel knot, it seems logical to suppose that the complex root shape is determined by one or more signaling pathways localized at the HERS level. The growth factors, IGF (insulin like growth factor) and BMP4 (Bone morphogenetic protein 4) are secreted by the mesenchymal tissues adjacent to Hertwig's epithelial root sheath and should activate their receptors within HERS. The artificial stimulation of IGF receptor produces a precocious elongation and an increase in the cell proliferation of the sheath (Yamashiro *et al.*). The overexpression of BMP4 factor produces a size reduction of Hertwig's sheath while its inhibition caused by Noggin stimulates the elongation of the root (Yamamoto *et al.*). The SHH factor seems to also play an important role in the epithelial mesenchymal interactions which control the sheath's growth. The inhibition of its receptor, Patched-1, induces a decrease in proliferation in the periphery of the sheath, provoking a delay in eruption and the formation of shorter roots (Nakatomi *et al.*).

The accelerated growth of the roots of the mouse with overexpressed RANK suggests that the RANK/RANKL pathway stimulates the signaling pathway which controls the growth of Hertwig's epithelial root sheath. The "induction" of the sheath's growth without altering its shape or morphologic integrity could suggest that the RANK/RANKL pathway stimulates the response of growth factors which control the tooth development but does not alter the molecular engram and the role of each one of them within the engram.

The increase of the cellular proliferation in the periodontal tissues adjacent to Hertwig's epithelial root sheath suggests that the overexpressed RANK, and through it, of its ligand RANKL, induce a molecular hyperactivity of the mesenchymal cells which could

stimulate molecular receptors in the epithelial cells of the sheath and in this way induce its growth. The strongly expressed P21 indicates that the dental follicle subjacent to Hertwig's epithelial root sheath could be the regulating center of its growth, in the same way as this protein is expressed in the enamel nodule during the formation of the tooth germ (Tummers *et al.*). The signaling pathways of these interactions could be the object of a subsequent research study.

The fact that the phenomenon of acceleration of eruption and root development due to an overexpressed RANK/RANKL pathway does not produce morphological alterations on the Hertwig's epithelial root sheath or on the integrity of the root of young molars, could be explained by the active presence of this "root formation regulating center" localized in the follicular tissue adjacent to the epithelial sheath, which is a vital cellular source of dental tissue. This hypothesis coincides with some studies concerning the effect of orthodontic movement on "immature" teeth, where it is suggested that the apical tissue creates a biological tolerance to orthodontic forces in teeth with an open foramen (Mavragani *et al.*; Thesleff *et al.*, 2001).

The use of the Keratin 14 marker showed that the identity of the epithelial cells of Hertwig's epithelial root sheath remained unaltered despite the stimulation coming from the adjacent mesenchymal cells. This shows that the activation of the peri-radicular tissues induces a greater speed in the response of the epithelial cells of the sheath without producing cellular transformation. The increase in number of epithelial cells in the root sheath of RANK^{Tg} molars at the start of the root formation (3 to 7 day age group) shows a response in cellular proliferation to the stimulus of the RANK/RANKL pathway. This is of importance during the crown-root transition stage and during the formation of the cervical third. The increased number of epithelial cell rests of Malassez in the 14 to 21 day age group, suggests that the regulation of the RANK/RANKL pathway could be important in periodontal homeostasis and integrity. More detailed studies of the epithelial cell rests of Malassez are needed to determine its role in hyper-osteolysis conditions.

The lack of expressed amelogenin in the HERS of the molars of 5 day old RANK^{Tg} mice and its strong expression at the crown-root transition level in the control molars allows the proposal of two hypotheses. The first hypothesis proposes the existence of a feedback relationship between the expressed RANKL and the

amelogenin, which has actually been previously proven by other studies (Hatakeyama *et al.*, 2003). The over-expressed RANKL in the tissues adjacent to Hertwig's epithelial root sheath and the peri-radicular osteoclastic hyperactivity would affect the expression of amelogenin and in this way, its regulating role in the early stages of root growth. Therefore, the over-activation of the RANK/RANKL pathway would inhibit the expression of the amelogenin in an earlier stage in transgenic mice as compared to the controls. The second hypothesis has to do with the acceleration of the root formation due to the overexpressed RANK. The overactivation of the RANK/RANKL pathway produces an acceleration of tooth eruption and formation. The presence of amelogenin in Hertwig's epithelial root sheath in 5 day old control molars and its absence in RANK molars of the same age could be explained by the acceleration in the formation process of these mice compared with the controls.

The question raised is then, does the RANK/RANKL pathway activate the cells in Hertwig's epithelial root sheath directly or is it indirectly through the cells in the apical follicular tissue? The results of this study show that the expressed RANK activates the pulp cells, the preodontoblasts, the ameloblasts, and the mesenchymal cells in the apical follicular tissue at 5 days of age, but a direct activation of HERS cells is not conclusive.

Evaluating the relationship with other factors such as IGF, BMP4, MSX2 and SHH, which have been directly tied to the activation of the epithelial cells in HERS through the RANK/RANKL pathway, would help recognize the variant in the epithelial mesenchymal molecular engram responsible for the accelerated root growth phenomenon present in the RANK^{Tg} mouse. A more detailed study of the signaling pathways could identify possible "target" elements for pharmacological treatments aimed to control the root growth and integrity in physiological and physiopathological cases of hyper-resorption of alveolar bone (osteolysis, juvenile Paget's disease, cherubism, periodontal disease, and early orthodontic movement among others).

CONCLUSIONS

The research on the RANK transgenic mouse evidences the impact on root growth of the bone micro-environment and specifically the RANK/RANKL/OPG osteoclastic pathway. The over-activity of the

osteoclasts during growth alters the structure of the alveolar bone. This osteolysis favors the growth response of Hertwig's epithelial root sheath and the dental cells without affecting the integrity of the root during its formation.

The RANK/RANKL pathway could play a very important role in the control of root growth during the transitional crown-root stage. Also, the cells in the dental follicle subjacent to the root could be its regulating growth center. RANK/RANKL could have a direct effect on the molecular IGF/SHH/BMP4/MSX2 engram, which controls dental growth in the crown at the level of the enamel nodules, in the root at the level of Hertwig's epithelial root sheath, and as suggested by this study, at the level of the dental follicular cells subjacent to the root in formation.

The evidence shows the implication of RANK/RANKL in root growth and, at a later stage, in the homeostasis and integrity of the periodontal tissues. AntiRANK agents are currently used as therapeutic agents for the control of tumoral and inflammatory pathologies. This study reinforces its importance as a future therapeutic agent for the regulation of root growth and root integrity under circumstances such as osteoclastic over-activity and orthodontic treatment.

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**BERDAL, A.; BETANCUR, J. J.; MORALES, M. C.; PIMIEN-
TA, C. & CASTAÑEDA, B.** Evaluación de la influencia de la
resorción ósea alveolar sobre la estructura radicular de
molares en ratones transgénicos con sobreexpresión de
RANK. *Int. J. Odontostomat.*, 9(3):357-372, 2015.

RESUMEN. Las alteraciones en el balance de la actividad osteoblástica-osteoclástica (osteopetrosis, osteolisis) tienen un impacto en el desarrollo dental. La activación de la vía sobre la osteoclastogénesis RANK/RANKL/OPG en ratones RANK^{Tg} produce una aceleración de la erupción de los dientes y elongación de las raíces, lo que sugiere que esta vía podría controlar la velocidad de formación de los dientes. El objetivo de este estudio consiste en evaluar el

efecto de la hiperactividad osteoclástica en la formación de las raíces de los molares en ratones RANK^{Tg}. Se realizó un estudio histológico descriptivo y comparativo del impacto de la hiper-reabsorción del hueso alveolar sobre la vaina epitelial radicular (de Hertwig - HERS) de molares en ratones RANK^{Tg} y controles. Se realizó la inmuno-detección de la proliferación matricial, epitelial y celular de proteínas, combinada con técnicas histológicas, inmunohistoquímicas e histoenzimológicas. La hiperactividad osteoclástica en el hueso alveolar no altera la estructura de la raíz dentaria y la integridad de los molares en ratones RANK^{Tg}; la aceleración de la formación de la raíz no altera la integridad de la misma. Se encontró un área de hiper-proliferación celular en el tejido folicular apical del HERS, que podría regular el crecimiento de la raíz en respuesta a la actividad osteoclástica. La sobreexpresión en los RANK produce una inhibición de la expresión de amelogenina a los 5 días de edad, lo que sugiere una regulación indirecta de estas células por RANK/RANKL. La exploración de otros factores moleculares expresados en HERS, y el engrama relacionado, haría posible el uso de nuevas terapias para el control de patologías óseas e inflamatorias durante la formación de la raíz dentaria.

PALABRAS CLAVE: resorción ósea, desarrollo de la raíz, células epiteliales.

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Correspondence to:
John Jairo Betancur P.
Specialist in Orthodontics
Associate Professor
School of Dentistry
University of Antioquia
Medellín
COLOMBIA

Email: betancurjohn@hotmail.com

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