

Isolation and characterization of a human cementocyte-like cell line, HCY-23

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Abstract: Cementum is the mineralized tissue covering the tooth root that functions in tooth attachment and post-eruptive adjustment of tooth position. It has been reported to be highly similar to bone in several respects but remains poorly understood in terms of development and regeneration. Here, we investigate whether cementocytes, the residing cells in cellular cementum, have the potential to be protagonist in cementum homeostasis, responding to endocrine signals and directing local cementum metabolism. Cells from healthy erupted human teeth were isolated using sequential collagenase/EDTA digestions, and maintained in standard cell culture conditions. A cementocyte-like cell line was cloned (HCY-23, for human cementocyte clone 23), which presented a cementocyte compatible gene expression signature, including the expression of dentin matrix protein 1 (*DMPI*), sclerostin (*SOST*), and E11/gp38/podoplanin (*E11*). In contrast, these cells did not express the odontoblast/dentin marker dentin sialoprotein (*DSPP*). HCY-23 cells produced mineral-like nodules *in vitro* under differentiation conditions, and were highly responsive to inorganic phosphate (Pi). Within the limits of the present study, it can be concluded that cementocytes are phosphate-responsive cells, and have the potential to play a key role in periodontal homeostasis and regeneration.

Keywords: Dental Cementum; Gene Expression; Cell Culture Techniques.

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Introduction

Dental cementum, a mineralized tissue covering the tooth root, is formed in acellular and cellular types.¹ Acellular cementum (acellular extrinsic fiber cementum, or primary cementum) has been described as a critical factor for tooth attachment and covers the most coronal area of the tooth root. In contrast, cellular cementum (cellular intrinsic fiber cementum, or secondary cementum) has been proposed to play a role in post-eruptive adjustment of tooth position,¹ and is located at the apical and furcation regions of roots. During cellular cementum development, cementoblasts secrete a layer of unmineralized extracellular matrix (ECM), called cementoid, and as cementoid deposition progresses, cementoblasts might be entrapped in the ECM and become cementocytes. Like bone, dental cementum ECM is primarily composed of collagen (predominantly type I and smaller

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amounts of types III, IV, V, XI, and XII), several non-collagenous proteins, including bone sialoprotein (BSP) and osteopontin (OPN), and proteoglycans, such as biglycan (BGN) and decorin (DCN).^{2,3} Unlike bone, dental cementum is non-innervated, avascular, and grows by apposition with no apparent physiological capacity for remodeling or turnover. While it is recognized that dental cementum regeneration is possible, current clinical strategies to regenerate the periodontal tissues often lack a biologic basis for treatment and have unpredictable outcomes with limited regeneration, especially for dental cementum.⁴ Studies using transgenic animals have identified novel regulators of cementogenesis,^{5,6,7} and comparative proteomic analysis of human dental cementum versus alveolar bone identified differentially expressed proteins associated with potential physiological differences between these two tissues.^{8,9} Although important progress has been made with regards to the understanding of dental cementum development, currently it is clear that a significant barrier to improved periodontal regenerative therapies is that cementum remains poorly understood.

Cementocytes, apparently terminally differentiated cells, are part of a select group of specialized cells that also include osteocytes. Although osteocytes comprise more than 95% of skeletal cells, they were just recently revealed as key regulators of bone homeostasis and remodeling, acting as mechanosensors, and direct endocrine regulators of mineral metabolism.¹⁰ A question may be posed whether the cementocyte is a dynamic actor in cementum in comparable fashion with the osteocyte in the skeleton, responding to changing tooth functions and endocrine signals, as well as actively directing local cementum metabolism. Here, we modified a previously reported protocol¹¹ to obtain a cementocyte-like cell line from human teeth. Once these human cementocyte (HCY)-23 clonal cells were established, they were characterized regarding their gene expression signature and potential to produce mineral nodules *in vitro*. In addition, as it is well established that dental cementum is highly sensitive to phosphate metabolism,^{12,13,14,15} we examined the effect of inorganic phosphate (Pi) on HCY-23 behavior.

Methodology

Biological samples

Completely erupted teeth (third molars and 1st upper premolars) of eleven subjects (three males and eight females, aged 14 to 30 years), with no clinical signs of caries and/or periodontal disease, were collected. Teeth extractions were performed at the School of Dentistry following a local IR-approved protocol (466/12 – CAAE # 42930915.9.0000.5418). Systemically healthy subjects (non-smokers, with no history of systemic medication in the last 6 months and/or pregnancy) provided written informed consent to participate in this study, and the study was performed between 2014 and 2016. Following tooth extraction, soft connective tissues adhering to the tooth surfaces were carefully scraped off using a sterile curette, and discarded. Teeth were then rinsed several times in sterile phosphate buffered saline (PBS - Gibco BRL, Life Technologies, USA), placed in biopsy medium composed of minimum essential Eagle medium - alpha modification (α -MEM, Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum, 250 mg/mL gentamicin sulfate, 5 mg/mL amphotericin B, and 1% penicillin/streptomycin (Gibco, Carlsbad, USA), and transferred to the laboratory facilities to be processed for cementocyte isolation.

Cell culture and cloning

Extracted teeth were removed from the biopsy medium and their root's apical portion detached for cementocyte isolation from the cellular dental cementum. Periodontal ligament and pulp tissues were eliminated from the root fragments by enzymatic digestion with 300 U/mL type I collagenase (Sigma-Aldrich, St. Louis, USA) for 30 minutes at 37°C (3x). Procedures used for cementocytes enzymatic isolation are outlined in Table 1. Obtained cells were cultured in standard medium (α -MEM with 10% FBS and antibiotics), which was changed every 3 days to obtain sufficient cell number for single cell cloning. All obtained clones (115) were assessed regarding their gene expression profile and only one clone with the expected signature (HCY-23, for human cementocyte clone 23), *e.g.*, DMP1⁺, SOST⁺, E11⁺, DSPP⁺, was used for further characterization. Cells between the second

Table 1. Description of the procedures used for isolating a human-derived cementocyte-like cell line.

Step 1	Teeth extracted (n=13), quickly rinsed in saline, and kept in biopsy medium (α -MEM added with 10% fetal bovine serum, 250 mg/mL gentamicin sulfate, 5 m/mL amphotericin B) at room temperature.
Step 2	Once at the lab, soft connective tissue adhering to the tooth surfaces were scraped off using a sterile curette, and the apical portions of teeth were dissected and used for cementocyte isolation from the cellular dental cementum.
Step 3	Additional removal of PDL and pulp tissues from tooth root fragments was achieved by enzymatic digestion (300 U/mL type I collagenase for 30 minutes at 37°C), and supernatant was discarded.
Step 4	Tooth root fragments were subjected to (3x) serial digestions (collagenase A, 300 U/mL and EDTA, 5 mM/ 0.1% BSA, for 20 minutes each at 37°C), and the supernatant was discarded.
Step 5	Tooth fragments were subjected to (9x) serial digestion cycles of collagenase A (300 U/mL) and EDTA (5mM, pH=7.4) for 25 minutes each, under agitation, at 37°C, in order to allow cementocyte release from the cementum matrix, and supernatants plated in biopsy media. Additionally, tooth fragments were rinsed with HBSS at 37°C (3x) and cultured in biopsy culture medium.
Step 6	Cell outgrowth monitored and <i>DMP1</i> ⁺ heterogeneous populations were subcloned. A single clone, HCY-23, exhibiting a cementocyte-like gene expression signature, was used for the subsequent studies.

and sixth passages were used for the experiments, which were always performed in triplicate.

Gene expression procedures

Total RNA was obtained using the RNeasy system (Qiagen, Valencia, USA) following the manufacturer's recommendations, and complimentary DNA (cDNA) synthesized from 1 μ g of total RNA (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostic Co., Mannheim, Germany), following the recommended protocol. Primer sequences were obtained with the LightCycler® Probe Design Software 2.0 (Roche Diagnostics GmbH, Germany) and are listed in Table 2. Quantitative real-time PCR (qPCR) reactions were optimized for each primer and water was used as a negative control for the reactions. qPCR reactions were performed using the SYBR Green System (FastStart Essential DNA Green Master; Roche Diagnostic Co., Indianapolis, USA) on the LightCycler 480 instrument (Roche Diagnostics GmbH, Netherland).

Immunofluorescence analysis

In order to determine the morphology of HCY-23 cells, Phalloidin 488 and Syto Green were used to stain the nucleus and cytoskeleton, respectively, and the images were obtained in a confocal microscope. Briefly, HCY-23 cells were seeded in glass slides at a density of 2×10^4 cells per slide and cultured in DMEM medium supplemented with FBS 10% for 24 hours. After 24 hours, cells culture medium was removed, and after three washes with phosphate-buffered

Table 2. Primers sequences.

<i>GAPDH</i>	
Forward	5' - AGCCACATCGCTCAGACAC -3'
Reverse	5' - GCTGTACTCGGACACGCTTTT -3'
<i>SOST</i>	
Forward	5' - AGCTGGAGAACAACAAGACCA -3'
Reverse	5' - GCTGTACTCGGACACGCTTTT -3'
<i>E11</i>	
Forward	5' - AAATGTCGGGAAGGTACTCG -3'
Reverse	5' - AGGGCACAGAGTCAGAAACG -3'
<i>DSPP</i>	
Forward	5' - GCAACATGCTGATGGGAAGA -3'
Reverse	5' - TTTACCTTCGTTGCCTTTCC -3'
<i>OPG</i>	
Forward	5' - GAAGGGCGCTACCTTGAGAT -3'
Reverse	5' - GCAAAGTATTCGCTCTGG -3'
<i>DMP1</i>	
Forward	5' - TTCTTTGTGAACACTCGGAGGGTA -3'
Reverse	5' - CAGGATAATCCCCAAAGGGAAC -3'
<i>OCN</i>	
Forward	5' - AGCTCAATCCGGACTGT -3'
Reverse	5' - GGAAGAGGAAGAAGGT -3'
<i>ALPL</i>	
Forward	5' - CCTGCCTTACTAACTCCTTAGTGC -3'
Reverse	5' - CGTTGGTGTGAGCTTCTGA -3'
<i>OPN</i>	
Forward	5' - GAGGGCTTGGTTGTCAGC -3'
Reverse	5' - CAATTCTCATGGTAGTGAGTTTTCC -3'

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SOST: sclerostin; E11: podoplanin; DSPP: dentin sialophosphoprotein; OPG: osteoprotegerin; DMP-1: dentin matrix protein 1; ALPL: alkaline phosphatase; OCN: osteocalcin.

saline (DPBS, Invitrogen™, Life Technologies, Gibco, Carlsbad, USA), cells were fixed in 4% formaldehyde solution with PBS for 20 minutes at room temperature. Next, cells were permeabilized using 0.1% Triton X-100 solution in PBS for 15 minutes, and blocked with a solution of BSA for 4 hours. Then, glass slides containing fixed cells were stained for 20 minutes at room temperature protected from light. Finally, cells were washed (2–3 times) in a phosphate-free buffer and visualized using the Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Mineralization assay, in vitro

The Von Kossa assay was used to assess mineralization capacity of the selected clone. Cells were seeded (5×10^4 cells/well) in 24-well plates and incubated for 24 hours in standard medium. Next, the medium was changed to mineralization induction medium (α -MEM added with 10% FBS and induction supplements containing 50 mg/mL ascorbic acid, 10 mM β -glycerophosphate, and 10^{-5} M dexamethasone), and the cultures were incubated for 28 days. The medium was changed every other day, and mineral nodules formation assessed after 21 days. Cells were washed with PBS and fixed in 100% ethanol for 30 minutes. Then, every 5 minutes, an ethanol solution was exchanged at concentrations of 100, 90, 70, and 50%. The ethanol was aspirated and the cells washed with distilled water. Silver nitrate 5% solution was added to each well and incubated at 37°C, 5% CO₂ for 1 hour protected from light. Cells were then washed four times with distilled water, and after 24 hours under the light of a negatoscope, the mineral nodules were photographed. In addition to the Von Kossa assay, HCY23 cells were cultured as described above and the impact of osteogenic induction on the expression of *DMP1*, *OPN*, *ALPL*, and *OCN* transcript levels assessed by qPCR at 3, 7, 14, and 21 days after osteogenic induction.

Inorganic phosphate (Pi) treatment

To determine the response pattern of HCY-23 to Pi treatment, a dose-response experiment was used (0.05, 0.25, 0.5, and 1 mM). Briefly, cells were seeded at 1.5×10^4 cells/well in 24-well cell culture plates and incubated for 24 hours (day 0) in culture media,

containing α -MEM, 2% FBS, 100 μ g/mL streptomycin and 100 U/mL penicillin, at 37°C and 5% CO₂. After 24 hours, HCY-23 cells were treated or not with Pi at 0.05, 0.25, 0.5 and 1 mM, and cell proliferation determined at 3, 7, 14, and 21 days. Total cell number and viability were obtained using a hemocytometer and trypan blue, respectively. Next, cells were plated at 1.5×10^4 cells/well in 24-well cell culture plates and incubated for 24 hours in culture media containing α -MEM, 10% FBS, 100 μ g/mL streptomycin and 100 U/mL penicillin, at 37°C and 5% CO₂. HCY-23 cells were then treated or not with Pi at 0.05, 0.25, 0.5 and 1 mM, and *DMP1*, *OPN*, *ALPL* and *OCN* transcript levels assessed by qPCR at 72 h after Pi treatment as described previously in this manuscript.

Statistical analysis

The effect of osteogenic conditions in gene expression profile was assessed by intergroup (student *t*-test) and intragroup (one-way ANOVA followed by the Bonferroni's post hoc test) analysis with significance level set at 5% (GraphPad Prism 6.0, GraphPad, La Jolla, USA). Moreover, one-way ANOVA followed by the Bonferroni's post hoc test was used to determine the impact of Pi treatment on cell number and gene expression. Experiments were performed at least twice with comparable results and quantitative data is presented as mean \pm standard deviation (SD) of one representative experiment performed in triplicate, unless otherwise stated.

RESULTS

Demographical findings and morphological characterization:

A total of 24 heterogeneous cell populations was obtained from 13 teeth. About 37% of these populations was *DMP1*⁺, and were subsequently cloned using the ring-cloning technique. A total of 115 clones were obtained and characterized according to the expression of known cementocytes markers, e.g. *DMP1*, *SOST*, and *E11*. We found that only one clone, HCY-23, presented the expected gene expression signature (e.g., *DMP1*⁺, *SOST*⁺, *E11*⁺, and *DSPP*), and was then used for further characterization. Figure 1 illustrates the representative morphology of HCY-23 cells.

A

Genes	HCY-23
DMP-1	+
SOST	+
E-11/GP38	+
OPG	+
DSPP	-

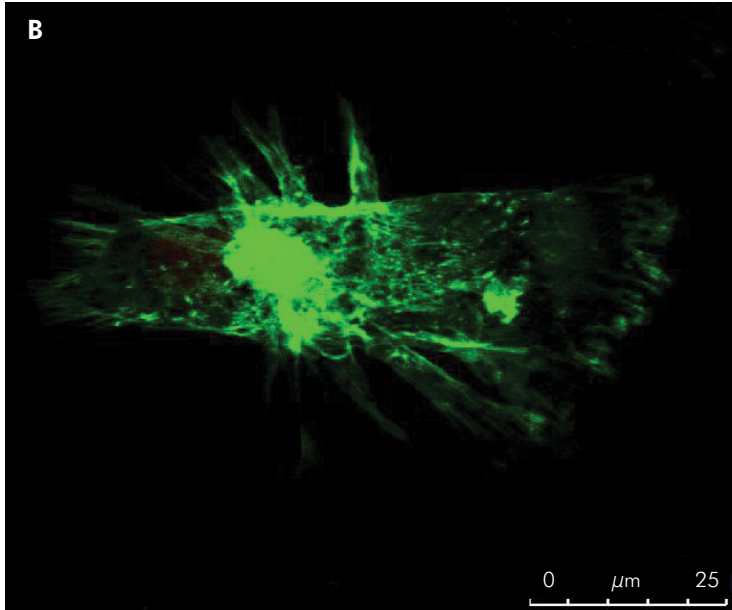


Figure 1. A) basal gene expression panel for HCY-23 cells; B) A representative confocal image of HCY-23 cells stained by Phalloidin 488 and Syto Green for nucleus and cytoskeleton, respectively.

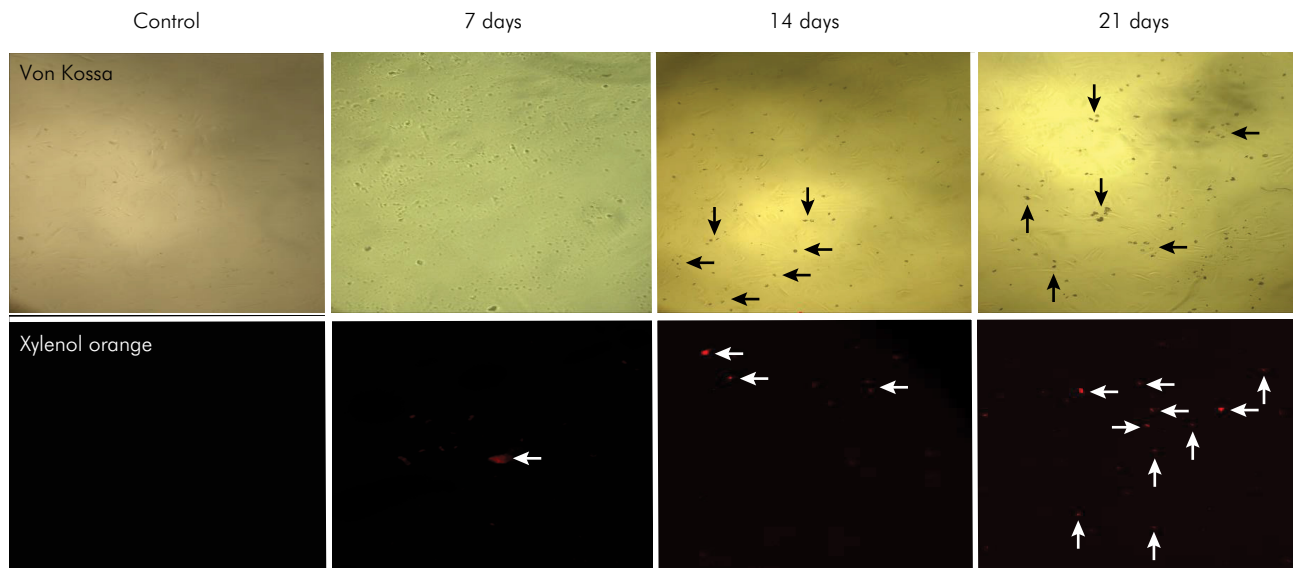


Figure 2. Representative illustration of the Von Kossa and xylenol orange assays at 7, 14, and 21 days after osteogenic induction. An increasing amount of mineral nodules is noted overtime (arrows).

Osteogenic conditions modulate HCY-23 cells gene expression and induce mineral nodule formation *in vitro*

An *in vitro* mineralization assay was performed to determine whether the human cementocyte-like cell line HCY-23 had the ability to promote mineral

nodule formation. Mineralizing conditions were created, and we found that HCY-23 cells presented the ability to form small amounts of mineral nodules *in vitro* as early as 21 days after the induction was started (Figure 2). Mineral nodule formation was visually increased by 28 days (data not shown). In the

current investigation, it was also assessed whether osteogenic conditions would affect *DMP1*, *OPN*, *ALPL*, and *OCN* transcript levels at 3, 7, 14, and 21 days after treatment. Intergroup data analysis showed that osteogenic induction resulted in increased levels of transcripts for *DMP1* and *ALPL* at 14 and 21 days, and *OCN* at day 14 ($p < 0.05$). In contrast, *OPN* mRNA levels were significantly diminished at day 14 and 21 after treatment ($p < 0.05$). Figure 3 illustrates gene expression findings under osteogenic conditions.

HCY-23 are Pi responsive cells

As dental cementum and cementoblasts have been shown to be highly sensitive to phosphate metabolism, we wanted to know whether the cementocyte-like cell line HCY-23 was affected by Pi treatment. First,

using a dose-response experiment, we found that, at 3 and 5 mM, Pi was highly toxic to HCY-23, whereas at 1 mM Pi did not affect cell viability up to 21 days as compared to the control group (data not shown). Guided by our preliminary findings, we decided to repeat the proliferation assay with Pi treatment at 0.05, 0.25, 0.5, and 1 mM up to 21 days. Data analysis demonstrated that cell number was increased in all the experimental groups compared to day 0, with no difference among treatments ($p > 0.05$) (Figure 4). Intriguingly, we found that Pi alone resulted in a dose-dependent mineral deposition by HCY-23 cells (data not shown), and decided to examine whether Pi treatment would affect the expression of mineralization-associated genes. Our findings showed that Pi treatment significantly increased

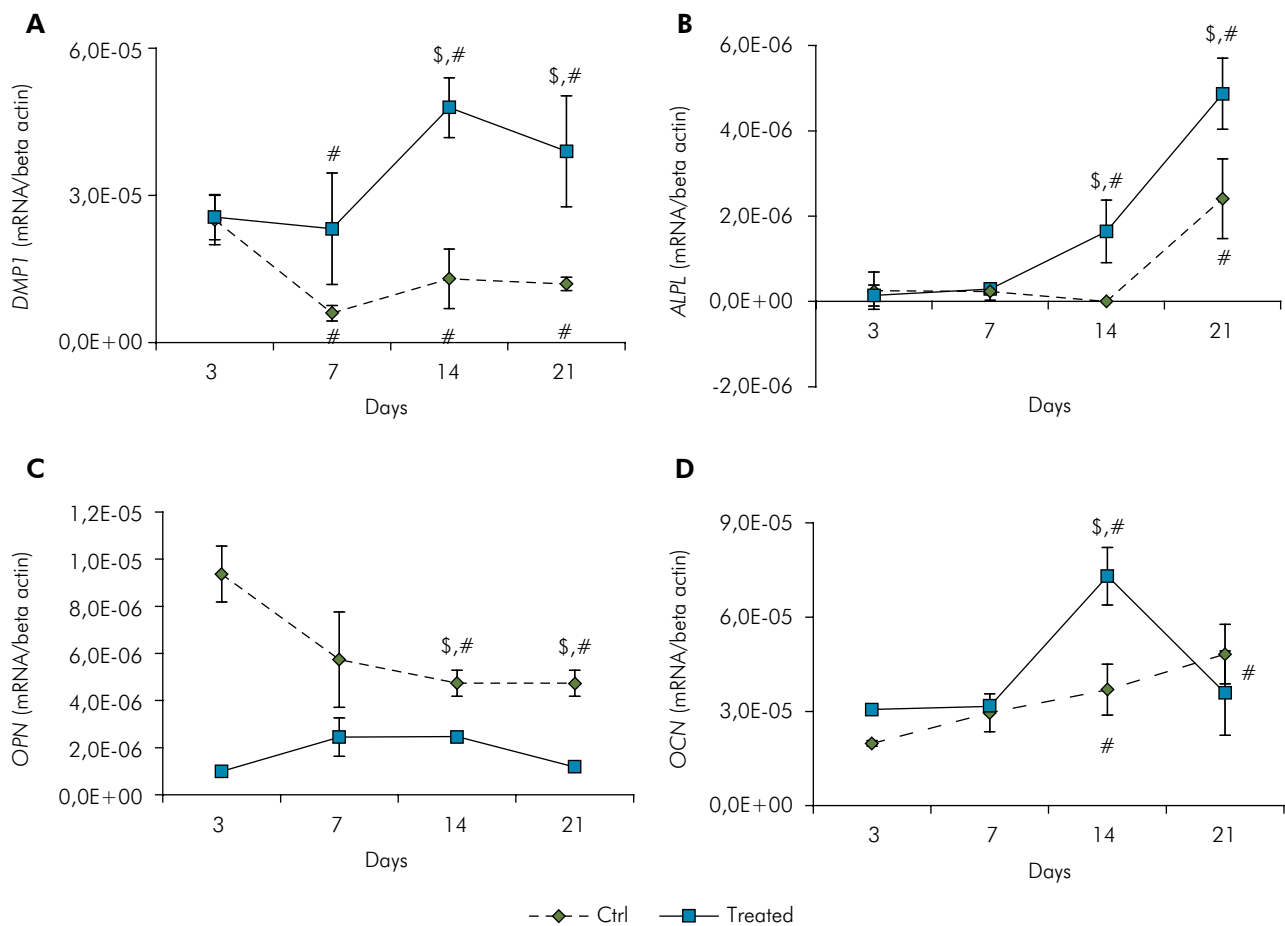


Figure 3. Effect of osteogenic induction on the expression of *DMP1*, *ALPL*, *OPN*, and *OCN* at 3, 7, 14, and 21 days after treatment (Pi-treated). #Intragroup difference by one-way ANOVA followed by the Bonferroni test versus day 3 with $\alpha = 0.05$. \$Intergroup difference by Student *t*-test with $\alpha = 0.05$.

transcript levels for *DMP1*, *OPN*, *OCN*, and *ALPL* starting at doses as low as 0.25 mM at three days after treatment ($p < 0.05$) (Figure 5).

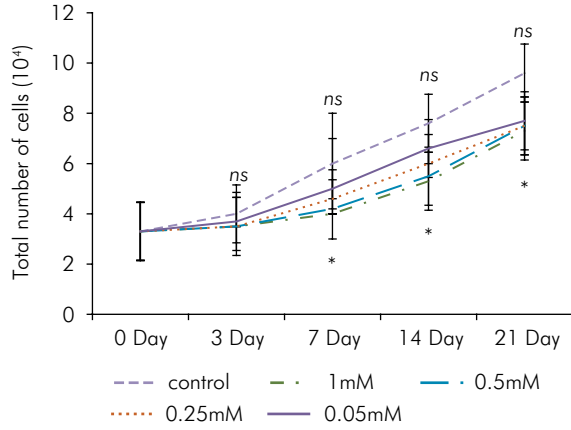


Figure 4. Effect of inorganic phosphate treatment (Pi) on the total number of cells at 3, 7, 14, and 21 days after treatment (Pi-treated). ns: non-significant intergroup differences by one-way ANOVA with $\alpha = 0.05$; *intragroup difference by one-way ANOVA followed by the Bonferroni test compared to day 0 for all the experimental groups with $\alpha = 0.05$.

Discussion

Teeth are made of two major parts, an enamel-covered crown and a cementum-covered root. Tooth development studies have focused on crown formation for several decades. However, a significant progress has been made in tooth root studies in the last two decades with the application of contemporaneous techniques such as transgenic animals.^{15,16} Although dental cementum is now recognized as a critical tissue for tooth attachment, it was only discovered and characterized on human teeth after advances in microscopy almost a full century later than enamel and dentin. The functional relevance of dental cementum started to be appreciated after Black and others, in the late nineteenth and early twentieth centuries, reported detailed morphological data of periodontal tissues. These early studies on cementum laid the foundation for more advanced understanding of cementum ultrastructure, composition, development, physiology, disease, genetics, repair, and regeneration throughout the

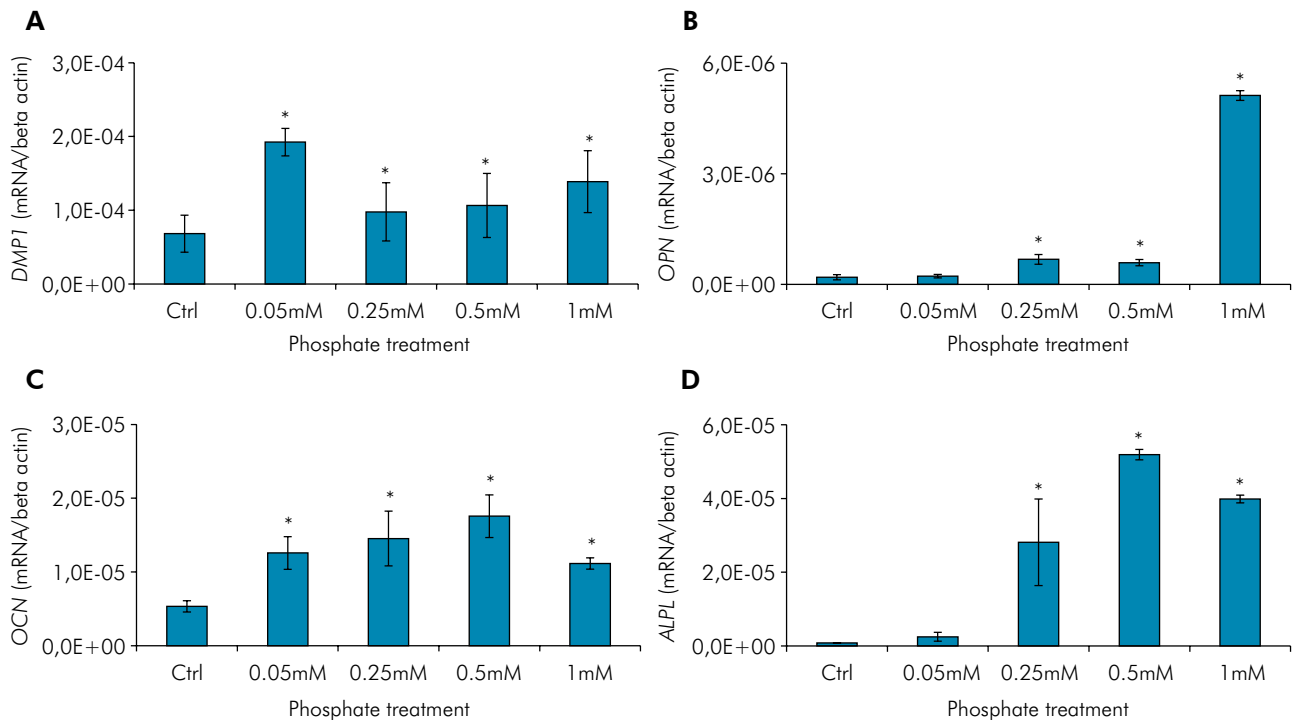


Figure 5. Effect of inorganic phosphate treatment (Pi) on the expression of *DMP1*, *ALPL*, *OPN*, and *OCN* at 3 days after treatment (Pi-treated). *Statistically different from the control group by one-way ANOVA followed by the Bonferroni test ($\alpha = 0.05$).

twentieth and into the twenty-first century. Foster¹⁷ has published a very nice and comprehensive description of the historical events from the discovery of the dental cementum to the recognition of its relevance as part of tooth-supporting structures. Dental cementum is now recognized as a mineralized tissue covering the tooth root, which functions in tooth attachment and post-eruptive adjustment of tooth position. Two major types of dental cementum have been reported in human teeth, acellular cementum is located on the cervical parts of the tooth root and has been reported to be critical for tooth attachment, and the so called cellular cementum, which is located on the apical portions of the tooth root and in the furcation region and has a role in post-eruptive adjustment of tooth position. During cellular cementum formation, cementoblasts, the tooth root lining cells, become entrapped in the cementoid matrix probably due to a rapid rate of extracellular matrix (ECM) deposition, and become cementocytes. Though, the event of cementogenesis is a critical aspect of periodontal homeostasis and regeneration, to date, it is not completely understood. Some fundamental questions remain regarding the process of cementogenesis, including the origin of cementoblastic cells, the factors directing cementum formation, and the potential functions for cementocytes during cementum formation and regeneration. In the current investigation, we investigated whether cementocytes have the potential to respond to endocrine signals and direct local cementum metabolism. We used a modified approach to obtain a primary cementocyte culture from human teeth that was comprehensively characterized regarding cell morphology, the expression of a panel of cementocyte-associated genes, their potential to produce mineral nodules *in vitro* and to respond to osteogenic induction and inorganic phosphate treatment. A cementocyte-like cell line was cloned (HCY-23), which presented a cementocyte compatible gene expression signature, including the expression of DMP1, SOST, E11, and OPG, whereas these cells did not express the odontoblast/dentin marker DSPP. Furthermore, we found that HCY-23 cells produced mineral-like nodules *in vitro* under differentiation conditions. Based on our results, HCY-23 cells were

found to present a similar phenotype, including the production of mineral nodules *in vitro* and gene expression profile, to a previously isolated murine cementocyte-like cell line.¹⁸

Zhao et al.¹⁸ focused on the potential role of their cementocyte-like cell line to respond to a mechanical demand, in the current study, we examined whether HCY-23 cells were responsive to Pi treatment. Examination of cementum development in mouse models for Pi imbalance, including the tiptoe walking (ttw or Enpp^{-/-}) mutant mouse carrying a mutation in the PC1 gene, the ank/ank transgenic mice, and the TNAP deficient mouse, revealed dental cementum to be exceptionally sensitive to Pi metabolism.^{5,6,12} Likewise, human studies confirmed the tooth phenotype found in mouse models for Pi imbalance,^{19,20} and therefore, the demonstrated sensitivity of dental cementum to Pi metabolism in both human and mouse teeth establishes this as a well-conserved and fundamental biological process directing cementogenesis across species. Foster et al.²¹ explored the potential mechanisms involved with the impact of Pi metabolism in cementum development, and demonstrated the effect of Pi treatment in an immortalized murine cementoblast cell line (OCCM-30) behavior. They found that Pi at 5 mM significantly upregulated genes including the SIBLING family genes *Opn* and *Dmp1*, whereas *Ocn* was found to be downregulated. In a later study, the same group⁵ defined the impact of an altered Pi environment on the composition of cementum ECM using immunohistochemistry and *in situ* hybridization approaches. They found that low extracellular levels of pyrophosphate (PPi) resulted in a marked increase of *Dmp1* mRNA and *Dmp1* protein levels, and transcript levels for *Opn* in the dental cementum ECM and cementoblasts. In addition, the authors reported a clear increase in *Dmp1* message in cementocytes along the molar root. In line with these findings, our study demonstrated that Pi treatment may significantly impact on the expression of key genes regulating mineralization, including DMP1, OPN, OCN, and TNAP, and therefore suggests that cementocyte may play a role in the establishment of cementum phenotypes resulting from an altered phosphate environment

during development and regeneration. Intriguingly, Pi treatment differentially regulates gene expression in cementoblasts versus cementocytes. While our findings of increased expression of DMP1 and OPN in cementocyte-like cells parallel with the increased expression of these differentiation/mineralization markers in cementoblasts⁵, our observation on OCN and TNAP mRNA levels are contrasting. We speculate that distinct functional commitment and differentiation state are responsible for the observed dissimilar response of cementoblasts and cementocytes to Pi. However, additional studies should be considered to further explore our hypothesis. The SIBLING proteins, such as DMP1 and OPN, standing for small integrin-binding ligand, N-linked glycoprotein, are components of the extracellular matrix of mineralized tissues including cementum, and evidence shows that these proteins play key roles in the mineralization of these tissues.^{22,23} In the present study, we found that an osteogenic induction also affected gene expression of HCY-23 cells. Similarly to Pi treatment, osteogenic induction significantly elevated DMP1,

TNAP, and OCN transcript levels, whereas OPN mRNA level was downregulated.

In summary, in addition to the already known effect of Pi on cementoblasts behavior, we showed for the first time that Pi regulates genes involved with Pi/PPi metabolism like TNAP, and those identified to be important in the differentiation and mineralization, such as SIBLING family genes. To the best of our knowledge, this is the first time that a human-derived cementocyte-like cell line has been generated, comprehensively characterized and shown to be highly sensitive to Pi treatment. These findings will assist in elucidating the critical mechanisms involved in cementum formation and accelerate future dental research and clinical translation for the development of more predictable regenerative therapies.

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