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Human Cementoblastoma- Derived Cells Express Cartilage- Like Phenotype *In vitro* and *In vivo* and Induce Bone Formation

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Abstract

The molecular mechanisms that regulate proliferation and differentiation of cementoblasts, have not been elucidated to date. In this paper, it is shown that human cementoblastoma-derived cells (HCDC) express greater levels of cartilage markers such as type II and X collagens, aggrecan (ACAN) and SRY-box 9 (SOX9) stem cell markers; MCAM (melanoma cell adhesion molecule; synonym: CD146) and STRO-1 than human gingival fibroblasts (HGF). Our *in vivo* studies demonstrate that HCDC induce bone formation through endochondral ossification as observed 14 days after HCDC were implanted in rat critical-size calvarial defects. At 30 and 60 days post-implantation, the defects treated with HCDC were filled with 70 ± 1.6 and 91 ± 1.3% of newly formed bone. To confirm the identity of this tissue, we analyzed the newly formed bone using histomorphology and immunostaining. The results showed the expression of bone sialoprotein (BSP) and osteocalcin (OCN). Immmunostaining of human periodontal structures showed that cementoblasts and periodontal ligament cells express cementum protein 1 (CEMP1), cartilage markers, type II and X collagens and CD146 which has been identified as a marker for chondroprogenitor cells. Altogether these results indicate that HCDC has the capacity of multilineage differentiation and induces regeneration of mineralized tissues other than cementum.

Keywords: Cementum; Cartilage; Bone regeneration; Cementoblasts; Cementoblastoma; Stem cells

Introduction

Regenerative medicine, and particularly tissue engineering, holds a tremendous economic and therapeutic potential [1]. In order to accomplish an efficient tissue engineering, a triad is required: cells that will give rise to new tissue, a biomaterial to act as a scaffold or matrix to hold the cells, and molecules that will direct the cell differentiation process to the expected or particular desired tissue type [2]. Cementum is a mineralized tissue with an extracellular matrix composed of calcified collagenous Sharpey's fibrils, type I and III collagen [3,4], glycosaminoglycans, proteoglycans and non-collagenous proteins such as fibronectin, osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), vitronectin (VtN), alkaline phosphatase (TNSALP), enamel-related proteins, amelogenin (AMEL), ameloblastin (AMBN) as well as growth factors such as TGF-β and BMP-2, platelet-derived growth factor (PDGF), osteoprotegerin (OPG), and cementum-derived growth factor (CGF) [5-8].

Cementum matrix contains novel cementum-derived molecules known as CEMP1 (cementum protein 1; GenBank Accession: NM_001048212; NM_001048216; GI: 313677962; HGNC: ID 32553) and a mRNA encoding a polypeptide of 140 amino acids (GenBank Accession Number: AAR22554.1; GI:38503520) that is a truncated isoform of 3-hydroxyacyl-CoA dehydratase 1 (HACD1) (GenBank Accession Number: AY455942.1; GI:38503519) and of which a gene synonym is the cementum attachment protein (CAP). These proteins have shown to be expressed by cementoblasts, some periodontal ligament (PDL) cell populations and mesenchymal stem cells located paravascularly in the PDL [9-11]. Therefore, cementum components and cementoblasts appear to hold the potential to achieve regeneration of the periodontium and other mineralized tissues [12-14]. A thorough understanding of the biological properties of cementum and cementoblasts is required to determine its role in periodontal formation and therefore, periodontal regeneration. Furthermore, the principles attributed to cementum regeneration might be used in the regeneration of other mineralized tissues. In this respect, cementum and bone share some structural, biochemical and functional features, however, there is limited knowledge regarding if cementoblasts and osteoblasts belong to the same cell lineage [15]. It is not clear if the tissue described histologically in several reports as a cementum-like or bone-like refers to the same tissue [15-17]. Nevertheless, the histological features of bone are very similar to the cellular intrinsic fiber cementum [CIFC] [18]. A study based on immunolabeling of osteoblast specific markers has suggested that cementoblasts of cellular cementum and osteoblasts share the same precursor cells [19]. This supports our current knowledge regarding the location in endosteal spaces of the alveolar bone and the perivascular location in the periodontal ligament of progenitors cells that will give rise to the progeny of periodontium cell populations named cementoblasts, osteoblasts and periodontal ligament fibroblasts [20,21] which have the potential to recreate the original periodontal tissues [22,23].

It has been shown that periodontal ligament cells cultured in a three-dimensional fashion have a multi-lineage potential since these cells differentiated into cementoblastic/osteoblastic and chondrogenic-like phenotypes [24]. Additionally, cementum extracts have shown

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to induce cartilage phenotype in mesenchymal cells [25]. *In vitro* and *in vivo*, human recombinant CEMP1 has proven to possess functional abilities for inducing chondrogenic and osteogenic activities [13,14,26,27]. CEMP1 induces periodontal ligament cells to express cartilage markers such as type II and X collagen, ACAN and SOX9 [24] and shares a similar sequence with types X and XI collagens. It also presents cross-reaction with type X collagen [3].

We hypothesized that human cementoblastoma-derived cells express some stem cell-like properties, express cartilage-like phenotype *in vitro* and *in vivo* and promote bone regeneration in critical-size defects in rat calvarium which implies a cartilage intermediary. To test this hypothesis, we determined the expression of stem cell and cartilage markers, CD146, STRO-1, type II and X collagen, ACAN and SOX9 *in vitro* and then transplanted human cementoblastoma-derived cells into critical-sized defects in a rat cranium model to study their role in bone regeneration.

Materials and Methods

Cell culture

Human cementoblastoma-derived cells (HCDC) and human gingival fibroblasts (HGF) were isolated and grown as described previously [28,29]. Cells between the 1st and 2nd passages were used for the experiment. The cells were grown in DMEM media supplemented with 10% fetal bovine serum and antibiotics (streptomycin 100 µg/ml, penicillin 100 units/ml). Cell cultures were maintained in an atmosphere of 95% air/5% CO_2 at 37°C with 100% humidity.

Western blot

HCDC and HGF cells were plated at a 5×10^4 density in 6/well culture plates and cultured for 3, 7 and 14 days. At the end of the experimental period, cells were scraped with a policeman and dissolved in lysis buffer containing 1% SDS and protease inhibitor cocktail [3]. Protein concentrations were determined as described elsewhere [30]. Western blot analyses were performed using rabbit polyclonal antibodies against human SOX9, type II and X collagen, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CEMP1, (produced in house) and mouse anti-human CD146, (Santa Cruz Biotech. USA). Proteins from HCDC and HGF-derived cells (10 µg/lane) were separated on SDS/12% PAGE (except for type II collagen and CD146 which were separated on SDS/10% PAGE), and electroblotted onto an Immobilon-P (PVDF) membrane (Millipore Corp.). Membranes were blocked using 5% non-fat milk for 1 h and then incubated with 1:300 diluted antibodies for 1 h. After washing, membranes were incubated with 1:1000 diluted HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG or goat anti-mouse secondary antibody for 1 h. Membranes were washed with PBS and developed as described elsewhere [31]. Blots were scanned and analyzed with a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290. The relative level of each protein was assessed by measuring the integrated intensity of all pixels in each band excluding the local background. Results are expressed as percentages of protein intensity.

Real-time PCR

HCDC and HGF cells were cultured during 3, 7 and 14 days as described above. At term, cells were collected and analyzed for mRNA expression of ACAN, SOX9, type II and X collagen and CEMP1 by real-time RT-PCR. Total RNAs were extracted according to the manufacturer's recommended protocols with Trizol Reagent (Invitrogen, Carlsbad, USA). Ten Nano grams were used per reaction and the level of mRNA expression was quantified by the one-step real-time RT-PCR method using Superscript® III Platinum® SYBR® Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA). Primer sequences for human genes encoding CEMP1, SOX9, types II and X collagens, ACAN and GAPDH were as follows (forward/reverse): CEMP1 (5′- ATGGGCACATCAAGCACCTGA -3′/5′-CCCCATTAGTGT-CATCCGC -3′); SOX9 (5′- GTAATCCGGGTGGTCCTTCT -3′/5′-GACGCTGGGCAAGCTCT -3′); type II collagen (5′- CGGCTTC-CACACATCCTTAT -3′/5′- CTGTCCTTCGGTGTCAGGG -3′); type X collagen (5′- GTGGACCAGGAGTACCTTGC -3′/5′- CATA-AAGCCCACTACCCA -3′); aggrecan (5′- ACAGCTGCAGTGAT-GCCCT -3′/5′- TTCTTGGAGAAGGGAGTCCA -3′); GAPDH (5′-CAACGGATTTGGTCGTATTGG-3′/5′- GCAACAATATCCACTT-TACCAAGAGTTAA -3′). A 25 µL reaction was set up with the following PCR conditions: (cDNA synthesis) 50°C for 3 min, denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and finally 40°C, 1 min. Amplifications were performed in a Corbett Rotor-Gene 6000 (Qiagen, Valencia, CA, USA). All experiments were performed in triplicate and expression levels of the abovementioned molecules were obtained using delta-delta ct method normalizing for GAPDH.

Flow cytometry

HGF and HCDC were cultured up to confluence and then incubated with DMEM supplemented as mentioned before and with the addition of brefeldin A (BFA) to a final concentration of 10 µg/ml, for 4 hr at 37°C, 5% CO_2, 95% air atmosphere. Cells were detached with 0.02% trypsin-0.1% EDTA, re-suspended in PBS with 0.05% bovine serum albumin (BSA) and then washed twice with PBS-0.05%-BSA. An indirect immunofluorescence technique was used for protein detection. Briefly, Non-specific binding was blocked by incubating the cells in PBS with 5% BSA for 10 min. For STRO-1 and CD146 detection, cells were incubated for 30 min at 4°C with STRO-1 or CD146 mouse anti-human monoclonal antibodies (Santa Cruz Biotechnology, CA, USA), diluted 1:100 in PBS, followed by FITC-labeled rabbit anti-mouse IgG antibody (Invitrogen, Carlsab, CA, USA) for 30 min at 4°C for CEMP1, type II and X collagen, SOX9 and ACAN. Cells were first fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson, CA, USA) for 15 min, followed by fluorochrome-labeled IgG Cy5 goat antibody against rabbit (Invitrogen, Carlsab, CA, USA) for 30 min at 4°C. For isotype controls monoclonal IgG2a-FITC (eBioscience, San Diego, CA, USA) was used. Cells were fixed with 1% paraformaldehyde at pH 7.4 and 3×10^4 events were acquired using a BD Biosciences FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA) and a BD Cell Quest, Pro v.5.1.1 software (Beckton Dickinson). The percentage of cells labelled with each Ab was calculated compared to cells stained with isotype control antibody. Cytometry data were analyzed using the WinMidi 2.8 software.

In vivo cranial defect surgery

***Ethics Statement*:** All animal procedures were approved by the Institutional Research Ethical and Animal Care and Use Committee (Facultad de Odontología, Universidad Nacional Autónoma de México). The animals were euthanized with carbon monoxide gas. For orthotopic implantation, 72 Wistar rats (average mass of 250 g and 6 weeks old) (Harlan Laboratories, México City, México) were used. Since in this study a critical-size calvarial defect was used, in order to prevent rejection, rats were given a single dose of cyclophosphamide immunosuppressant (100 mg/kg) two days after human cells implantation [32,33]. Three dimensional gelatin matrix scaffolds with

or without cells (HCDC or HGF) were implanted under anesthesia (0.2 mL/100 g body weight via intramuscular injection of a pre-mixed solution composed of ketamine-hydrochloric acid (HCL) (64 mg/mL) (Keta-Sthetic, Boehringer Ingelheim, St. Joseph, MO), xylazine (3.6 mg/mL) (Rompun, Miles, Shawnee Mission, KS) and atropine sulfate (0.07 mg/mL), (Elkins-Sinn, Cherry Hill, NJ). Hair over the calvarium was shaved and cleaned with a depilator. Xylocaine (1%, 0.5 mL) was injected intradermally in the middle of the calvarium. A 3 cm midline calvarial incision was made, cranial skin flaps were elevated, the subcutaneous fascia was divided and periosteal flaps were reflected bilaterally. Using a trephine drill (Fine Science Tools, Inc., Foster City, CA), a 9 mm hole was drilled, penetrating through the calvarial bone. Constant saline irrigation was used during this process and extreme care was taken not to damage the dura mater. The animals were randomly assigned to 4 groups; two groups acted as control with 6 animals each. A trephine burr was used to create a standardized defect. The various groups were treated as follows: Group 1: The defect was implanted with gelatin matrix scaffold without cells. Group 2: The defect was left empty. Group 3: The defect was implanted with gelatin matrix carrier scaffold with a 5×10^5 HCDC suspension. Group 4: The defect was implanted with gelatin matrix scaffold containing 5×10^5 HGF suspensions. Each subgroup consisted of 6 rats. The scaffolds containing HCDC or HGF were then placed into the cranial defects. Cranial skin was sutured with Vicryl 5-0 sutures. All animals in each group were euthanized with carbon monoxide at 14, 30 and 60 days post-implantation and the calvariae were harvested. All animals survived throughout the experimental terms.

Histomorphometric analysis

After 14, 30 and 60 days the animals were euthanized and the whole calvarium was removed and fixed in 10% paraformaldehyde for 24 h. decalcified in 0.5% formaldehyde containing 10% EDTA, pH 7.4, at 4°C for 5 wks. The samples were dehydrated in a graded alcohol series, embedded in paraffin, and 5 μm thick sections were prepared and stained with H&E (hematoxylin and eosin). Sections from 14 days specimens were axially oriented in order to produce a plane of analysis through the center of the defect. Three central section's/defects were used for histometric/histological analysis. Additionally, three central sections were stained with Masson's trichrome for cross-reference. Sections were stained with safranin-O to detect sulfated GAG. Briefly, slides were stained 0.1% Safranin-O for 5 min with a brief rinse in 1% acetic acid in water. In order to stain acidic polysaccharides such as glycosaminoglycans in cartilage slides were stained with Alcian Blue 8GX 1% (w/v), pH 2.5 (Sigma) for 30 min at room temperature. Slides were washed, dehydrated and cleared with xylene before covering with mounting medium and cover slipped. Assessment under light microscopy was conducted to complete morphological evaluation of the tissue's response in the different groups. Furthermore, on all sections from the specimens that were implanted for 30 and 60 days image analysis was performed for quantitative analysis of the newly formed bone. The outline of the implant area was defined. Finally, using the image analysis program, surface area of bone and percentage of bone area were assessed and an average value was calculated (Zen Lite, Carl Zeiss, Germany).

Immunostaining in human periodontal tissues

Immunoexpression of ACAN, SOX9, type II and X collagen, CEMP1, and CD146 was determined in human dental tissues which were obtained from autopsy specimens (Departmento de Patología, Facultad de Odontología, Universidad Nacional Autónoma de México), following the policies of the Ethics and Research Review Board of the Facultad de Odontología, Universidad Nacional Autónoma de México. The specimens containing periodontal structures were fixed, decalcified, embedded in paraffin, sectioned and mounted on glass salinized slides as described elsewhere [4]. Double-immunofluorescence staining was performed using mouse anti-human CD146 monoclonal antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal antibodies against human ACAN, SOX9, type II and X and XI collagen (Santa Cruz Biotechnology, Inc.), and CEMP1 (produced in house) as described elsewhere [3]. Briefly, sections were pre-treated with 10% BSA in PBS for 1 hour at room temperature and incubated with primary monoclonal antibodies (diluted 1:300 in PBS) and polyclonal antibodies (diluted 1:500 in PBS) for 12 h at 4°C. Sections were then incubated with 1:50 diluted FITC-conjugated anti-mouse IgG (Molecular Probes; Eugene, OR) and Alexa-Fluor-594-conjugated anti-rabbit IgG (Molecular Probes), diluted 1:400 as secondary antibodies for 1 h at room temperature. Samples were analyzed using a fluorescent microscope (Axioskope 2, Carl Zeiss, and Germany) with the appropriate filter combinations. Sections incubated with normal rabbit or mouse serum or lacking first antibody were used as controls.

Statistical analysis

Seventy two animals were used for control and experimental groups and values are expressed as mean ± S. E. Multiple comparisons between treatment groups were made with the Neuman-Keuls post-hoc test with a two-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. Statistical analyses were performed with Sigma Stat V 3.1 software (Jandel Scientific Ashburn, VA).

Results

Expression of molecules associated with cartilage phenotype

Gene expression: Gene expression profiling of isolated cell fractions from HGF and HCDC is shown in Figure 1. Quantitative PCR demonstrated that HCDC expressed ACAN ($P<0.001$), collagen type II, ($P<0.001$) and collagen type X ($P<0.003$) at 3, 7 and 14 days of culture. However, SOX9 mRNA expression showed no differences between HGF and HCDC. There was also significant enrichment of CEMP1 mRNA in the cell population representing HCDC at 7 and 14 days of culture as compared to HGF mRNA ($P<0.001$) (Figure 1).

FACS: Figure 2 shows that CEMP1 cementum molecule is expressed by HCDC approximately 5 fold when compared with HGF. The expression of cartilage markers revealed that there is a high expression of HCDC; SOX9 was expressed 4 fold, ACAN 3.5 fold, type II collagen 3-fold and importantly, type X collagen was expressed 7-fold when compared to HGF. The expression of STRO-1 and CD-146 stem cell markers was analyzed in both HGF and HCDC. The results show that STRO-1 is incipiently expressed in HGF and HCDC. Nevertheless, CD-146 is expressed 2.5 fold in HCDC when compared to HGF.

Western Blot: Total protein extracted from HCDC and HGF was subjected to western blotting and used to determine the expression of cementum and cartilage-related proteins The results shown in Figure 3 indicate that cementum protein 1 (CEMP1) is expressed in HCDC in 3, 7 and 14 days by 2, 1 and 2.2-fold when compared to HGF. Cartilage-related proteins such as type X collagen were expressed by 5, 3.2 and 4-fold at 3, 7 and 14 days of culture respectively. CD146 was expressed 1, 3 and 7-fold at 3, 7 and 14 days of culture respectively, type II collagen was expressed 0.5 fold at 3 days of culture when compared to HGF, and SOX 9 showed an incipient expression in both HCDC and HGF.

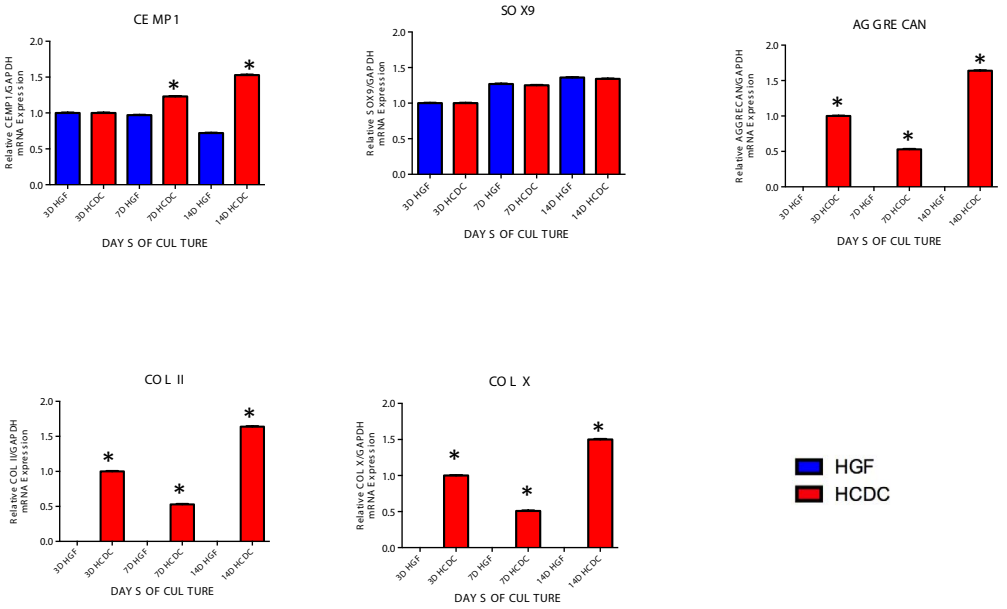

Figure 1: Gene expression of cartilage-related molecules in HCDC and HGF. Quantitative real time PCR shows that the expression of ACAN, types II and X collagens is restricted to HCDC. CEMP1 expression was higher at 7 and 14 days in HCDC. SOX 9 show that there is no difference between HCDC and HGF at this expression level. HCDC show CEMP1' higher expression at 7 and 14 days when compared to HGF (*p<0.05).

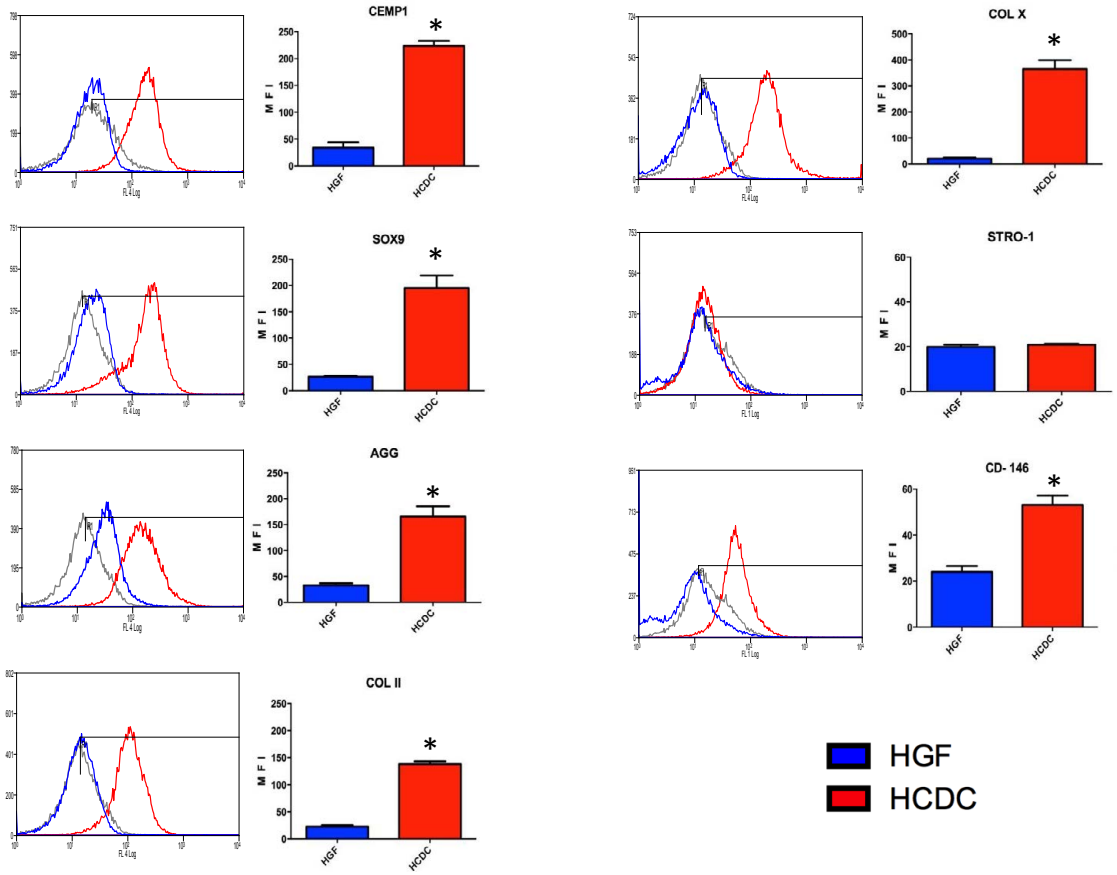

Figure 2: FACS analyses of cartilage-related molecules and STRO-1 and CD146 stem cell markers in HCDC and HGF. A strong expression of SOX9, ACAN, types II and X collagens, CD146 and CEMP1 is revealed in comparison with HGF. Results indicate that the population of human cementoblastoma-derived cells, highly express these markers when compared to HGF. STRO-1 expression was similar between both cell types. (*p<0.05).

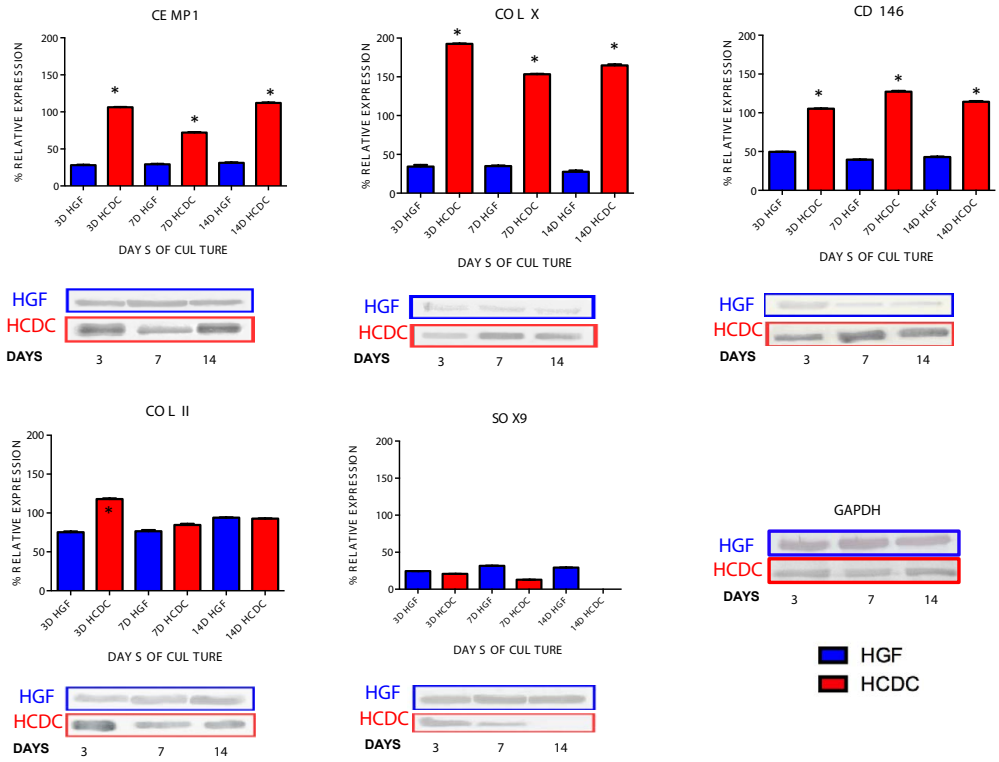

Figure 3: Western Blot analysis. CEMP1 and CD146 are noticeably expressed when compared to HGF. Type X collagen is strongly expressed at 3, 7 and 14 days of culture. SOX9 and type II collagen show no statistical differences between HCDC and HGF (*p<0.05).

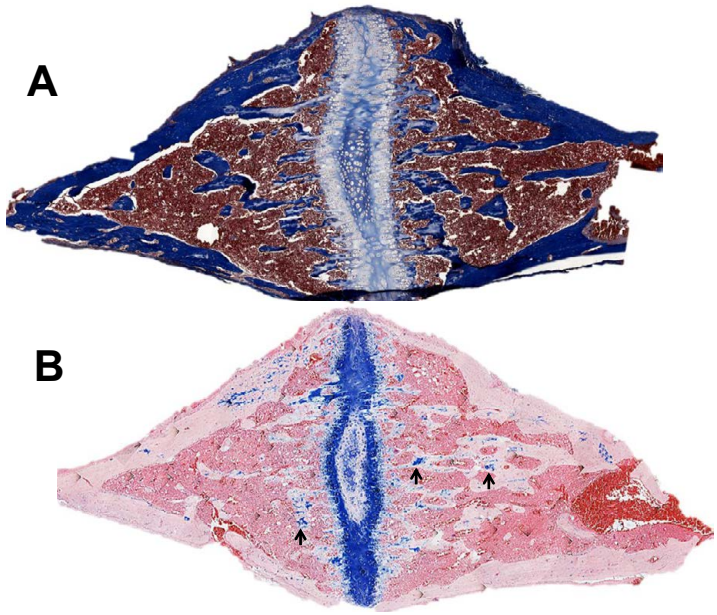

Figure 4: Histological analysis of regenerated tissue in rat calvaria at 14 days post-implantation shows a center of cartilage-like tissue in the center with hypertrophic cartilage in the periphery and strips of new bone formation stained with Masson's Thrichrome (A). Axial section representing cartilage-like tissue revealed to be strongly stained by alcian blue as well as some islands on the extension of the defect (Arrow), (B). Magnification 10x.

Cartilage and bone formation *in vivo* by HCDC: In order to determine if HCDC or HGF could induce bone formation, we used a standardized model of critical-sized defects in rat calvaria. For this purpose a gelatin scaffold containing either HCDC or HGF or a gelatin blank scaffold was used. Calvarial rat samples were collected after 14, 30 and 60 days. Samples obtained at 14 days revealed that those defects treated with HCDC embedded in gelatin matrix scaffold showed tissue with histomorphological characteristics resembling cartilage-like tissue surrounded by strips of bone (Figure 4A). A panoramic section stained with alcian blue revealed the presence of

acid mucopolysaccharides around the cartilage-like tissue (Figure 4B). In Figure 5 it is observed at major detail the tissue representing cartilage. The cells showed the characteristics of chondrocytes and hyperthrophic cartilage becoming bone-like tissue. The staining of cartilage-like tissue with Masson´s trichrome stain reveals in detail the cellular characteristics of cartilage, hypertrophic cartilage and newly formed bone (Figure 5A). Safranin O and alcian blue reveal its identity as a cartilage (Figure 5B and 5C). Defects treated with HGF showed only connective tissue (data not shown). Calvarial defects showed that control specimens (gelatin matrix) analyzed 30-days post-surgery displayed a band of thin connective fibrous tissue bridging the extremes of the defect without evidence of any sign of bone growth (Figure 6A). Defects that were implanted with HGF embedded in gelatin matrix showed a band of fibrous connective tissue connecting both extremes of the defect (Figure 6B). Experimental scaffolds of gelatin matrix containing HCDC showed an almost complete filling of the cranial defect. Interestingly the bone-like formed tissue was located in the middle of the defect. The bone-like tissue was connected to the extremes of the defect by a thick bundle of fibrous connective tissue (Figure 6C). At sixty days post-surgery, control specimens (blank defect) showed a thin fibrous bridge of connective tissue (Figure 7A). Defect treated with HGF embedded in gelatin matrix showed a disorganized and amorphous mineralized tissue which represented 23% ± 2.3% of new bone (Figure 7B). Experimental defects treated with HCDC embedded into the gelatin matrix showed a complete filling of the defect with bone-like tissue; the newly formed bone-like tissue showed morphological characteristics of normal bone with osteocytes embedded in their lacuna and osteoblasts lining the outer edge of

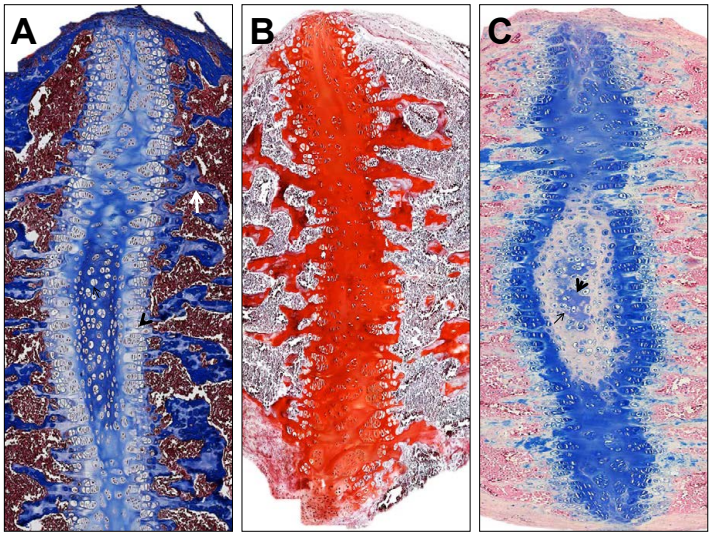

Figure 5: A detailed histological analysis of the center of the calvarial defect is shown. A. Staining of the center of the cartilage-like-tissue with Masson's Thrichrome shows that the center of the defect is filled with chondroblast-like cells with a "mirror" (arrow) appearance, the periphery composed by hypertrophic cartilage (arrowhead) and strips of mineralized bone (thick arrow). B. Staining with Safranin O revealed the identity of cartilage-like tissue by staining the tissue formed in the center of the defect. Detailed alcian blue staining shows that cells located in the center of the defect are stained (arrow) as is the band of tissue surrounding it where chondrocytes nucleus are also stained (arrowhead). The periphery of this tissue is strongly stained with alcian blue (thick arrow) Magnification 20x.

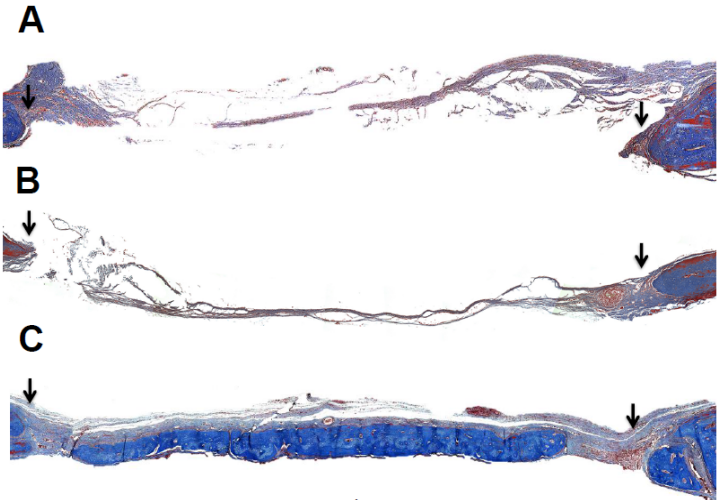

Figure 6: Histological analysis at 30 days post-implantation. Sections were stained with Masson´s trichrome. Critical-sized calvarial defects treated either with gelatin scaffold or without treatment show a bridge of dense connective tissue fiber bundles (A). Defects treated with HGF shows a bridge of connective tissue (B). Defects treated with HCDC show bone-like tissue occupying most of the defect. Notoriously, bone connection was not observed with the margins of the defect (C) Magnification 10x.

Figure 7: Histological analysis at 60 days post-implantation. (A) The figure shows the control defect which was either empty or treated with gelatin scaffold. A band of connective tissue connects the borders of the defect; no bone formation is evident. (B) Defects treated with HGF show the formation of amorphous and disorganized bone-like tissue (ossicles) in the center of the defect. (C) Defects treated with HCDC show almost complete filling up to 91% of the defect with bone-like tissue. Magnification 10x.

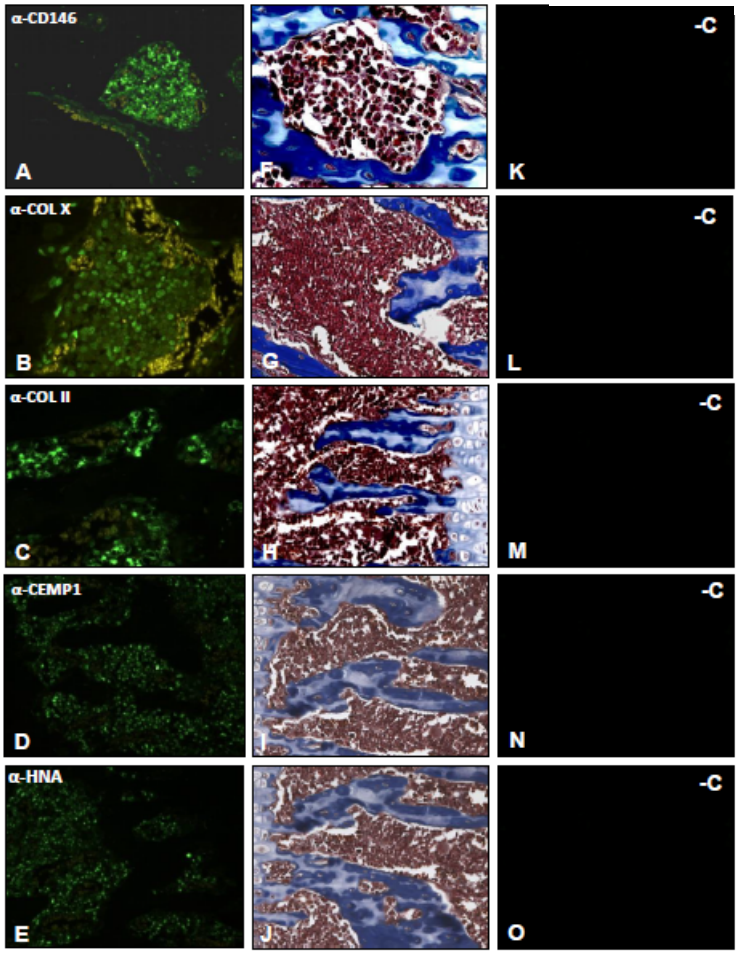

Figure 8: Immunostaining of cells in the calvarial defect. At 14 days cells located in the vicinity of bone and cartilage-like tissue express CD146 strongly (A). Immuno-expression of type X (B) and II (C) collagens is observed in bone-marrow-like tissue. Some cell populations express these cartilage markers as well. Cells located in these spaces strongly express CEMP1 (D) and human nuclear antigen which implies that the cells that deposit cartilage-like tissue are from human origin (E). Magnification 20x A, B and C. 10x B and C. Specimens stained with Masson´s thrichrome for anatomical orientation (F-J), magnification 20X. Negative controls (K-O).

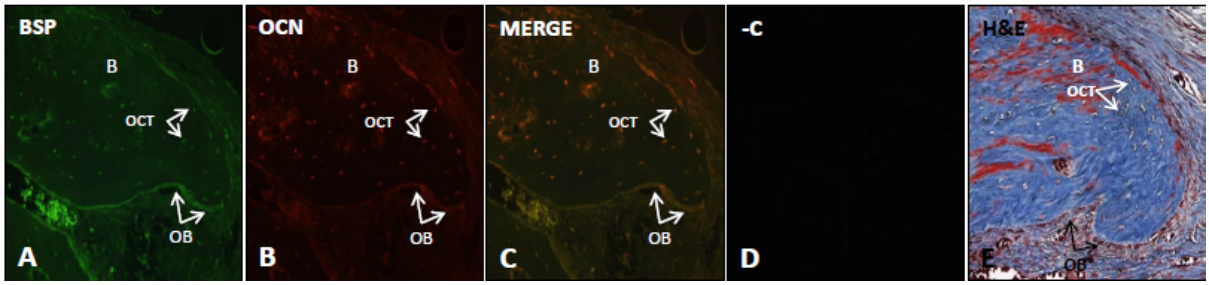

Figure 9: Molecular analysis of bone-like tissue by double immunostaining. Osteoblast cells facing the border of bone (OB) and osteocytes (OCT) express BSP. Arrows (A). Osteocalcin shows similar expression in osteoblasts (OB) and osteocytes (OCT) and stains intensively (B). Co-localization of both proteins is shown in osteoblasts (OB) and osteocytes (OCT) (C). Negative control using rabbit or mouse preimmune serum (D). H&E staining for anatomical orientation (E). Magnification 20x.

the bony tissue (Figure 7C). There was no evidence of inflammatory response in the experimental or control groups. Histomorphometric analysis was performed using H&E stained sections. Cranial critical-size defects treated with HCDC demonstrated a 70% ± 1.6% and 91% ± 1.3% of newly formed bone at 30 and 60 days respectively. This was statistically significant as compared to the gelatin-only matrix scaffold and the blank cranial defect. Control specimens, blank defect and gelatin matrix alone, were filled with fibrous tissue only.

Molecular analysis of the filled defect: In order to assess the molecular identity of the filled defect, the presence and expression of cartilage related molecules was analyzed by immunofluorescence tissue sections from 14 days. Our results revealed that CD146, a stem cell marker for chondroprogenitors, is strongly expressed by cells located in bone-marrow like spaces (Figure 8A). Type X and II collagen had a similar location; however there was evidence that some cell subpopulations expressed these cartilage-related molecules (Figure 8B and 8C respectively). Cells surrounding the cartilage-like tissue and surrounded by bone-like tissue expressed CEMP1 which was observed widely distributed among cells located into the spaces between bone strips (Figure 8D). Human nuclear antigen reveals that cells located around the cartilage-like tissue in spaces that resembled bone marrow cross-reacted with this antibody, establishing that the cells responsible of cartilage and bone regeneration are induced by HCDC (Figure 8E). Double-immunofluorescence revealed that bone related molecules such as BSP and OCN were expressed by osteoblasts facing the mineralized front of bony tissue as well as osteocytes immersed into the mineralized matrix and both co-localized in the same cellular structures (Figure 9A-9C). However, OCN showed a more intense expression in osteocytes. Controls using pre-immune mouse and rabbit serum were negative.

Immunolocalization of cartilage-related molecules in human periodontal tissues: Figure 10 shows the immunolocalization of CD146 on human periodontal tissues revealed that it was expressed by cementoblasts facing the cementoid phase as well as some periodontal ligament cell subpopulations. CEMP1 showed a similar tissue distribution and was strongly expressed by cementoblasts. Both proteins were localized in the same tissue components. Periodontal ligament cells and some cementoblasts expressed type II collagen. Type X collagen was lightly expressed by cementoblasts facing the cemented phase of cementum and a few cells located into the periodontal ligament. SOX9 was strongly expressed by cells in the periodontal ligament and highly expressed in cementoblasts. ACAN was also expressed by cementoblasts facing the cementum. Periodontal ligament cells also expressed this molecule. CD146 and the molecules tested in this study immunoreacted with cells around blood vessels in the periodontal ligament which were strongly positive. Controls using pre-immune mouse and rabbit serum were negative.

Discussion

In this research human benign cementoblastoma-derived cells have been used. Previously, these cells were characterized as putative cementoblasts [29]. Although cementoblastoma is a rare ectomesenchymal odontogenic tumor that originates from the root of the tooth, it is characterized by the formation of cementum-like tissue and recapitulates cementum deposition similar to that during root formation in the later stages of odontogenesis. Furthermore, cementoblastoma has continuity with the cementum layer of the apical third of the tooth root and remains separated from bone by a continuation of the periodontal ligament, all of which supports an odontogenic origin [33]. Therefore these cells have been used in the past as putative cementoblasts-like cells [29].

Our results show that HCDC express cartilage-related molecules *in vitro* and promote bone formation in critical-sized defects in rat cranium through endochondral ossification. From our findings we infer that HCDC can give rise to cartilage, which eventually becomes hypertrophic and turns to bone. We have shown that HCDC express CD146 which is a 113 kDa to 119 kDa transmembrane glycoprotein expressed in normal adult tissues by vascular endothelium and bone marrow cells and it was recently identified as a marker of isolated chondroprogenitor cell sub-populations localized near and around blood vessels. It also identifies osteoprogenitors [34]. Since $CD146^{+}$ chondroprogenitors exhibit high potential for cartilage formation [35], our findings demonstrate that pericytes and cells in the periodontal ligament with paravascular location are $CD146^{+}$. Perycytes expressing $CD146^{+}$ have shown to yield osteocytes, chondrocytes and adypocytes [36], thus indicating that perycites and paravascular cells have multi-lineage potential including the ability to differentiate into cementoblasts. From these results we infer that cementoblasts might maintain their "ancestral memory" with chondrogenic/osteogenic differentiation potential.

Cell populations in HGF and HCDC show similarity in STRO-1 expression. This is not an uncommon finding since there is evidence that human gingival fibroblasts-derived cells possess a heterogeneous mesenchymal stem cell population which can differentiate *in vitro* into osteoblasts, chondroblasts and adypocytes [37-41]. Therefore they are able to form bone in calvarial defects [42]. Interestingly, it has been shown that $STRO\text{-}1^{+}$ gingival MSCs induce regeneration of the periodontal structures, including bone, cementum and periodontal ligament [43]. Isolated mRNA from HCDC and HGF showed the expression of key chondrogenic genes, including type II and X collagen, SOX9 and ACAN [44]. Type II collagen is present in native cartilage and is a marker for chondrocyte differentiation [45-47]. Our results show that type II collagen (the primary structural protein

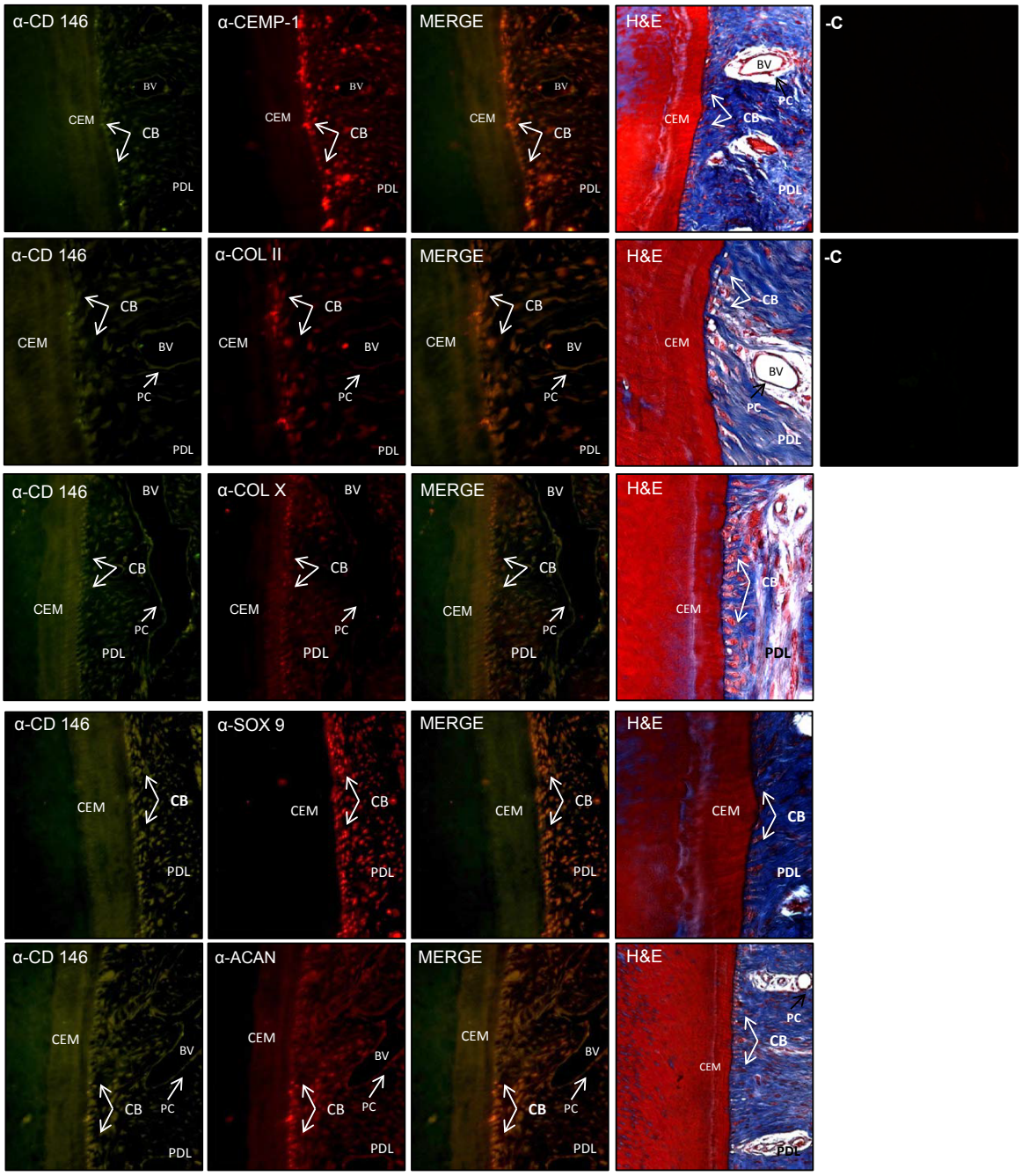

Figure 10: Immunofluorescence identification of CD146, cartilage markers and CEMP1 on human periodontal tissues. Triplets of microphotographs show the distribution and co-localization of CD146 with CEMP1, types II and X collagens, SOX9 and ACAN. Masson´s trichome staining sections for anatomical orientation are shown. The row at the right is a representative negative control using normal rabbit or mouse serum, which was negative. Notice the strong SOX9 and CEMP1 expression in cells lining the cementum and weak expression of type II and X collagens and ACAN. CEM=cementum; CB=cementoblasts; BV=blood vessel; PC=perycites; PDL=periodontal ligament. Magnification 20x.

for cartilage) is only expressed by HCDC and not by HGF. HCDC contained a higher cell population that express type II collagen, ~3-fold relative to HGF. The expression of SOX9, a transcription factor which plays a regulatory role during chondrogenesis and type II collagen expression, showed no differences between HCDC and HGF. Nevertheless, FACS analysis revealed that SOX9 was expressed 3.5 fold more by HCDC when compared to HGF. ACAN is a structural glycoprotein important in mediating chondrocyte-chondrocyte and chondrocyte-matrix interaction and it is a marker for pre-hypertrophic chondrocytes [48,49]. It also regulates chondrocyte morphology [50,51]. This molecule was only expressed by HCDC. Type X collagen is specific to cartilage and is synthesized by terminally differentiating chondrocytes, mainly hypertrophic chondrocytes. It is thought to facilitate the calcification process. Thus, it is of major importance in endochondral bone growth and development [52-56] and invariably precedes the onset of ossification [57,58]. However, a hypertrophic cartilage template contains all necessary signals to initiate bone tissue formation, vascularization, remodeling, and establishment of bone. The

central construct core is progressively filled with matrix and ultimately remodeled into trabecular-like bone structures [59]. In our model, bone regeneration was probably engineered by activating HCDC toward an endochondral ossification route and thus by invoking a developmental engineering paradigm [59]. CEMP1 a specific marker for cementum has been shown to induce periodontal ligament cells towards osteogenic/chondrogenic phenotype and it is thought to play a role during mineralization *in vitro* and induce bone regeneration *in vivo* [13]. It is expressed at high levels in HCDC relative to HGF; it is therefore possible that HCDC producing CEMP1 have the potential to differentiate to osteoblasts-like and chondroblast-like cells.

Our *in vivo* studies using a standard critical-size model in rat cranium clearly demonstrate that HCDC induces bone formation through endochondral ossification. Our results show that cartilage-like tissue formed after 14 days contains core cells morphologically similar to chondrocytes and a hypertrophic zone of cartilage on the periphery. Molecular analysis reflects the nature of cartilage based on expression of type II and X collagen and CD146. We believe that these cells are chondroprogenitors. However, there was heterogeneity in the expression of cartilage markers in cells facing cementum thus indicating that this is not a unique cementoblast phenotype. This phenomenon has also been described for osteoblasts [60]. More importantly these cells exhibited immunoreaction with anti-human nuclear antigen indicating that HCDC possess the potential to differentiate towards osteoblasts and chondroblasts. The histological and molecular characteristics of the formed bone reflected mineralized tissue with lamellar organization, bone marrow spaces; osteoblasts bordering bone surface and osteocytes within bone which immunoreacted with antibodies against BSP and OCN. This observation confirms the identity of bone. HGF showed a disorganized mass composed mainly by ossicles without the apparent presence of bone marrow spaces. As demonstrated previously, a single administration of cyclophosphamide (100 mg/kg) soon after antigenic stimulation duplicates the administration of multiple injection [31,32].

Taken together, all these data indicate that HCDC possess some stem cell-like properties and the capacity of lineage differentiation at least to chondroblastas and osteoblasts. Our data adds to the hypothesis that chondroblasts, cementoblasts and osteoblasts might have their origin in an ancestral source of progenitor cells located paravascularly and in perycites bordering blood vessels in the periodontal ligament [9,22,61,62]. Therefore, possibly, cementoblasts hold the potential to maintain homeostasis in the periodontium and act as progenitor cells themselves. However, it is important to consider that HCDC may not represent a homogeneous cell population. In this respect HCDC possess some characteristics of stem cells which have the ability to divide asymmetrically giving rise to two daughter cells, one with self-renewal capability and the other, a progenitor cell which has the ability of differentiation but not self-renewal [63]. Nevertheless it appears highly plausible that within each neoplasm there is a small fraction of cells with stem cell property which are the responsible for initiating and maintaining a tumor like it might happen in the case of human cementoblastoma [64]. Based on this statement and according to our findings HCDC contains some cell populations with stem cell potential.

In conclusion, the present study has demonstrated that HCDC are a highly potent population of progenitor cells retaining some stem cell-like properties, with ability to differentiate into specialized lineages and regenerate functional tissues. Together, our findings provide a greater insight into HCDC phenotype and illustrate their ability to regenerate mineralized tissues.

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References

1. Persidis A (1999) Tissue engineering. Nat Biotechnol 17: 508-510.
2. Ivanovski S, Gronthos S, Shi S, Bartold PM (2006) Stem cells in the periodontal ligament. Oral Dis 12: 358-363.
3. Alvarez-Pérez MA, Narayanan S, Zeichner-David M, Rodríguez Carmona B, Arzate H (2006) Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein (CP-23). Bone 38: 409-419.
4. Valdés De Hoyos A, Hoz-Rodríguez L, Arzate H, Narayanan AS (2012) Isolation of protein-tyrosine phosphatase-like member-a variant from cementum. J Dent Res 91: 203-209.
5. Komaki M, Iwasaki K, Arzate H, Narayanan AS, Izumi Y, et al. (2012) Cementum protein1 (CEMP1) induces a cementoblastic phenotype and reduces osteoblastic differentiation in periodontal ligament cells. J Cell Physiol 227: 649-657.
6. Arzate H, Zeichner-David M, Mercado-Celis G (2015) Cementum proteins: role in cementogenesis, biomineralization, periodontium formation and regeneration. Periodontol 67: 211-233.
7. Nuñez J, Sanz M, Hoz-Rodríguez L, Zeichner-David M, Arzate H (2010) Human cementoblasts express enamel-associated molecules in vitro and in vivo. J Periodontal Res 45: 809-814.
8. Paula-Silva FW, Ghosh A, Arzate H, Kapila S, da Silva LA, et al. (2010) Calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1- and ERK-dependent manner. Calcif Tissue Int 87: 144-1457.
9. Melcher AH, McCulloch CA, Cheong T, Nemeth E, Shiga A (1987) Cells from bone synthesize cementum-like and bone-like tissue in vitro and may migrate into periodontal ligament in vivo. J Periodontal Res 22: 246-247.
10. Nuñez J, Sanz-Blasco S, Vignoletti F, Muñoz F, Arzate H, et al. (2012) Periodontal regeneration following implantation of cementum and periodontal ligament-derived cells. J Periodontal Res 47: 33-44.
11. MacNeil RL, Thomas HF (1993) Development of the murine periodontium. II. Role of the epithelial root sheath in formation of the periodontal attachment. J Periodontol 64: 285-291.
12. Grzesik WJ, Kuzentsov SA, Uzawa K, Mankani M, Robey PG, et al. (1998) Normal human cementum-derived cells: isolation, clonal expansion, and in vitro and in vivo characterization. J Bone Miner Res 13: 1547-1554.
13. Serrano J, Romo E, Bermúdez M, Narayanan AS, Zeichner-David M, et al. (2013) Bone regeneration in rat cranium critical-size defects induced by Cementum Protein 1 (CEMP1). PLoS One 8: e78807.
14. Montoya G, Arenas J, Romo E, Zeichner-David M, Alvarez M, et al. (2014) Human recombinant cementum attachment protein (hrPTPLa/CAP) promotes hydroxyapatite crystal formation in vitro and bone healing in vivo. Bone 69: 154-164.
15. Bosshardt DD (2005) Are cementoblasts a subpopulation of osteoblasts or a unique phenotype?. J Dent Res 84: 390-406.
16. Sculean A, Donos N, Windisch P, Brecx M, Gera I, et al. (1999) Healing of human intrabony defects following treatment with enamel matrix proteins or guided tissue regeneration. J Periodontal Res 34: 310-322.
17. Yukna RA, Mellonig JT (2000) Histologic evaluation of periodontal healing in humans following regenerative therapy with enamel matrix derivative. A 10-case series. J Periodontol 71: 752-759.
18. Bosshardt DD, Sculean A, Windisch P, Pjetursson BE, Lang NP (2005) Effects of enamel matrix proteins on tissue formation along the roots of human teeth. J Periodontal Res 40: 158-167.
19. Tenorio D, Cruchley A, Hughes FJ (1993) Immunocytochemical investigation of the rat cementoblast phenotype. J Periodontal Res 28: 411-419.
20. Pitaru S, McCulloch CA, Narayanan SA (1994) Cellular origins and differentiation control mechanisms during periodontal development and wound healing. J Periodontal Res 29: 81-94.

21. McCulloch CA (1995) Origins and functions of cells essential for periodontal repair: the role of fibroblasts in tissue homeostasis. Oral Dis 1: 271-278.

22. Gould TR, Melcher AH, Brunette DM (1980) Migration and division of progenitor cell populations in periodontal ligament after wounding. J Periodontal Res 15: 20-42.

23. McCulloch CA, Melcher AH (1983) Cell migration in the periodontal ligament of mice. J Periodontal Res 18: 339-352.

24. Hoz L, Romo E, Zeichner-David M, Sanz M, Nuñez J, et al. (2012) H. Cementum protein 1 (CEMP1) induces differentiation by human periodontal ligament cells under three-dimensional culture conditions. Cell Biol Int 36: 129-136.

25. Arzate H, Chimal-Monroy J, Hernández-Lagunas L, Díaz de León L (1996) Human cementum protein extract promotes chondrogenesis and mineralization in mesenchymal cells. J Periodontal Res 31: 144-148.

26. Carmona-Rodríguez B, Alvarez M, Narayanan AS, Zeichner-David M, Reyes-Gasga J, et al. (2007) Human Cementum Protein 1 induces expression of bone and cementum proteins by human gingival fibroblasts. Biochem Biophys Res Commun 358: 763-769.

27. Villarreal-Ramírez E, Moreno A, Mas-Oliva J, Chávez-Pacheco JL, Narayanan AS, et al. (2009) Characterization of recombinant human cementum protein 1 (hrCEMP1): primary role in biomineralization. Biochem Biophys Res Commun 384: 49-54.

28. Narayanan AS, Page RC (1976) Biochemical characterization of collagens synthesized by fibroblasts derived from normal and diseased human gingiva. J Biol Chem 251: 5464-5471.

29. Arzate H, Alvarez-Pérez MA, Aguilar-Mendoza ME, Alvarez-Fregoso O (1998) Human cementum tumor cells have different features from human osteoblastic cells in vitro. J Periodontal Res 33: 249-258.

30. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

31. Paterson PY, Hanson MA (1969) Studies of cyclophosphamide suppression of experimental allergic encephalomyelitis in Wistar rats. J Immunol 103: 795-803.

32. Hou FX, Yang HF, Yu T, Chen W (2007) The immunosuppressive effects of 10mg/kg cyclophosphamide in Wistar rats. Environ Toxicol Pharmacol 24: 30-36.

33. Sapp JP, Eversole LR, Wysocki GP (1997) Contemporary Oral and Maxillofacial Pathology. St. Louis: Mosby, Michigan.

34. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, et al. (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131: 324-336.

35. Su X, Zuo W, Wu Z, Chen J, Wu N, et al. (2015) CD146 as a new marker for an increased chondroprogenitor cell sub-population in the later stages of osteoarthritis. J Orthop Res 33: 84-91.

36. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, et al. (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3: 301-313.

37. Fournier BP, Larjava H, Häkkinen L (2013) Gingiva as a source of stem cells with therapeutic potential. Stem Cells Dev 22: 3157-3177.

38. Fournier BP, Ferre FC, Couty L, Lataillade JJ, Gourven M, et al. (2010) Multipotent progenitor cells in gingival connective tissue. Tissue Eng Part A 16: 2891-2899.

39. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, et al. (2009) Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 183: 7787-7798.

40. Tang L, Li N, Xie H, Jin Y (2011) Characterization of mesenchymal stem cells from human normal and hyperplastic gingiva. J Cell Physiol 226: 832-842.

41. Li N, Liu N, Zhou J, Tang L, Ding B, et al. (2013) Inflammatory environment induces gingival tissue specific mesenchymal stem cells to differentiate towards a pro-fibrotic phenotype. Biol Cell 105: 261-275.

42. Wang F, Yu M, Yan X, Wen Y, Zeng Q, et al. (2011) Gingiva-derived mesenchymal stem cell-mediated therapeutic approach for bone tissue regeneration. Stem Cells Dev 20: 2093-2102.

43. El-Sayed Fawzy KM, Paris S, Becker ST, Neuschl M, De Buhr W, et al. (2012) Periodontal regeneration employing gingival margin-derived stem/progenitor cells: an animal study. J Clin Periodontol 39: 861-870.

44. Wu L, Bluguermann C, Kyupelyan L, Latour B, Gonzalez S, et al. (2013) Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. Stem Cell Reports 1: 575-589.

45. Risbud M, Ringe J, Bhonde R, Sittinger M (2001) In vitro expression of cartilage-specific markers by chondrocytes on a biocompatible hydrogel: implications for engineering cartilage tissue. Cell Transplant. 10: 755-763.

46. Kolettas E, Buluwela L, Bayliss MT, Muir HI (1995) Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. J Cell Sci 108: 1991-1999.

47. Seghatoleslami MR, Lichtler AC, Upholt WB, Kosher RA, Clark SH, et al. (1995) Differential regulation of COL2A1 expression in developing and mature chondrocytes. Matrix Biol 14: 753-764.

48. Muir H (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 17: 1039-1048.

49. Dean DD, Lohmann CH, Sylvia VL, Cochran DL, Liu Y, et al. (2002) Effect of porcine fetal enamel matrix derivative on chondrocyte proliferation, differentiation, and local factor production is dependent on cell maturation state. Cells Tissue Organs 171: 117-127.

50. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB (2002) Structure and function of aggrecan. Cell Res 12: 19-32.

51. Lauing KL, Cortes M, Domowicz MS, Henry JG, Baria AT, et al. (2014) Aggrecan is required for growth plate cytoarchitecture and differentiation. Dev Biol 396: 224-236.

52. Shen G (2005) The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthod Craniofac Res 8: 11-17.

53. Schmid TM, Linsenmayer TF (1985) Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. J Cell Biol 100: 598-605.

54. Kwan AP, Cummings CE, Chapman JA, Grant ME (1991) Macromolecular organization of chicken type X collagen in vitro. J Cell Biol 114: 597-604.

55. Bohme K, Conscience-Egli M, Tschan T, Winterhalter KH, Bruckner P (1992) Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol 116: 1035-1042.

56. Ninomiya Y, Gordon M, van der Rest M, Schmid T, Linsenmayer T, et al. (1986) The developmentally regulated type X collagen gene contains a long open reading frame without introns. J Biol Chem 261: 5041-5050.

57. Schmid TM, Linsenmayer TF (1987) Type X collagen. In: Mayne R, Burgeson RE (Eds) Structure and Function of Collagen Types. FL Academic Press, Orlando.

58. Mwale F, Billinghurst C, Wu W, Alini M, Webber C, et al. (2000) Selective assembly and remodelling of collagens II and IX associated with expression of the chondrocyte hypertrophic phenotype. Dev Dyn 218: 648-662.

59. Scotti C, Piccinini E, Takizawa H, Todorov A, Bourgine P, et al. (2013) Engineering of a functional bone organ through endochondral ossification. Proc Natl Acad Sci USA 110: 3997-4002.

60. Candeliere GA, Liu F, Aubin JE (2001) Individual osteoblasts in the developing calvaria express different gene repertoires. Bone 28: 351-361.

61. Melcher AH (1976) On the repair potential of periodontal tissues. J Periodontol 47: 256-260.

62. Gould TR, Melcher AH, Brunette DM (1977) Location of progenitor cells in periodontal ligament of mouse molar stimulated by wounding. Anat Rec 188: 133-141.

63. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105-111.

64. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ (2005) Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 5: 899-904.