# Cellular Aging Significantly Promotes CEMP1 Expression in a STRO-1+ Enriched PDL Cell Population

**By**

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# Chapter 1-Introduction

The term 'Periodontium,' describes the tissues supporting and investing the tooth. It is comprised of the periodontal ligament (PDL), root cementum, alveolar bone and that part of the gingiva facing the tooth (Nanci et al., 2006). Each of the periodontal components has its very specialized structure and these structural characteristics directly define function. The periodontium protects, supports, and provides nourishment to the tooth. The PDL is a connective tissue embedded between the cementum and the inner wall of the alveolar bone socket. It is composed mainly of collagen fibers, ground substance and cells. The cells include fibroblasts, osteoblasts, osteoclasts, epithelial rest cells of Malassez, macrophages, undifferentiated mesenchymal cells, axons with Schwann cells, endothelial cells and cementoblasts (Marchesan et al., 2011). They all contribute to the development, repair and regeneration of the PDL and the surrounding structures. The main mechanical function of the PDL is to provide an elastic resistance to the matiscative forces. It also plays an important role in the regeneration of cementum, bone, as well as maintaining itself. Proper functioning of the periodontium is only achieved through structural integrity and interaction between its components. Disease and/or injury to the periodontium can result from microbial insult as well as affected by other factors including, systemic disease, aging, trauma, genetics, and iatrogenic activity (Nanci et al., 2006). Achieving periodontal regeneration is difficult, requiring several tissues to be reconstructed. One of the early steps in this process is the attachment of new connective tissue fibers to the root surface. The cementum covering the root has an important

role in this process as it invests and securely attaches the PDL fibers to the root surface and therefore, cementogenesis is a critical process for homeostasis and regeneration of the periodontium (Bosshardt et al., 1996).

The complex processes that regulate cementogenesis and normal cementum metabolism remain unclear to date. Recent evidence indicates that cementum formation is critical for appropriate maturation of the periodontium (Villarreal-Ramirez et al., 2009). Although cementum was first described in 1835 and its recognition probably dates back to 1667 (Zander et al., 1958), only until recently has it remained a poorly defined tissue at the cellular and molecular level. Classification of cementum via light and electron microscopy has defined several subtypes based on the presence (cellular) or absence (acellular) of cells and the source of collagen fibers (extrinsic vs intrinsic). Acellular extrinsic fiber or primary cementum is found on the cervical half to two thirds of the root, whereas cellular intrinsic fiber or secondary cementum is distributed along the apical third of the root and considered less well mineralized than acellular extrinsic fiber cementum. All of these subtypes are quite different from bone, in that they lack a direct blood supply, lymph drainage, and innervation as well as exhibit little to no remodeling (Saygin et al., 2000). Despite these differences, cementum is still very similar to bone. Unlike dentin and enamel in which there are clear differences in the proteins present in these tissues as well as factors present regulating their function compared with bone, cementum on the other hand appears to contain factors in common with bone. The composition of cementum is approximately 50% hydroxyapatite (mineral) as well as 50% collagenous (organic) and noncollagenous proteins. Cementum matrix contains collagen types I and III and possesses proteins similar to bone. More specifically, these include bone

sialoprotein, osteopontin, vitronectin, fibronectin, osteocalcin, osteonectin, proteoglycans and growth factors such as transforming growth factor (TGF-β) and bone morphogenetic protein-3 (BMP-3) (Saygin et al., 2000). Osteopontin (OPN) and bone sialoprotein (BSP) have been implicated in mineral deposition as well as cell- and matrix-matrix interactions. OPN has a poly-Asp and BSP two poly-Glu domains, whose repetitive sequences are known to bind calcium to mineral surfaces. OPN is a molecule associated with early stages of mineralization and its expression by cementum tumor cells suggests that this molecule could be associated with the initial process of mineralization observed in cementum tumor cells (Arzate et al., 1998).

Research through the years has demonstrated that cementum is a unique tissue histologically however definitive identification of proteins specific to cementum, have not been revealed. Although they seem to function similarly in both bone and cementum one challenge presented in studying cementoblastic differentiation is due in part from the inability to identify fully differentiated cementoblasts because most molecules expressed by cementoblasts are pleiotropic and only few cementum-specific markers having been described (Paula-Silva et al., 2010). An adhesion molecule, cementum-derived attachment protein (CAP), and a growth factor, cementum protein (CP), are present in the mature cementum. CAP is characterized as a collagen-like protein with a molecular weight of 56 kDa and restricted to cementum and periodontally-derived cells, and although CAP and osteopontin are generally considered to be different proteins, it is not possible to rule out the possibility that CAP may be a variant of osteopontin formed by differential splicing of its mRNA (Arzate et al., 1992; Wu et al., 1996). CAP and CP appear to be immunologically related to each other and an extensive investigation revealing the expression of a novel cDNA obtained from a human cementoblastic-derived cell

line isolated a tissue-specific-gene product, CP-23, which was expressed by periodontal ligament cell subpopulations and cementoblasts (Alvarez-Perez et al., 2006). With the lack of specific cementum markers and an inherent difficulty obtaining a pure cell population, viable cementoblasts have not yet been isolated. Attempts have been made to isolate cells that express the cementoblast phenotype (Arzate et al., 1992). Arzate et al (1992) proposed culturing cementum tumor cells for at least three reasons: first, they could be used to obtain sufficient quantities of biologically active cementum components due to the paucity of cementum in human teeth and intensive dissection procedures necessary; second, the cells can be used to study the biosynthesis and processing of CAP and other cementum proteins; and third, early cementogenesis is believed to involve deposition of extracellular matrix produced by Hertwig's epithelial root sheath (HERS) cells on dentin, as well as disruption of the HERS, migration and attachment of ectomesenchymal cells from dental follicle onto the matrix, their differentiation into cementoblasts, and laying down of cementum. The Arzate group in 2000, isolated and characterized a human cementoblastoma-derived cell line that expressed the cementum phenotype and went further to reveal via energy dispersive X-ray microanalysis (EDX) in a time course fashion, that the extracellular matrix material deposited had similar Ca/P ratios for both cementoblastoma-derived and osteoblastic cells of 1.6 and 1.628 respectively (1.97 for human cementum). These values were similar to those reported for hydroxyapatite in enamel and bone. Strong immunostaining for CAP in cementum tumor-derived cells indicated this was a significant source for this protein yet, several osteoblastic cells also immunoreacted, which implies cementoblasts and osteoblasts share a common ancestor (Arzate et al., 1998; Arzate et al., 2000). Arzate' s group via immunolocalization, showed CP was expressed

specifically in precementum as well as cells paravascular to the blood vessels in the rest of the PDL, but cells in the PDL were only lightly stained; therefore, CP is periodontal ligament and cementum-specific. This protein was termed “cementum protein-23” (CP-23). The group revealed 98% of putative cementoblasts and 15% of periodontal ligament cells cultured in vitro expressed the CP-23 gene product (Alvarez-Perez et al., 2006).

Subsequently, Arzate and his group (Arzate et al., 2007) demonstrated that transfection of a human cementoblastoma-derived protein named Cementum Protein 1 (CEMP1) into human gingival fibroblasts induced mineralization and expression of bone and cementum-matrix proteins. This protein like CP-23 was expressed by cementoblasts and progenitor cells localized in the periodontal ligament. The transfected human gingival fibroblasts had higher alkaline phosphatase activity and expressed genes for bone sialoprotein, osteocalcin, osteopontin and also produced biological-type hydroxyapatite (Carmona-Rodriguez et al., 2007). The findings of this study indicated that CEMP1 may participate not only in differentiation and mineralization of nonosteogenic cells, but function in cementum and bone formation as well. Since CEMP1 is synthesized by cementoblast cells and a restricted periodontal ligament cell subpopulation (cementoblast precursors), it has been suggested that this protein is a cementum-specific biological marker. Utilizing this theory, Arzate’s group in 2009 produced a full length human recombinant CEMP1 protein in human gingival fibroblasts (Arzate et al., 2009). The identity of the human recombinant protein was determined by Western blot analysis using two specific polyclonal antibodies and a single protein species of 50 kDa was recognized; almost twice the theoretical molecular mass calculated from the cDNA sequence of 25.9 kDa (Villarreal-Ramirez et al., 2009).

The human body has the remarkable capacity for regeneration. The unique cells that give rise to specialized tissues are termed stem cells. These extraordinary cells have the capacity for self-renewal and have the ability to give rise to one or more different cell types. In mature tissue, adult stem cells play a major role in homeostasis and tissue repair while even more remarkable, is the developmental capacity of embryonic stem cells. These cells can, in theory, give rise to all tissue types and are often referred to as pluripotent cells. Due to regulatory and ethical issues associated with the use of embryonic stem cells, attention has been redirected to stem cells derived from adult tissues. The regenerative potential of adult stem cells has been recognized for several decades. Adult stem cells can be categorized into Hematopoietic and Mesenchymal stem cells depending on their origin. Hematopoietic stem cell literature has been well established however, within the diverse population of bone marrow stromal cells, there exists a subset of stem cells called mesenchymal stem cells capable of self-renewal and they can differentiate into several phenotypes including bone, cartilage, adipocytes and hematopoiesis-supporting stroma (Krebsbach and Robey, 2002). Since the discovery of residential stem cells in the periodontal ligament, recent studies have focused on the potential role of these cells in cementum formation, which is essential for periodontal regeneration. Periodontal ligament cells have the potential to differentiate into cementoblasts (Seo et al., 2004; Fuji et al., 2008). The cementogenic potential of these cells to form cementum/bone-like structure has been evaluated by in vivo transplantation assays using immunocompromised rodents (Seo et al., 2004, Gay et al., 2007). In addition, polymerase chain reaction (PCR) analysis and mineralization assays are widely used for in vitro evaluation of cementogenesis (Gay et al., 2007; Flores et al., 2008). In light of this, a previous study isolated so called multipotent

postnatal stem cells from the periodontal ligament using one of the early mesenchymal stem cell surface markers STRO-1. These stem cells were designated PDLSCs and the results of this work revealed that most colony-forming cells were contained within the STRO-1 positive cell population, confirming STRO-1 as an early progenitor marker for PDLSCs (Seo et al., 2004). Xu et al went on to quantify the percentage of PDLSCs in the adult human periodontal ligament using cultured periodontal ligament cells from freshly extracted teeth. Magnetic bead sorting and flow cytometric data revealed that on average, 2.6% of periodontal ligament cells present were positive for the stem cells surface markers STRO-1 and CD146. This small population of cells was capable of multilineage differentiation toward a chondrogenic, adipogenic and osteoblastic phenotype thereby verifying the multipotent nature of these cells (Xu et al., 2009).

Paula-Silva and colleagues (2010) were the first to link STRO-1 and CEMP1 markers to cementogenesis. Prior to their work, molecules responsible for recruiting mesenchymal cells and inducing their differentiation into cementoblasts had not been identified. The results of their work provided evidence that CEMP1 could be one of those molecules that performs these functions. The group's in vivo experiment involving exposed root canals in dogs showed that calcium hydroxide promoted cementogenesis and induced cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1 and ERK-dependent manner. These data suggest that clinical treatment with calcium hydroxide recruits mesenchymal STRO-1 positive PDL cells via a CEMP1 gradient that in turn, promotes mesenchymal cell differentiation toward cementogenesis. They further showed that in vivo calcium hydroxide treatment formed neocementum at the dental root apex of these repaired teeth in a STRO-1 positive and CEMP1 positive cellular environment. Due to the proximity of the staining of these two markers, they

proposed that cells positive for STRO-1 may also be positive for CEMP1 (Paula-Silva et al., 2010). This concept was expanded upon recently (Aminlari et al., 2011, unpublished) as STRO-1 positive PDL cells that are also CEMP1 positive, were shown to have properties consistent with cementoblasts and can be directed toward cementogenesis. This group revealed the presence of STRO-1 and CEMP1 markers on postnatal stem cells from the PDL and went further through magnetic bead sorting, flow cytometric analysis, immunofluorescence analyses, dilution cloning, mRNA isolation, and mineralized tissue analysis to test whether STRO-1/CEMP1 double positive clones can be both successfully differentiated into cementoblast-like cells and produce cementum-like tissue. The results of their study showed successful cloning of STRO-1/CEMP1 positive cells able to produce mineralized-like tissue with features similar to those of cementum; more specifically, a Ca/P ratio of 1.89 which as mentioned previously, is very close to human cementum at 1.97 (Arzate, et al., 2000). Despite these findings however, mRNA expression levels of CEMP1 were highest in the osteogenically induced parental PDL cells, not necessarily in the STRO-1 positive cells (which were CEMP1 positive as well) as anticipated. The authors predicted that the STRO-1 positive cells (which were CEMP1 positive as well) would express the highest levels of CEMP1, and hypothesized that they did not due in part, to the inherent “young age” and early passage of the parental PDL cells as opposed to the serially diluted and late passage (“old age”) of the STRO-1/CEMP1 positive cells. Perhaps CEMP1 expression in fact occurs much earlier than the 14-day time course assay performed. The temporal expression and regulation of CEMP1 expression during differentiation toward mineralization is not known. Therefore, additional time point assays were performed on osteogenically induced parental PDL cells and magnetic bead isolated STRO-1 positive cells

whereby CEMP1 expression was examined at 3 and 7 days and it was found that CEMP1 expression was very low at both 3 and 7 days. The authors suggested that perhaps earlier and later time points would best serve to examine the optimal time to assay for CEMP1 expression during differentiation processes. (Aminlari 2011, unpublished).

**Cellular Senescence and Cementum Markers**

In addition to identifying optimal time points to assay for high expression of CEMP1, the concept of cellular senescence became important since cell experiments often required multiple cell passages. Senescence or biological aging is defined as the endogenous and hereditary process of accumulative changes to molecular and cellular structure disrupting metabolism and resulting in death. Senescence occurs at both the level of the entire organism as well as at the level of its individual cells (Hayflick 1977). Once cells have entered senescence, they cease to divide and undergo a series of dramatic morphologic and metabolic changes. Aged cells accumulate age-related damage and become post-mitotic when they can no longer replicate. They also can no longer express the full gamut of markers they once could at an earlier age or passage. Viewed historically as a cell-intrinsic mechanism, senescence functions to restrict unlimited cell proliferation. This concept originated from classic experiments performed by Leonard Hayflick in the early 1960's, who found normal human fibroblasts cease to divide following a period of proliferation in culture (Hayflick et al., 1961). However, the physiologic role of senescence remains poorly understood (Ben-Porath, Weinberg, '05). It is quite possible for serially diluted cells or cells undergoing multiple passages to become 'tired' and unable to express certain markers. Another reason is that cells develop multiple mutations

over time, and in order to 'weed-out' potentially cancerous-like cells, the cells will no longer continue to divide and pass on these mutations. These cells undergo senescence and stop dividing. Most recently, Komaki et al investigated the change in CEMP1 expression during the differentiation of human periodontal ligament cells. Their objective was to investigate if CEMP1 is a regulator of cementoblasts. Immunocytochemical analyses and RT-qPCR revealed CEMP1 expression was reduced when periodontal ligament cells differentiated into osteoblasts in vitro; more specifically, they found that CEMP1 expression was reduced when periodontal ligament cells were cultured under osteogenic conditions, i.e., cultured with osteogenic induction medium. The results went on to reveal CEMP1 expression declines when cells are committed to osteo-/chondro-blastic lineages in an in vivo diffusion chamber assay, which suggests that CEMP1 expression may be differentially regulated in osteoblasts and cementoblasts (Komaki et al., 2012).

It is believed the degree of periodontal tissue breakdown and tooth loss increase with age. The most important elements of the periodontal ligament are the principal fibers, which are collagenous, arranged in bundles, and the terminal portions insert into cementum and alveolar bone. Therefore the periodontal ligament not only has a primary function of tooth support, but also an important function in the tissue regeneration of the periodontal ligament itself, alveolar bone and cementum (Nanci et al., 2006). While some studies describing the changes of the periodontal ligament with age have been reported, they were limited to histological investigations. Therefore clarifying biological changes of the periodontal ligament with the process of aging would be useful to elucidate a relationship (if any) between periodontal breakdown and age. This may prove useful in maintaining periodontal health over a

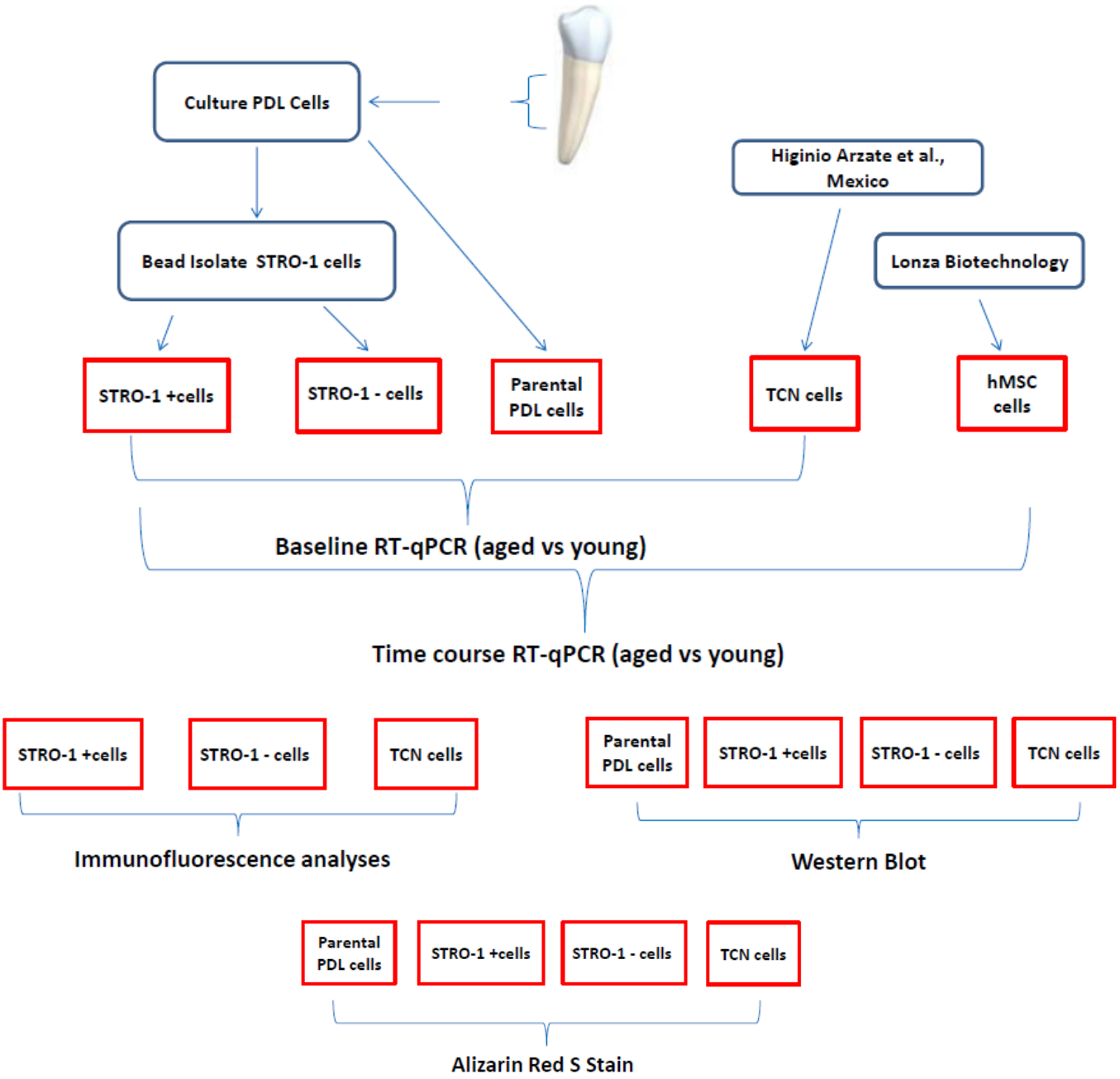
lifetime (Goseki et al., 1996). Healthy periodontal ligament from old subjects for biochemical assay however, is difficult to obtain because healthy teeth are rarely removed from the aged.

**Hypothesis and Specific Aims**

Reports from the literature conveying the notion that human fibroblast cultures derived from a variety of tissues types have a limited proliferative life-span thereby defining the measurable parameter of a finite number of population doublings in tissue culture and has been termed 'cellular aging' (Hayflick et al., 1961; Hayflick, 1977); therefore the life-span of certain cultured cells including human fibroblasts, indeed has a correlation with that of each species and the concept of a direct relationship between cellular life span and species longevity is supported (Rohme, 1981). As mentioned earlier, Paula-Silva et al showed that calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal PDL cells in a CEMP1 and ERK-dependent manner. They proposed that CEMP1 might be a mediator in periodontal regeneration as it stimulates PDL cell proliferation and migration. However, to date, no one has examined CEMP1 expression in the periodontal ligament cell with relation to cell aging. Specifically it is not known whether: 1) STRO-1 positive PDL cells that are also CEMP1 positive, express CEMP1 after the cell has aged 2) STRO-1 positive PDL cells that are also CEMP1 positive, can be driven toward a cementogenic phenotype after the cell has aged and 3) there is a specific time point at which CEMP1 expression is highest during differentiation to a cementoblast. In these studies we therefore tested the hypothesis that STRO-1 positive PDL cells that are also CEMP1 positive will have variable expression of cementum protein-one as the cell ages with potential peak expression at a younger age.

To test our hypothesis, we used human PDL cells obtained from the teeth of 12-16 year-old patients and investigated the effects of in vitro cellular aging on cementum protein 1 (CEMP1), Collagen 1 (Col 1), and Osteopontin (OPN) all of which may play important roles in periodontal tissue function. To confirm that STRO-1 and CEMP1 markers are present on postnatal stem cells from the PDL, and that these markers can be used to successfully isolate PDL cells from freshly extracted teeth for further differentiation to cementoblasts, we used magnetic bead sorting, immunofluorescence analyses, Western blotting and a baseline RT-qPCR to detect and quantify these markers. To test whether STRO-1/CEMP1 double positive PDL cells could be differentiated into cementoblast-like cells after they age, we differentiated these aged cells as well as aged parental PDL, double negative control counterparts, cementoblastoma controls, and human mesenchymal stem cells in a time course fashion toward a mineralization phenotype. Evaluation of cementoblast-like characteristics was assessed via mRNA expression/RT-qPCR for CEMP1, Col 1 and OPN. Confirmation of STRO-1/CEMP1 double positive and double negative controls was performed using both immunocytochemistry and Western blotting. Mineral production and quantification was confirmed using Alizarin Red S staining. Student's t-test was performed for statistical analysis when indicated. We expect to see a decrease or downregulation in CEMP1, Collagen 1 and Osteopontin expression with cellular aging.

# Chapter 2- Methods and Materials




Flow chart of experimental design and assays performed

### *PDL Cell Isolation*

Freshly extracted maxillary and mandibular premolars from human subjects ages 12-16 were obtained from the Oral Surgery Department under informed consent at the University of Michigan, School of Dentistry. Teeth were quickly placed in media containing α-minimal essential media (α-MEM) with L-Glutamine and without Ribonucleosides or Deoxyribonucleotides. Under sterile conditions, the teeth were washed twice with PBS. The PDL tissues from the middle third of the roots were scraped using a sterile blade as previously described (Xu, Wang et al. '09). The tissues were minced and covered with sterile glass coverslips, held down by Vaseline at three points, in 35mm tissue culture dishes in α-MEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and Fungizone (Invitrogen Corp., Carlsbad, CA, USA). The plates were labeled ART PDL-1, ART PDL-2, and so on. The plates were incubated in a humidified atmosphere (37°C, 5% $CO_2$), and the media was replaced every two to three days for up to a month. PDL cells that grew out of the PDL tissue were passaged into 10-cm plates by first washing with 5mL PBS, then incubating with 1 mL of 0.05% Trypsin-EDTA for 5 minutes until the cells detached from the plate and were floating in the media. Finally the Trypsin was inactivated with 5 mL of α-MEM medium containing the 1% FBS and 1% P/S (Hereinafter "PDL Growth Media"). The cells were centrifuged at 1000 rpm for 5 min, the supernatant removed and the cells resuspended in new PDL Growth Media and plated. Once confluent, they were again passaged. Passage 2 and 3 cells were used for cell isolation and subsequent experiments. Human cementoblastoma (TCN) cells were kindly provided by Dr. Higinio Arzate (UNAM, Mexico City, Mexico) and were cultured in αMEM with

10% fetal bovine serum plus 1% penicillin/streptomycin. Human mesenchymal stem cells (hMSCs) purchased from Lonza (Lonza, MD, USA), were kindly provided by Jin Ping Xu from the University of Michigan and cultured in mesenchymal stem cell media. Both TCN and hMSC cell strains were prepared to passage 2 and 3 and then used for subsequent experiments.

## *Magnetic Bead Isolation*

In order to isolate STRO-1 positive cells for experimentation, the CELLectin Biotin Binder Kit (Life Technologies, Grand Island, NY) was utilized. Fresh buffers were made and adjusted to pH of 7.0-7.4. Passage 3 PDL cells were washed twice with 5 mL of PBS. Cells were then incubated with 5mL of Cell Dissociation buffer at 37°C, 5% $CO_2$ for up to 15 min. Once the cells detached from the plate, 10 mL of the PDL Growth Media was added and centrifuged at 1000 rpm for 5 min. The pellet was then resuspended in 400 µL of Buffer 2 (PBS, 1% BSA, 0.6% Na Citrate). The cells were incubated on ice for 60min in the presence of Mouse Anti-human STRO-1 IgM antibody at 1:20 dilution.

While the cells were incubating, 25 µL of the magnetic Dynabeads were transferred to a new test tube and washed twice with Buffer 1 (PBS, 1% BSA) by placing in the Dynal MPC-1 Magnetic Particle Concentrator for 1 min. The Dynabeads were then resuspended in 25 µL of Buffer 1 and incubated in the presence of 20 µg of Biotin conjugated Goat anti-mouse IgG for 30 min at room temperature with occasional agitation.

The cells were washed twice with Buffer 2 by centrifugation at 1000 rpm for 5 min. They were resuspended in 1 mL of Buffer 1. The Dynabeads were washed three times as above and resuspended in the initial 25 µL of Buffer 1. The cells and the Dynabeads were mixed together and incubated for 20 min at 4°C with gentle mixing. The mixture was placed in the magnet for 2 min and the bead-unbound cells and supernatant removed (these were saved as "Sup" cells). The cell-bead complex was washed twice in Buffer 2 and resuspended in 200 µL of pre-warmed Buffer 3 (RPMI 1640, 1% FBS, 1% CaCl2, 1% MgCl2). Finally 6 µL of DNAse Releasing Buffer was added to the mixture and allowed to incubate for 15 min at room temperature. This step would help release the cells from the Dynabeads. The test tube was placed in the magnet again and the unbound cells were removed after 2 minutes. These cells would theoretically be the STRO-1 positive cells. The last step was repeated two more times along with pipetting up and down using a 200-µL pipetter to fully unbind the cells. All other cells were also grown; DNAsed STRO-1+ cells (STRO-1), Bead-bound cells (Beads), Pre-DNAse cells in the supernatant (Sup), and parental PDL cells.

## *Immunofluorescence*

STRO-1 negative, STRO-1/CEMP1 positive, parental PDL, and cementoblastoma (TCN) cells were subjected to staining for STRO-1 and CEMP1. Cells at passage 3-4 and passage 11-12 were washed with 5 mL PBS and released from plates using 3 mL of Trypsin for 5 minutes at 37°C. Cells were counted and plated in a 48 well plate at 5000 cells/well. The cells were allowed

to attach to the plate over night at 37°C. The next day, the cells were washed twice with 200 µL of PBS. To fix the cells, they were incubated in 200 µL of 4% paraformaldehyde for 15 minutes at room temperature. The wells were washed twice with 200 µL PBS and then blocked with 200 µL 5% BSA in PBS with 0.3% Triton X-100 for 1 hr at room temperature. This was to prevent the nonspecific binding of the primary antibody. The Triton X-100 allowed the permeability of the cells. The wells were washed twice with PBS and 200 µL of the primary antibody buffer (PBS, 0.3% Triton X-100, 1:200 dilution of Mouse anti-human STRO-1 monoclonal IgM antibody (BioFx Labs, MD), and 1:300 dilution of hCEMP1 antibody (Kindly provided by Dr. H Arzate, UNAM, Mexico City, Mexico) was added. Negative controls were incubated with either no antibody or Normal mouse IgG at 1:150 dilutions. The plates were wrapped in paraffin to prevent evaporation and incubated overnight in 4°C.

On the third day, the wells were washed twice with 200 µL PBS and incubated for 2 hours in the dark with the secondary antibody buffer (PBS, 0.3% Triton X-100, 1:300 dilution FITC conjugated Goat anti-Mouse IgM antibody, 1:300 dilution Texas Red conjugated donkey anti-Rat IgG antibody). The wells were washed twice with PBS. To stain the nuclei, DAPI staining was used by incubating the cells in 200 µL of 1:5000 dilution of DAPI in PBS for 1 minute. The cells were washed again and finally observed under the light microscope with the appropriate filters for FITC, Texas Red, and DAPI. Pictures were taken using the Q-Capture Pro software and Evolution MP digital camera attached to a Nikon TS 100 microscope.

## *Cellular Aging of PDL, STRO-1 positive, STRO-1 negative, TCN and hMSC cells*

All cell strains were cultured in α-MEM, hMSC medium, or osteogenic medium as previously described, on 10cm plates and allowed to reach 90% confluency (~$2.5x10^6$ cells). Cellular aging was performed by subcultivation according to Goseki and Hayflick (Goseki et al., 1996), (Hayflick, 1977). Briefly, cells were incubated on 10cm plates as either: PDL isolated, STRO-1 positive or STRO-1 negative bead isolated, hMSC or TCN donated cells at 5% $CO_2$ and 37° C while changing the medium every 3 days. Once cellular confluence was achieved, cells were passaged with 0.05% Trypsin as previously described. Cells plated as P2 or P3 were either used immediately or passaged again to appropriate plates (6cm or 6 well) for specific assays and considered 'young' cells (P 3-4) for each experiment thereafter. When cells had reached confluence at around one-week intervals, they were subcultivated in a 4:1 split ratio so that two population doublings per subcultivation would result. After the $10^{th}$ to $11^{th}$ passage, the growth rate of the cells decreased (certain cell strains more markedly than others), thereby increasing the time required to reach confluence (up to two weeks for hMSC and TCN cells). The resulting cells were then split into a ratio of 2:1 if necessary in order to achieve passage 12 or more, and at this late passage the cells were considered 'aged' (P 11-13).

## *Osteogenic/Cementogenic differentiation*

All cell strains young and aged, were osteogenically induced in either a time course fashion for mRNA analysis or for Alizarin Red S staining. Young and aged Parental PDL cells, supernatant or STRO-1 negative cells, STRO-1/CEMP1 positive cells, human mesenchymal stem

cells, and cementoblastoma cells (TCN cells) were washed and detached using 0.05% trypsin for 5 min and the trypsin deactivated with MSC growth media. The cells were counted, centrifuged, and resuspended in an appropriate type and amount of MSC growth media. A total of $5X10^5$ cells were transferred to each 6cm plate or $3x10^4$ cells were transferred to each well of a 6-well plate. 90 total 6cm plates and 8 total 6-well plates were seeded as seen in figure 13 and figures 17 - 18 respectively. Each 6-well plate was a single cell strain performed in triplicate for Alizarin Red staining using osteogenic medium and control medium. RNA analysis involved nine 6cm plates for each cell strain in a time course fashion (0hr, 6hr, 24hr, 72hr and 1wk) for both young and aged cells also using osteogenic medium and control medium. 3 wells of each 6-well plate were osteogenically induced, and the other 3 wells served as controls. Forty 6cm plates were also osteogenically induced and fifty 6cm plates then served as controls after which, RNA isolation would be performed at each specific time point for treated and untreated cells (0hr, 6hr, 24hr, 72hr, and 1wk). To induce differentiation, the control media was replaced with the osteogenic induction media containing L-glutamine, ascorbate, dexamethasone, and β-glycerophosphate according to the manufacturer's instructions (Lonza, MD USA). The media was replaced every 3-4 days. At the end of one week, the 6cm plates were designated for RNA extraction, reverse transcription and subsequent RT-qPCR and at the end of two weeks, the 6 well plates were designated for Alizarin Red S staining.

## *Alizarin Red S Staining*

Upon the completion of the osteogenic induction at two weeks, eight 6-well plates were stained for mineral content using the Alizarin Red S staining protocol. The solution was freshly made adding 0.5 mg of Alizarin red dye to 100 mL sterile deionized water ($dH_2O$) and mixed well. The media was discarded from each well and the cells washed two times with PBS. The cells were then fixed with 2 mL of 70% ethanol for 10 min. The ethanol was then removed and wells allowed to air dry. The cells were then stained with 2 mL of the Alizarin red dye solution for 5 min. Finally, they were washed two times with sterile $dH_2O$; air dried, and scanned using an Epson double-sided radiograph-scanning machine and Apple Mac computer.

To quantify the amount of Alizarin Red S staining, the plates were incubated for 30 min in 1 mL of 100 mM cetylpyridinium chloride. At this point, 200 μL of the now blue solution was placed in triplicate in a 96-well plate and read using a spectrophotometer at an OD reading of 570nm. Student's *t*-test was used for analysis of the results comparing aged vs young cells. $P < 0.05$ was considered significant.

## *RNA Extraction*

Upon completion of young and aged cell culture for both baseline, and time course evaluation with osteogenic induction at the end of one week, forty (40) osteogenic 6cm plates and seventy four (74) control 6cm plates were dedicated to RNA isolation and qRT-PCR. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, USA). The cells were washed twice with cold PBS. The cells were then coated with 350 μL of RLT lyses buffer with ethanol for

10 min at room temperature before they were scraped and pipetted into a Q1A-Shredder mini-spin column and centrifuged at 13,000 rpm for 2 min. Subsequently, 350-500 μL of fresh biologic grade 70% ethanol was added and mixed by pipetting. This mixture was placed in a mini column with a collecting tube and centrifuged for 15 sec at 13,000 rpm. At this point, the RNA theoretically stayed in the spin column. The eluent was discarded. The column was then washed once with 350 μL of Washing Buffer RWL. At this time, the DNase step was performed with the DNase Kit (Qiagen, USA) by adding 10 μL of DNase stock solution to 70 μL of Buffer RDD. Once gently mixed, add the DNase incubation mix (80 μL) was added directly to the RNeasy spin column membrane, and placed on the bench top (20-30° C) for 15 minutes. Then 350 μL of Washing Buffer RWL was added and the sample centrifuged for 15 sec at 13,000 rpm. Then the sample was washed with 500 μL of Washing Buffer RPE twice, and centrifuged at 13,000 rpm for 15 sec. At this time, the collecting tube was changed to a new one and the column spun for 2 min at 13,000 rpm. Finally, the column was transferred to a new eppendorf tube and 35 μL of RNase-free water, provided with the kit was added to the column and centrifuged at 13,000 rpm for 1 min. The RNA at this point had dissolved in the water and transferred to the eppendorf tube. To measure the purity of the RNA, 1:25 dilution was made using 4 μL of RNA and 96 μL of RNase-free water, placed in a 6 cuvette compartment, and the absorbance at a ratio of 260/280 was measured using a DU-640 Spectrophotometer (Table 1 and 2). A total of 98 samples (baseline and time course young/aged) were stored in the - 80°C freezer until further processing.

## *Reverse Transcription*

In order to perform RT-qPCR, the RNA samples required reverse transcription (RT) to produce complementary DNA (cDNA). Calculations were performed to allow for reverse transcription reactions to be performed for the time course experiment involving 45 samples of 'young cell' RNA and 45 samples of 'aged cell' RNA as well as 8 samples of "young-, aged cell" RNA for the baseline experiment. A 40 µl RT reaction with 0.5µg total RNA and random hexamers was performed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA USA). This kit included all the necessary components for the transcription and was utilized after removing from the ⁻20°C freezer and slowly thawed on ice. The thermal condition for RT was: 25°C 10 min, 37°C 120 min and 85°C 5 min and kept at 4°C until the samples were removed from the machine. Within 24 hours or sooner, the samples were stored in the ⁻20°C freezer.

## *Quantitative Real-Time Polymerase Chain Reaction*

Real time polymerase chain reaction (qPCR) was performed in quadruplicate on the cDNA samples from each of the five cell strains in each time course group compiling ninety samples total and each of the four cell types in the baseline group compiling eight samples total. The reaction was performed by using TaqMan® Universal PCR Master Mix (Life Technologies, Carlsbad, CA) and Vii7 Applied Biosystems PCR system (Applied Biosystems, Foster City, CA). Briefly, a 30 µl PCR reaction was prepared with 1µl cDNA (RT product) and

1.5µl gene specific probe and primers mixture from Life Technologies TaqMan® Gene Expression Assays. The sequences for the probes are listed in Table 3. The real-time PCR thermal condition was: 50°C 2 min, 95°C 10 min followed by *40* cycles of 95°C 15 sec and 60°C 1 min. Relative quantification was performed using the ΔΔCt method (Livak '01), data were normalized to the expression of GAPDH and Student's *t*-test was applied to compare statistically significant differences between groups (young vs aged cells). *P* values < 0.05 were considered significant.

### *Baseline Messenger RNA Expression*

Baseline expression of CEMP1, osteopontin, and collagen 1 in 'young' and 'aged' parental PDL cells, STRO-1/CEMP1 positive cells, STRO-1 negative cells, and cementoblastoma (TCN) cells, was evaluated and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using RT-qPCR with TaqMan primer/probes. The relative mRNA expression from this baseline experiment was then compared with the relative mRNA expression of the same genes in the same 'young and 'aged' cells osteogenically induced in a time course fashion with the addition of human mesenchymal stem cells (hMSCs) in the time course experiment.

### *Time Course Messenger RNA Expression*

Expression of CEMP1, osteopontin, and collagen I in 'young' and 'aged' parental PDL cells, STRO-1/CEMP1 positive cells, STRO-1 negative cells, cementoblastoma (TCN) cells, and

human mesenchymal stem cells (hMSCs) was evaluated and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a time course fashion using RT-qPCR with TaqMan primer/probes. Briefly, cells were plated young and aged as previously described on nine 6cm plates to ~70-80% confluency ($1x10^6$ cells). The first plate was designated 'zero hour' (0hr) and remained untreated through the one week time frame. Two subsequent plates were then used for each time point thereafter at six hours (6h), twenty four hours (24h), seventy two hours (72h), and one week (1wk), remaining either untreated as a control or treated with osteogenic induction media 'UT' or 'Tx' (Fig 13).

## *Western blot*

For further confirmation of the CEMP1 marker on a protein level, western blot analysis was performed on parental PDL, STRO-1 positive, STRO-1 negative and cementoblastoma cells. Samples were cultivated on 10cm plates to ~90% confluency ($2\text{-}2.5x10^6$ cells). Plates were scraped, cells were detached and medium pipetted into 15mL tubes, then centrifuged at 1000rpm for 5min at room temperature. The medium was then aspirated and the pellet resuspended in 500µL PBS, transferred to a 1.5mL eppendorf tube, and centrifuged @ 1000rpm for 5min at room temperature. The PBS was then aspirated and remaining pellet resuspended in 60µL RIPA lysis buffer (SIGMA, St Louis, MO) and protease inhibitor and incubated on ice for 1hr. The lysis buffer was then centrifuged @ 10,000rpm for 10min at 4°C. The supernatant was then transferred to a new eppendorf tube and protein quantity measured via BCA protein assay (Thermo Scientific, Rockford, IL) with a 96 well plate and the Spectra Max M2 machine

(Molecular Devices, Sunnyvale, CA) using Soft Max Pro 2 software. Calculations for protein concentration were then made and 20µg of each sample was added to the appropriate volume of laemmli sample buffer and 2-mercaptoethanol (Bio-Rad, Hercules, CA) in a 475µL: 25µL ratio. After the mix was prepared, each sample was boiled @ 95°C for 5 minutes and electrophoretically resolved using 4-15% Tris/Glycine Mini-Protean TGX gel (Bio-Rad, Hercules, CA), then electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked in Starting block TBS blocking buffer (Thermo Scientific, Rockford, IL) for 1 hour and incubated overnight with 1:300 diluted primary antibody CEMP1 (T-15) (Santa Cruz Biotechnology). Secondary antibody was donkey anti-goat IgG-HRP conjugated to horseradish peroxidase (Santa Cruz Biotechnology), diluted to 1:2000 and incubated for 2 hours and then washing with TBS buffer and Tween 20 (BioExpress, Baltimore, MD). Immunoblot bands were visualized by enhanced chemiluminescence using the West-Pico ECL detection system (Pierce Biotechnology).

# Chapter 3- Results

## *PDL Isolation*

PDL cells grew out of the PDL tissues isolated from the freshly extracted teeth. On average PDL cells grew from 57% of the 30 teeth collected. Cell growth from the tissues was seen anywhere from five days to two weeks from the initial PDL extraction. Marked cell growth was noticed from extracted premolars compared to third molars. No PDL cells grew from the carious or periodontally involved teeth. Teeth not immediately placed in media, resulted in a very low success of PDL cell growth. The plated tissues were kept and their media replaced regularly for up to a month. If no growth was seen at that point, they were discarded. Under light microscopy at 10X magnification, the cells were spindle or stellate shaped with a single large nucleus, resembling the morphology of a typical fibroblast. Pictures were taken of the first cells to grow from the PDL tissue (Fig. 1). When confluent, the cells were arranged in parallel arrays. Once cells in the second to third passage looked approximately 90 percent confluent they were used for magnetic bead isolation.

## *Magnetic Bead Isolation*

A magnetic bead isolation technique was used to isolate STRO-1+ PDL cells. Approximately 1.4% of the total cells initially subjected to the magnetic bead isolation released by the DNase were considered to be STRO-1 positive (Aminlari, Kapila et al., 2011). A smaller percentage of cells that were still bound to the Dynabeads were considered STRO-1 positive

and grown as well. Figure 2 shows a phase contrast microscopic image of STRO-1 positive cells with bound beads creating a colony-forming unit. The cell is multipolar, stellate shaped, and able to form colonies. Figure 3 shows a phase contrast microscopic image of bead isolated aged STRO-1 positive cells (P 11) displaying discernible changes in morphology.

### *Immunofluorescence*

In addition to magnetic bead isolation, immunocytochemistry was performed to further show that the magnetic bead isolated cells were positive for the STRO-1 and CEMP1 marker. STRO-1 expression was identified with green fluorescence (FITC). CEMP1 expression was identified with red fluorescence (Texas Red). The isolated cell population expressed the cell surface molecule STRO-1 in both young (P 3) and aged (P 11) cells. A portion of the parental PDL cells stained green, while almost none of the Supernatant or STRO-1 negative cells stained green. Also note, the merged images w/ FITC and Texas Red as a yellowish green color indicating cells positive for STRO-1 are simultaneously positive for CEMP1 displaying expression for both markers in both young and aged cells (Fig. 4-6).

### *Western blot*

To further demonstrate on a protein level PDL cells were isolated for the CEMP1 marker, western blotting for CEMP1 was performed on parental PDL, STRO-1/CEMP1 double positive, STRO-1 double negative, and cementoblastoma cells. CEMP1 expression was evident

in parental PDL, STRO-1 positive and cementoblastoma (TCN) cells but not in STRO-1 negative cells. Bands are observed for CEMP1 at approximately 50 KDa as expected. All cell strain samples were normalized to Beta-actin with bands visible at approximately 42 KDa (Fig. 7).

## *Osteogenic/Cementogenic Induction*

Parental PDL cells, STRO-1/CEMP1 positive cells, STRO-1 negative cells, Cementoblastoma cells, and human mesenchymal stem cells were osteogenically induced in a time course fashion for mRNA analysis and for Alizarin red S staining. The cell strains were induced in both a 'young' (P 3-4) and 'aged' (P 11-13) state and at 0 hr, 6hr, 24hr, 72hr and 1 week, RNA isolation was performed. By one week, hMSC, STRO-1/CEMP1 positive, and STRO-1 negative cells revealed a pyramidal shape with multiple spicule-like nodules. This was progressive starting on average, at around 72hr for both young and aged cells with more nodule formation in an aged state. Young parental PDL and STRO-1/CEMP1 positive cells appeared to be more confluent yet both as well as the STRO-1 negative cells kept their bipolar shapes with parallel clusters. This was not the case for the TCN cells in both regards; as time progressed, these cells morphologically evolved into cuboidal-shaped clusters of cells. The PDL, STRO-1/CEMP1 positive and TCN cells had nodules while the STRO-1 negative cells had few to none. Cell morphology for the aged cells on the other hand was quite different for parental PDL, STRO-1/CEMP1 positive, and TCN cells as their shapes became more flattened with increased volume. They were also less confluent and not arranged in parallel clusters. The

STRO-1 negative cells however maintained their confluency and parallel array. It was also noted that nodule formation appeared to increase in aged PDL, STRO-1/CEMP1 positive, STRO-1 negative and TCN cells. Images for young and aged parental PDL, STRO-1/CEMP1 positive, STRO-1 negative and cementoblastoma cells are shown in figures 8 and 9 respectively.

Osteogenic induction of parental PDL, STRO-1/CEMP1 positive, STRO-1 negative and cementoblastoma cells for quantitative analysis was also performed after two weeks; after two weeks of induction, cells were then stained with Alizarin Red S stain and evaluated. Visually, the osteoinduced young parental PDL, STRO-1/CEMP1 positive, and TCN cells had more appreciable nodule staining than the STRO-1 negative group however, once the cells aged, a visibly recognizable change occurred with nodule formation staining for the STRO-1 negative cells (Fig. 17 and 18). Quantification with cetylpyridinium chloride and spectrophotometry showed certain similar results. All the osteoinduced cell strains young or aged had significantly more nodule staining than their control counterpart ($P < 0.05$). The induced aged STRO-1 negative cells had significantly more nodule staining than the induced young counterparts and the induced young PDL cells had significantly more staining than the induced aged counterparts ($P < 0.05$). There was no significant difference in Alizarin red S staining between the young and aged STRO-1/CEMP1 positive and TCN cells ($P > 0.05$).

***Messenger RNA Expression*** (Baseline Study and Time Course Study)

A baseline expression of CEMP1, osteopontin, and collagen I in 'young' and 'aged' parental PDL, STRO-1/CEMP1 positive, STRO-1 negative, and cementoblastoma (TCN) cells was evaluated and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Comparing young versus aged cells, the results revealed a 208-fold upregulation of CEMP1 expression in aged STRO-1/CEMP1 positive cells ($P< 0.05$). Also revealed was a downregulation of CEMP1 expression in aged parental PDL, STRO-1 negative, and TCN cells which was not considered significant ($P> 0.05$). Relative expression for osteopontin showed a 10.2- and 12.9-fold upregulation in both aged PDL and STRO-1 positive cells respectively ($P< 0.05$), while a down regulation occurred in both TCN and STRO-1 negative cells which was not significant ($P> 0.05$). Relative expression for collagen 1 showed a 2.1-, 3.0- and 2.0-fold upregulation in aged PDL, STRO-1 positive and STRO-1 negative cells respectively ($P< 0.05$). A downregulation of expression for collagen 1 in aged TCN cells was noted as well however this was not considered significant ($P> 0.05$), (Fig. 10-12).

A time course experiment with and without osteogenic induction evaluating mRNA expression of CEMP1, osteopontin, and collagen 1 in 'aged' and 'young' parental PDL, STRO-1/CEMP1 positive, STRO-1 negative, cementoblastoma, and human mesenchymal stem cells (hMSCs) was also performed and normalized to GAPDH. Absorbance ratio and concentration of mRNA for young and aged cells are listed in tables 1 and 2. Calculations for fold increase/decrease and P values are listed in tables 4-6. The results revealed a progressive increase in CEMP1 expression for aged PDL cells with a 3 fold upregulation for induced and

untreated cells at 24 hours, a 7-fold upregulation for induced cells at 72 hours, and a peak 12-fold upregulation at 1 week for untreated (UT) aged cells ($P < 0.05$). An increase in CEMP1 expression was also noted for aged STRO-1/CEMP1 positive cells at 72 hours of osteogenic induction with a 2-fold and 5-fold upregulation at one week for the aged induced and untreated cells respectively ($P< 0.05$). CEMP1 expression in the aged cementoblastoma (TCN) cells revealed a shift from 0 - 24 hours of induction, to 24hr through one week. A 4-fold and 2-fold downregulation of CEMP1 expression for aged TCN cells at 6 hours and 24 hours was noted respectively ($P< 0.05$), yet at 72 hours and one week, a 2-fold upregulation of CEMP1 expression occurred for untreated and induced (UT, Tx) cells respectively ($P< 0.05$). CEMP1 expression for STRO-1 negative young and aged cells was less overall compared to all the other cell strains. A significant 3-fold downregulation for CEMP1 was noted for aged untreated STRO-1 negative cells at 24 hours (UT, $P< 0.05$) and a 19-fold and 2.9-fold downregulation at one week for both Tx and UT groups occurred respectively ($P < 0.05$). CEMP1 expression for aged hMSC cells revealed a noticeable downregulation for untreated cells at 72 hours and induced cells at one week however this was not significant ($P< 0.05$). No significant differences were noted between young and aged cells from 0hr through 24 hours. To summarize, CEMP1 expression was significantly upregulated for aged treated and untreated parental PDL and STRO-1/CEMP1 positive cells. A bi-phasic shift however occurred in CEMP1 upregulation for TCN cells from young to aged cells beginning at 6 hours through 24 hours (young cells), then shifting from 72 hours to one week (aged cells), (Fig. 14).

Collagen 1 expression for parental PDL cells showed a progressive increase for aged cells beginning at 24 hours with a 7-fold upregulation at one week for untreated cells ($P < 0.05$), and a 2-fold upregulation for induced cells ($P < 0.05$). For one week induced young and aged PDL cell groups, a dramatic decrease in relative expression for collagen 1 occurred overall. STRO-1/CEMP1 positive aged cells revealed a progressive significant upregulation of collagen 1 expression through time for aged cells treated and untreated, beginning at 6 hours and peaking at one week (UT) with an 18-fold increase in expression ($P < 0.05$). Upregulation of collagen 1 at 72 hours for untreated aged STRO-1/CEMP1 positive cells however was not considered significant ($P > 0.05$). Collagen 1 expression for aged induced TCN cells was significantly upregulated 4-fold and 5-fold for induced cells at 72 hours and one week respectively ($P < 0.05$) and significantly downregulated 3.5-fold for untreated (UT) control cells at one week as well ($P < 0.05$). In STRO-1 negative cells, collagen 1 expression showed a significant downregulation for aged cells throughout the time course with a peak 3-fold downregulation at 72 hours (UT, $P < 0.05$) and then a 4-fold upregulation for treated aged cells at one week (Tx, $P < 0.05$). Collagen 1 expression for aged hMSC cells showed a significant upregulation beginning at 24 hours (2-fold increase) and continuing throughout the time course peaking with a 10-fold increase at one week for untreated control cells ($P < 0.05$) and a 45-fold increase in expression at one week ($P < 0.05$) for induced cells. To summarize, a significant upregulation of collagen 1 occurred for aged treated and untreated parental PDL, STRO-1/CEMP1 positive, and hMSC cells beginning as early as 6 hours (STRO-1/CEMP1 positive cells) and spanning throughout the one week time course study (Fig. 15).

Osteopontin expression for aged parental PDL cells showed a 2-fold and 3-fold upregulation in expression at 72 hours and one week respectively for induced cells (Tx, $P < 0.05$). At all other time points, there was no significant difference between groups. STRO-1/CEMP1 positive aged cells showed a progressive significant increase in osteopontin expression from 24 hours to one week with a peak 55-fold upregulation at one week for induced cells (Tx, $P < 0.05$) as well as a 62-fold upregulation at one week for untreated control cells (UT, $P < 0.015$). Relative expression of osteopontin in STRO-1/CEMP1 positive cells was greatest for induced cells at one week. TCN cells showed the highest relative expression for aged untreated cells at 72 hours and at which time a significant 3.9-fold downregulation occurred for aged induced cells (Tx, $P < 0.05$). Relative expression of osteopontin for STRO-1 negative cells increased for aged cells through 72 hours at which time a 4-fold increase in expression occurred for induced cells however, this was not significant ($P > 0.05$). At one week, a significant 6.8-fold downregulation had occurred for aged, induced cells (Tx, $P < 0.05$). Osteopontin relative expression for hMSC cells remained higher for young cells from 24 hours to one week with no significant differences between young and aged cells prior to that point in time and peaking at one week for young cells as well as revealing a significant 6-fold and 8-fold downregulation for control and induced aged cells ($P < 0.05$) respectively. To summarize, osteopontin expression was upregulated in aged induced STRO-1 negative cells and significantly upregulated in aged induced parental PDL and STRO-1/CEMP1 positive cells, between 72 hours and one week (Fig. 16).

# Chapter 4-Discussion

Despite the many methods proposed for periodontal regeneration ie; guided tissue regeneration, guided bone regeneration, the use of membranes, bone morphogenetic proteins, and enamel matrix proteins, none have targeted cementogenesis specifically and sought to reliably initiate the regeneration of cementum and subsequent attachment of the periodontium. Part of this is due to the lack of knowledge about the process of cementogenesis and about the potent capability of cementoblasts with particular interest to their precursors or origin. Unpublished work by Aminlari et al. 2011, has confirmed with the use of cementum protein 1 (CEMP1) as a cementum marker, that STRO-1/CEMP1 double positive cells from the PDL appear to be possible precursors to cementoblasts. Their study went on to demonstrate successful expression of both markers, STRO-1 and CEMP1 on these double positive cells via immunofluorescence as well as compositional data suggesting that the mineralized-like tissue formed by these osteogenically induced double positive cells displayed a Ca/P ratio of 1.89 which is very close to cellular cementum at 1.97 (Arzate et al. 2000). In light of this nonetheless, mRNA expression levels for CEMP1 however were highest in the osteogenically induced parental PDL group, not the STRO-1/CEMP1 double positive group as expected. It was theorized, that these “younger” early passage parental PDL cells possessed all the growth factors and features necessary to initiate cementogenesis as opposed to the late passage, serially diluted and aged STRO-1/CEMP1 double positive cells. Perhaps a younger, earlier passage STRO-1/CEMP1 double positive PDL cell osteogenically induced and evaluated sooner than a 14 day observation period, would not only reveal the temporal expression of cementum

protein 1, but possibly higher levels as well. Aminlari was able to conclude that STRO-1/CEMP1 double positive cells from the PDL exhibit characteristics consistent with precursors to cementoblasts.

In this study, we investigated the effect of cellular aging on the temporal expression of CEMP1. This was organized as an initial baseline and then subsequent time course experiment including four different cell strains in the baseline study and an additional cell strain or a total of five different cell strains in the time course study. The cell strains included: parental periodontal ligament cells, cementoblastoma cells (TCN), STRO-1/CEMP1 double positive cells (CEMP1 positive), STRO-1 double negative cells and human mesenchymal stem cells (hMSC). We successfully isolated periodontal ligament cells from the PDL tissue of freshly extracted teeth as demonstrated in previous studies (Xu et al., 2009; Aminlari et al., 2011). We then showed that it is possible to isolate STRO-1 positive cells through magnetic bead isolation using a mouse antibody against human STRO-1. Several studies suggest that STRO-1 is an early marker of undifferentiated stem cells, and the expression may decrease over time with each passage (Zhou et al., ’08; Itaya et al., ’09; Yamato et al., ’10). Immunofluorescence performed on both passage 3 as well as passage 11 Dynabead isolated (STRO-1 positive) cells using the STRO-1 antibody and FITC however, showed significant staining even in the aged cells (Fig. 4 and 5 respectively). Through immunofluorescence, we also demonstrated successful expression of both STRO-1 and CEMP1 markers on aged STRO-1/CEMP1 double positive cells using the STRO-1 antibody and FITC as well as the CEMP1 antibody and Texas Red by merging the two images (Fig. 5).

The culturing, maintenance and aging of any cell strain can be challenging. Multiple variables coupled with operator error may wreak havoc with the simple plating of a specific cell strain or the ongoing maintenance of a particular time course experiment prior to its fruition. Hayflick defines and clarifies the terms “cell line” and “cell strain” and would argue the term “cell line” to be inapplicable to the type of cells described in this study. The term “cell line” is confined to only those cells grown in vitro for extended periods of time (years), which as a result, presumes potential “immortality” when serially cultivated in vitro. The cells described in this study are therefore assumed to lack this characteristic of potential immortality and exclude them from being referred to as “cell lines” rather, a more appropriate term such as “cell strains.” A cell strain therefore, is a population of cells derived from animal tissue, subcultivated more than once in vitro, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin (Hayflick et al., 1961). Once decidedly operating in full force with a particular cell strain, the method of ‘aging’ this cell strain may be achieved in several ways. A cell may age through multiple passages or through a substantial amount of time that it takes to achieve confluency, not to mention the stresses induced to the cell during the process.

Several investigators have performed in vitro cellular aging via sequential subcultivation techniques according to Hayflick (Hayflick, 1977) and/or variations thereof. For example, Goseki et al transferred PDL cells from freshly extracted human premolars onto 35mm culture dishes and when the cells had reached confluence, they were designated “passage 1.” The cells were then detached w/ 0.05% trypsin and then subcultured in 75cm$^2$ culture flasks until confluent because the number of PDL cells at confluence in this flask they calculated to be approximately

1 x $10^6$ cells. When the PDL cells reached confluency at one-week intervals, they were subcultivated in a 4:1 split ratio (2.5 x $10^5$ cells/flask) so that two population doublings per subcultivation would result. After the $15^{th}$ passage, the growth rate of the cells decreased markedly with the doubling time requiring 10 or more days to reach confluence and therefore a 2:1 split ratio was then performed in order to both effectively and efficiently age the cells in a more timely fashion. Goseki and his group considered cells at an early passage (less than 30% of the number of population doublings, or passage 6 to 7) to be 'young' and at late passage (more than 75% of the number of population doublings, or passage 17 to 20) to be 'old.' Goseki considers human PDL cells, which are fibroblasts with limited proliferation, suitable as an in vitro aging model for studying the biochemical basis of cellular aging. When considering morphological criterion in young versus old or aged cell cultures, PDL cells displayed increased cell volume with advancing cell passages, and the aging cells grew much more slowly. Increase in cell size with aging as well as reduced replicative properties are considered to be typical parameters of cellular aging (Goseki, Shimizu et al., 1996). In our study which involved a time course model, we isolated PDL cells in the very same manner however chose to subcultivate on 10cm culture plates until confluent or approximately 2-2.5 x $10^6$ cells. Each passage consisted of a 4:1 ratio split onto 10cm culture plates until passage 11-13 at which time a 9:1 split ratio was performed onto nine 6cm plates to allow for five different time points (0hr, 6h, 24h, 72h and 1wk) of RNA isolation evaluating induced and non-induced cells. Unlike Goseki's experiment with PDL cells only, we also included two additional cell strains in our study; one of which (cementoblastoma or "TCN" cell) was terminally differentiated and therefore challenging to age in a timely fashion as this cell strain not only grew slowly, but by passage 8, became increasingly

difficult to maintain. It is believed that the cultured cementum tumor cells (TCN cells) in this study would be useful for at least three reasons: First, they could be used to obtain sufficient quantities of biologically active cementum components as this has been difficult so far due to the paucity of cementum in human teeth and labor intensive dissection procedures necessary. Second, the cells could be used to study the biosynthesis and processing of CAP and other cementum proteins. Third, early cementogenesis is believed to involve deposition of extracellular matrix produced by Hertwig's epithelial root sheath (HERS) cells on dentin, disruption of the HERS, migration and attachment of ectomesenchymal cells from dental follicle on to the matrix, their differentiation into cementoblasts and laying down of cementum (Arzate et al., 1992).

A baseline investigation of CEMP1 expression via reverse transcription real-time polymerase chain reaction comparing young versus aged PDL cells provided a reference for intended results that were far from expected. Significant upregulation of CEMP1 in aged STRO-1/CEMP1 positive cells was quite opposite of what was anticipated. In addition, significant upregulation for Collagen 1 was also revealed in aged parental PDL, STRO-1/CEMP1 positive and STRO-1 negative cells as well. Finally, results of a significant increase for osteopontin expression in aged parental PDL and STRO-1/CEMP1 positive cells prompted a more in-depth time course study to: A) confirm or at least coincide with the baseline results, and B) establish at certain time points when induced and control cells (young and aged) peak with a specific gene expression. Results of the time course experiment not only coincided with the baseline study, but also exceeded certain expectations. CEMP1 expression was indeed upregulated in aged parental PDL, STRO-1/CEMP1 positive and TCN cells but not in human mesenchymal stem cells

and it stands to reason as hMSC cells are not differentiated or 'programmed' well enough to allow for such expression. CEMP1 relative expression in young and aged STRO-1 negative (and theoretically CEMP1 negative) cells was considerably lower throughout the time course as expected and significantly downregulated in aged cells after one week of treatment with osteogenic media. Attempting to induce these cells toward a mineral producing phenotype without the CEMP1 gene or little to no CEMP1 expression was not effective; they displayed a significant downregulation in aged cells (19 fold), but also, little to no mineral nodule formation in young cells. On the other hand, CEMP1 expression in young, as well as aged PDL, STRO-1/CEMP1 positive, and TCN cells revealed nodule formation in the young osteogenically induced PDL, STRO-1/CEMP1 positive and TCN cells and increased mineral formation in the same aged cells (Fig. 7 and 8). Results of a study by Goseki et al showed a decrease in collagen expression of PDL cells with cellular aging (Goseki et al., 1996). Their assays for collagen secretion were performed using Sirius Red stain and quantified with spectrophotometry at 540nm. This in fact, was not the case with our study and our results using RT-qPCR were quite different. Relative expression for collagen generally speaking, was upregulated for every aged cell strain except the STRO-1 negative cells. The upregulation of collagen occurred as early as 6 hours (STRO-1/CEMP1 positive cells) and continued throughout the one week time course experiment. The authors of Goseki's article comment on the decreased expression of collagen in PDL cells with respect to aging perhaps correlating clinically in a decreased regenerative capacity of the PDL cell itself and adjacent tissues. This decreased regenerative capacity however is overshadowed by the microenvironment with which these cells reside enduring constant trauma and insult. Examination in vitro without the threat of microbiologic

destruction, allows the investigator to admire the potential of these cells in an otherwise healthy environment as they age. Perhaps the STRO-1 negative cells which are theoretically CEMP1 negative because they express minimal to no CEMP1, and also express no upregulation of collagen in aged cells, require CEMP1 as a signaling agent or vehicle not only for mineral production, but regeneration and repair as well. We see the same influential shift for TCN cells as they continue to age through the time course experiment. Increased collagen 1 expression with the aged TCN cells is observed as time progresses, possibly due to the upregulation of CEMP1 occurring at the same time at the same shift point between 24 and 72 hours. It is at this time point and thereafter that we notice a significant upregulation of collagen 1 for induced aged cells (4 fold at 72hr and 5 fold at one week).

It might be suggested that Collagen 1 and CEMP1 are tied together sharing a common pathway with one requiring the other, and as the STRO-1/CEMP1 positive cell ages, this pathway is either initiated or better yet, upregulated. Perhaps for the STRO-1 negative cell, this pathway no longer functions as the cell ages; due to possibly a manifestation of the Collagen 1 and CEMP1 expression no longer being tied together. Aminlari showed that when isolating STRO-1 positive PDL cells through magnetic bead isolation, only approximately 1.4% of the total cells along with a smaller percentage bound to the Dynabeads, were considered STRO-1 positive and therefore a far greater population of STRO-1 negative cells exist (Aminlari et al., 2011). Theoretically, a parental PDL cell population will have STRO-1 positive and STRO-1 negative cells but as you age the PDL cells, a predominant cell strain will rise and this might be the STRO-1 negative cell. This stands to reason because if a STRO-1/CEMP1 positive cell population dominates, cementum deposition would be in excess producing routine pathologic

cementoblastomas; therefore a 'negative feedback' type of regulation must prevail. This would allow for cementum deposition with age, but not to the point of pathologic deposition. Also of interest to note, the untreated control aged human mesenchymal stem cells at one week revealed a 10 fold upregulation of collagen however, *inducing* the same aged cells by one week also revealed a *45 fold* increase in collagen secretion. They also show a progressive upregulation of CEMP1 (aged vs young) throughout the time course to nearly a 2 fold increase by one week as well which draws attention once again to both CEMP1 and Collagen 1 being tied together in a common pathway. The hMSC cells, which are less differentiated than a PDL or cementoblastoma cell, show tremendous potential to differentiate into collagen secreting and mineral producing cells especially in the aged state; perhaps in a regenerative or repair capacity. This is indeed revealed after one week of induction with osteogenic media.

Osteopontin (OPN) is a molecule associated with early stages of mineralization. Its expression by cementum tumor cells (TCN cells) suggests that this molecule could be associated with the initial process of mineralization observed in these particular cells. The synthesis of this molecule may be regarded as the expression of a cell with an osteoblast-like phenotype and confirms its role as one of the molecules regulating the process of cementogenesis (Arzate et al., 1998). An upregulation of OPN is demonstrated for aged PDL, STRO-1/CEMP1 positive and STRO-1 negative cells however at one week a shift occurs in the ability to express OPN for STRO-1 negative cells with osteogenic media compared with STRO-1/CEMP1 positive cells. It is important to bear in mind that OPN is an early maker (osteogenic marker) in mineral deposition, not a marker present in early or young cells. We expected to see more expression of OPN for TCN cells with treatment as the time course progressed and this was not the case.

Untreated young and aged cells up to 72 hours revealed a progressive increase in OPN expression and then began to decrease thereafter. The cementoblastoma cells did in fact respond but at different times and to differential treatment. Perhaps a 'generic' type of osteogenic treatment as was used, may actually suppress OPN in these cells especially as they age. This might be necessary again as a possible type of negative feedback or rather, these terminally differentiated cells simply prefer laying down cementum rather than a bone-like mineralized material and the osteogenic media was quite possibly inhibiting the process. Lee et al evaluated the effects of aging on human dental pulp cells (HDPCs) with regard to dentinogenesis and observed a decrease in OPN expression with aging. To better understand the mechanism of the aging process, stress-induced premature senescence (SIPS) was performed by treating the HDP cells with $H_2O_2$. Odontogenic and osteogenic functions impaired by senescence were assayed via several experiments including Western blotting, reverse transcriptase polymerase chain reaction and Alizarin Red S staining. Results of oxidative stress-mediated premature senescence with simple replicative senescence appeared to be similar manifesting a decrease of OPN expression (Lee et al., 2013). Environmental stresses can markedly differ from the pulp to the periodontium however, with one influencing the other as well. Inflammation, oxidative stress along with replicative senescence provided an environment in vitro that produced such results for Lee and his group; an environment that was not created in our study. To summarize for OPN expression, the STRO-1/CEMP1 positive cells were very responsive to mineralization treatment while the cementoblastoma cells were counter-responsive to mineralization treatment and our data supports the findings of Arzate et al with regard to OPN upregulation in the presence of CEMP1 as a potential inducer.

The Arzate group not only showed CEMP1 regulates mineral deposition as demonstrated with anti-cementoblastoma protein antibodies thereby decreasing OPN and BSP levels, but they also showed using immunolocalization that this cementoblastoma protein was expressed specifically in the precementum of acellular and cellular cementum (Arzate et al., 1998, 2006). The concept of CEMP1 regulating mineral deposition is therefore not new, nor is the concept of increased cementum deposition with aging. Kuttler studied the microscopic basis of the anatomy of the dental apex using 402 extracted teeth from patients ranging in age with two groups: 18 - 25 and 55 – death. They concluded that the foramen of the root deviates more and more from the apical center with an increase of age caused by a thickening of the apical cementum. Also the diameter of the foramen increased with age due to the apposition of new layers of cementum (Kuttler, 1955). Determinations of cementum thickness on 233 single-rooted teeth with healthy supporting tissues showed a straight-line relationship between age and cementum thickness. The thickness of cementum was approximately tripled between the ages of 11 and 76 years. This occurred more in the apical region and less around the cementoenamel junction (Zander et al., 1958). Stein et al. also examined the microscopic structure of the root apex to ascertain what changes, if any, occur over time. They studied 111 extracted teeth both vital and non-vital from 47 patients ranging from 26-77 years old. It was noted that as age increased, the width of the cementum appeared to increase as well. They indicate that Nitzan et al. ascribed to the increase in the width of cementum to the aging process, not to physiologic changes (Stein et al., 1990). Our data supports this finding via RT-qPCR, time course osteoinduction and Alizarin Red staining which reveal an upregulation of not only CEMP1 expression, but OPN and Collagen expression as well. This was seen in parental

PDL, STRO-1/CEMP1 positive, and cementoblastoma cells, but not consistently or at all in hMSC, and STRO-1 negative cells. It was interesting to observe via spectrophotometry, significantly more nodule formation with Alizarin Red S staining for aged STRO-1 negative cells compared to young STRO-1 negative cells however not similarly with aged STRO-1/CEMP1 positive, PDL, and TCN cells despite the visibly increased staining. This may be due in part, to the differences in osteoinduction time of one week for the time course vs two weeks of induction for the Alizarin Red S staining. Confining the aged cells to a 6 well plate for two weeks could have dramatically altered the capabilities of these particular cell strains and perhaps warrants further investigation.

To conclude, we have demonstrated successful aging of parental PDL, STRO-1/CEMP1 positive, STRO-1 negative, human mesenchymal stem and cementoblastoma cells, which were able to produce mineralized-like tissue in an 'aged' state. CEMP1 expression was significantly upregulated during the aging process for several cell strains including STRO-1/CEMP1 positive cells and these cells continued to exhibit characteristics consistent with precursors to cementoblasts. This will aid in advancing our knowledge of the mechanisms involved with cementogenesis, cellular aging and ultimately periodontal regeneration as well as helping improve current methodologies for treating patients with periodontitis and attachment loss.

# Tables and Figures

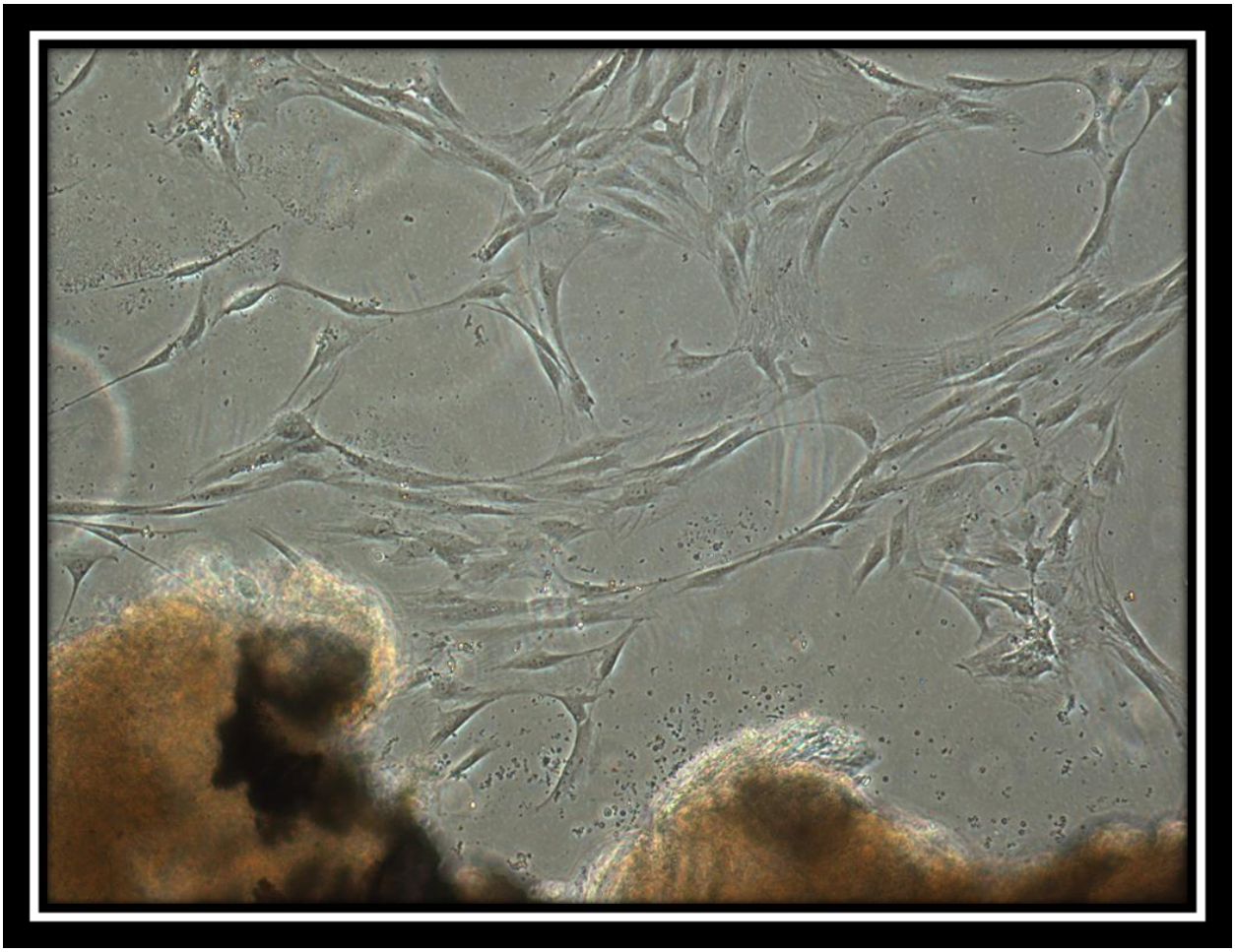


**Figure 1**: Phase contrast image of PDL tissue (Bottom) giving rise to PDL cells (top).

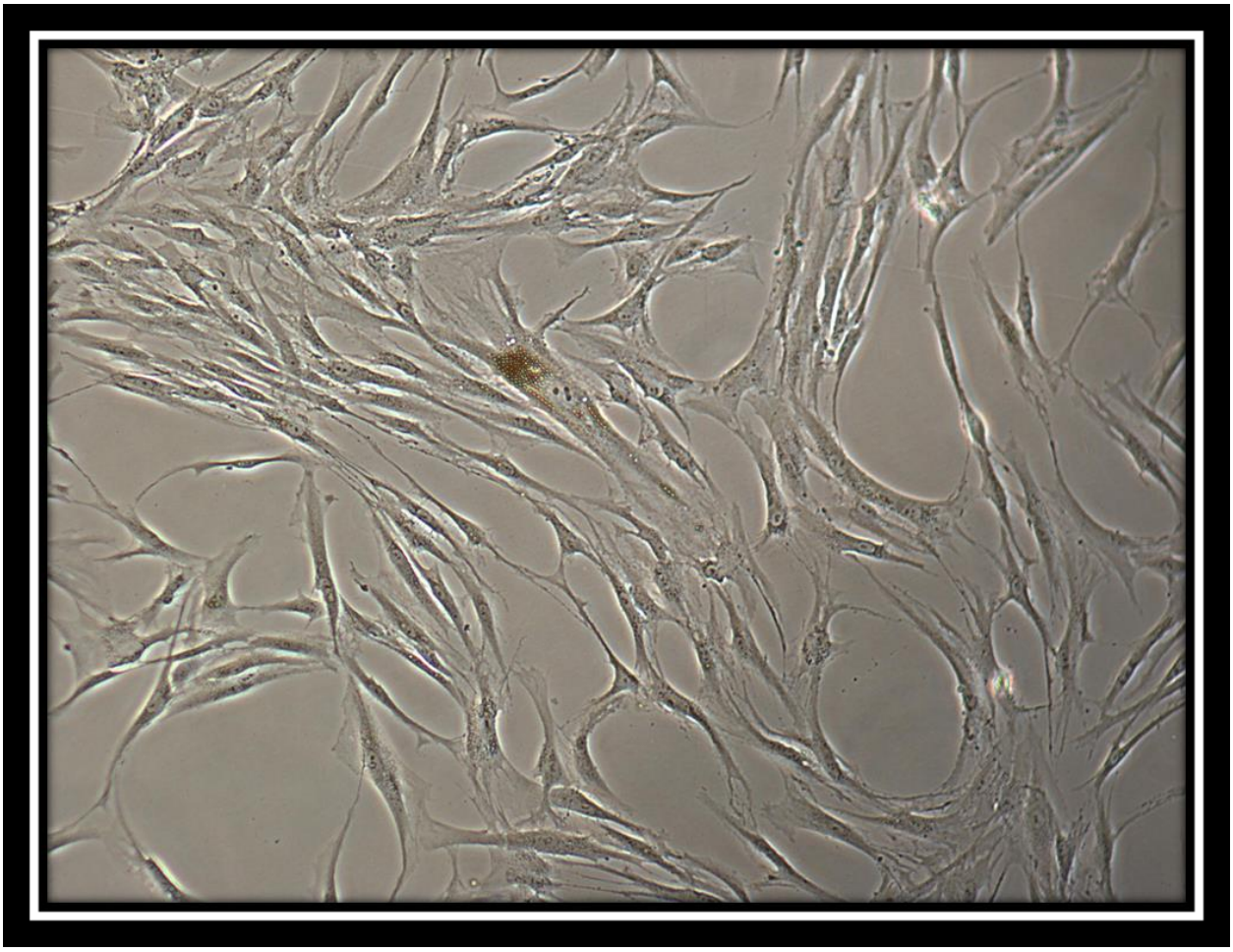


**Figure 2:** Phase contrast image of post-bead isolation of PDL cells using human STRO-1 antibody. Note the STRO-1 positive cells attached to the gold Dynabeads.

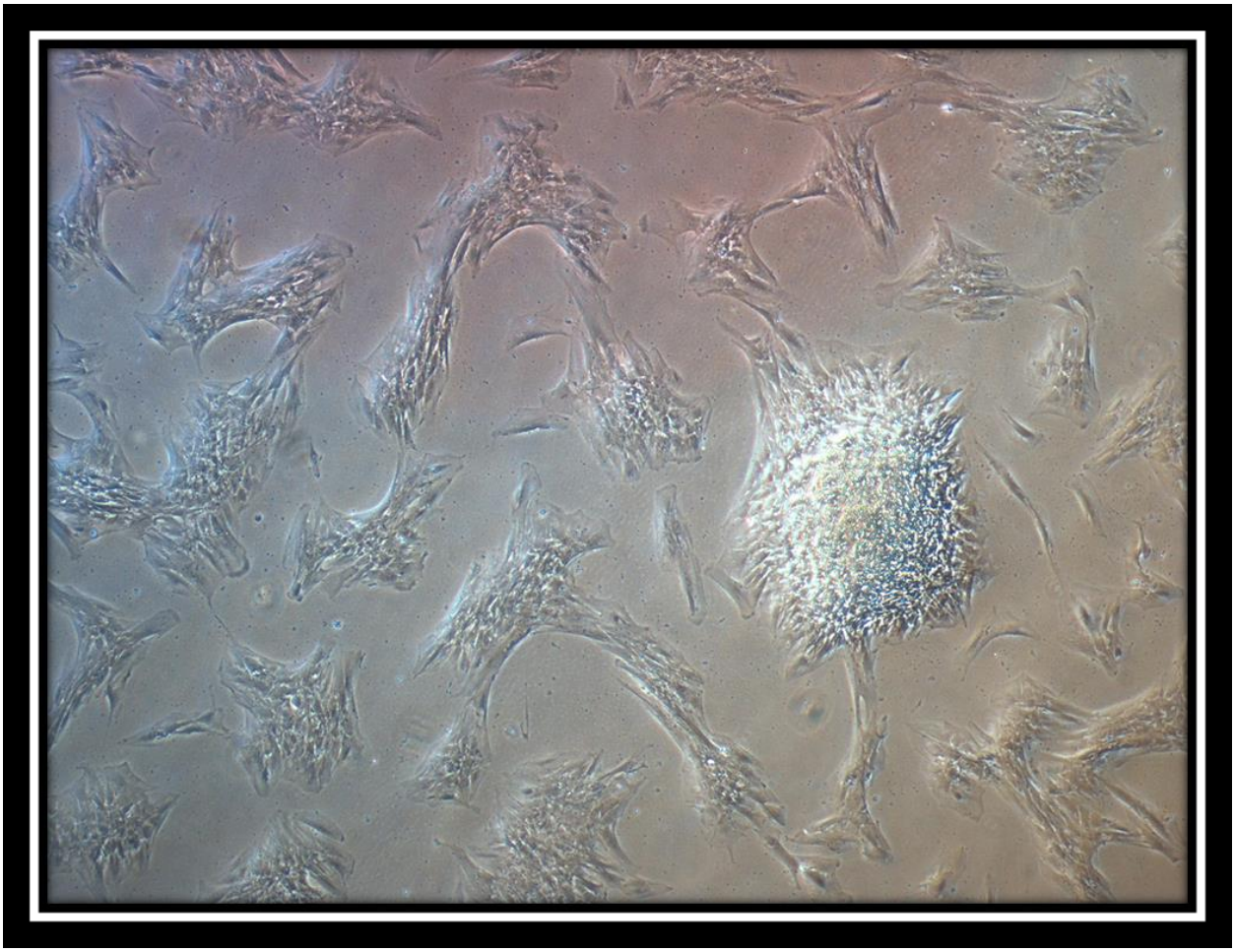


**Figure 3:** Phase contrast image of aged (P 11) STRO-1 positive bead-isolated cells. Note the changes in morphology from spindle shapes to more flattened shapes with increased cell volume.

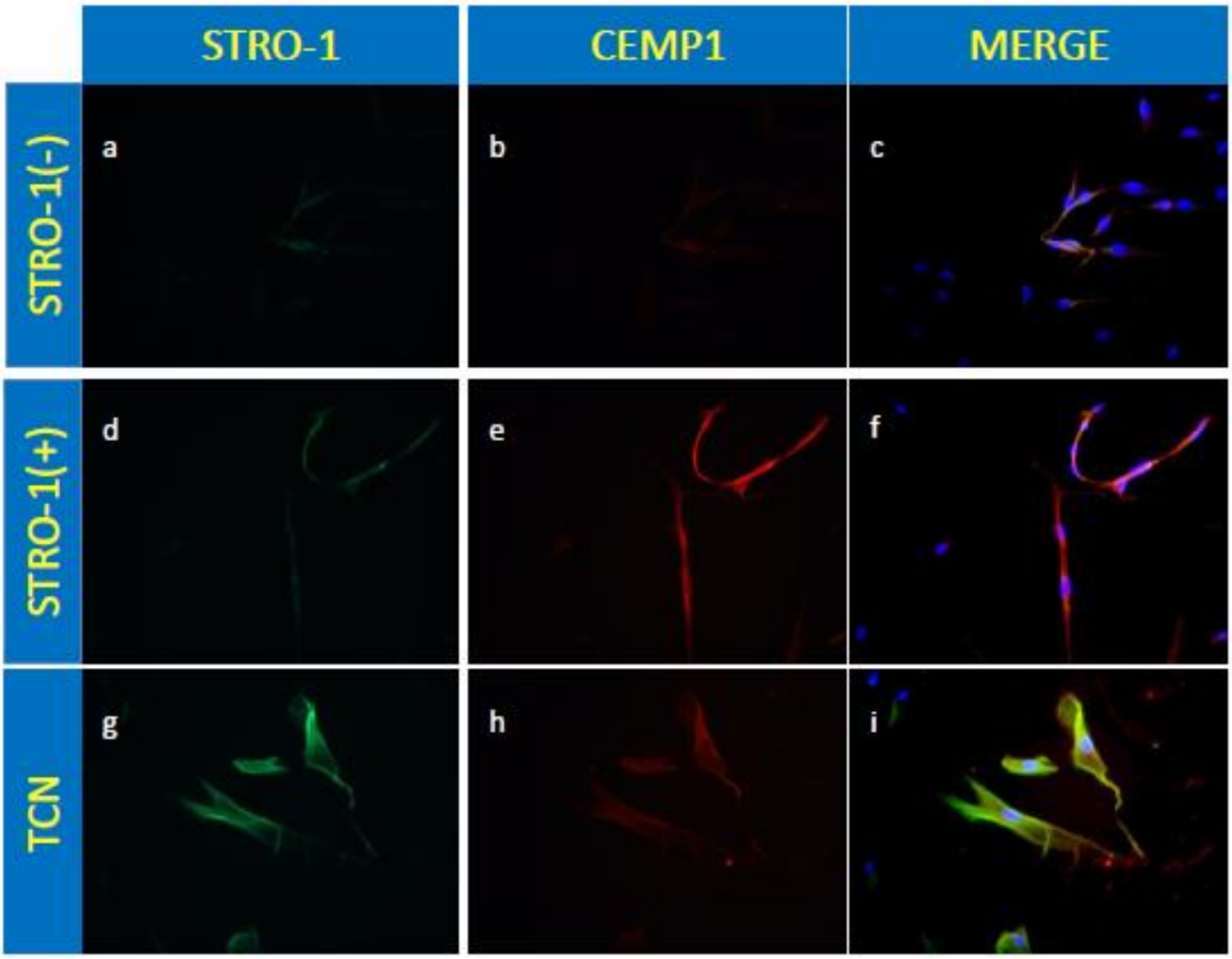



**Figure 4**: Immunofluorescent images staining for STRO-1, CEMP1, and the nucleus with P-3 cells. The blue color represents DAPI staining of the nucleus. The green color represents staining of the STRO-1 marker with FITC. The Red color represents staining of the CEMP1 marker with Texas Red. **a**,**d**,**g** show FITC staining of STRO-1 on STRO-1-, STRO-1+, and TCN cells respectively. **b**,**e**,**h** show Texas Red staining of CEMP1 marker on STRO-1-, STRO-1+, and TCN cells respectively. **c**,**f**,**I** demonstrate merging of STRO-1 and CEMP1 staining where both markers are present.

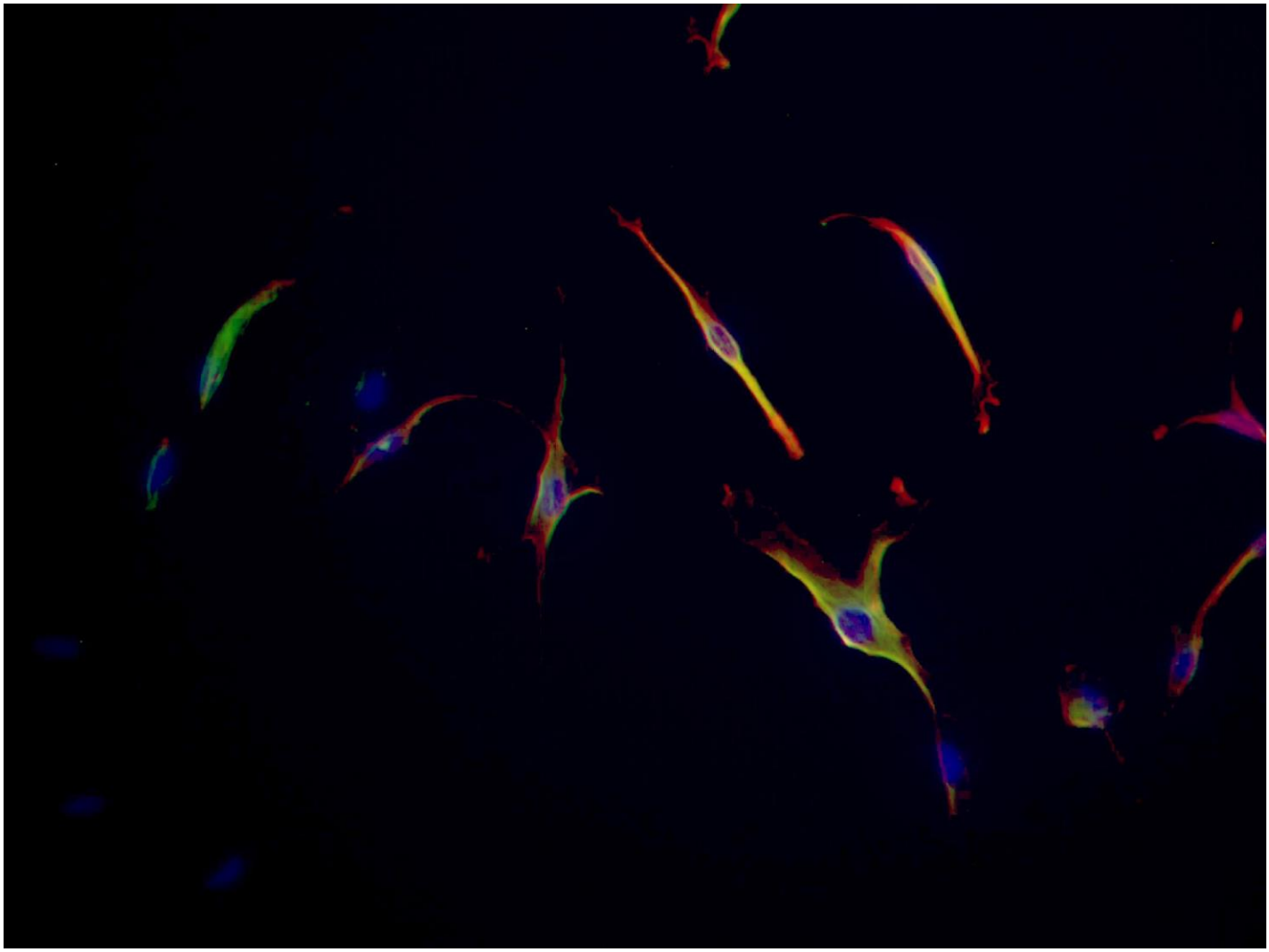


**Figure 5:** Merged microscopic image of aged P-11 STRO-1 positive bead isolated PDL cells stained for STRO-1 with FITC, CEMP1 with Texas Red, and the nucleus with DAPI.

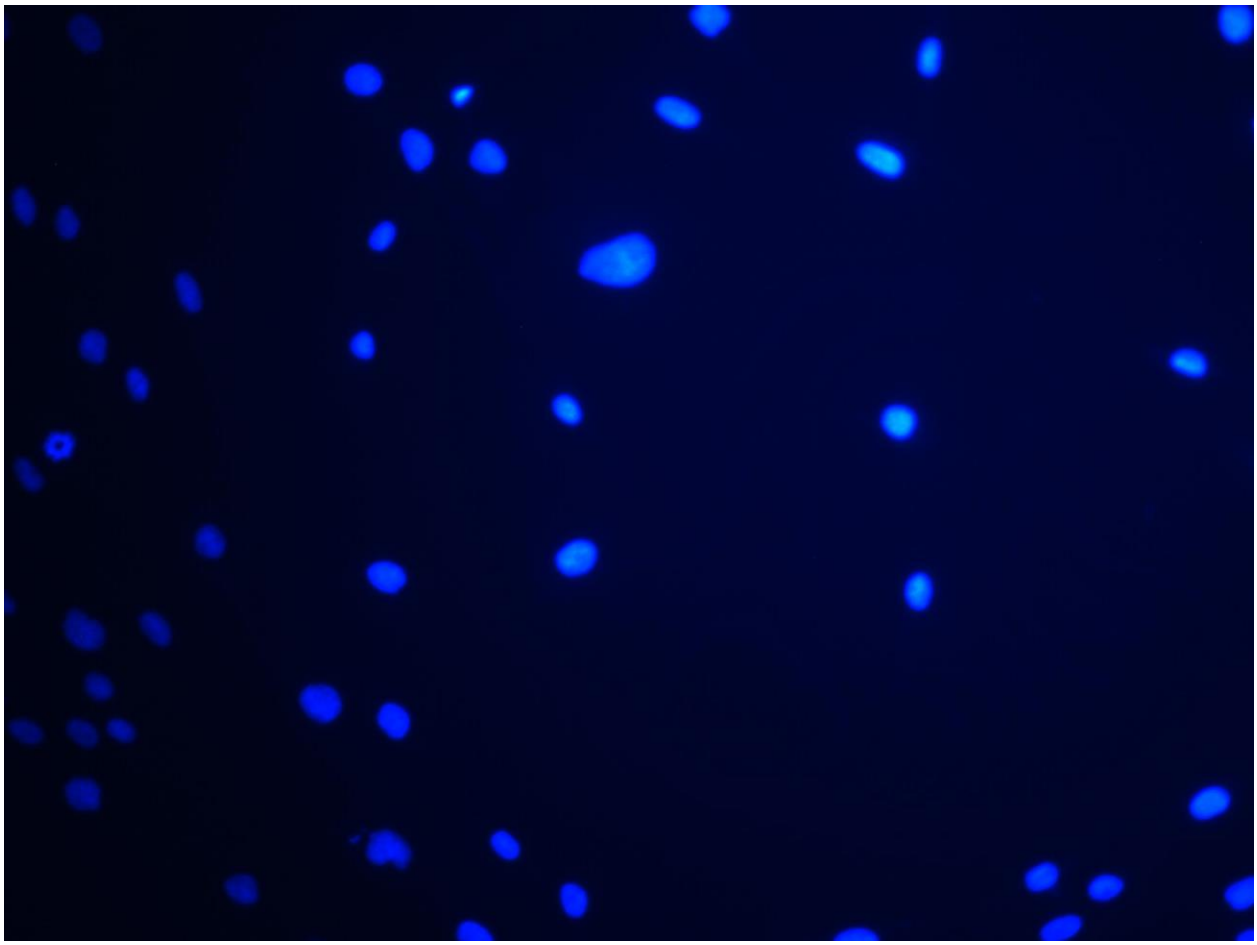


**Figure 6:** STRO-1 negative P-11 (Supernatant or non-bead isolated PDL cells) cells stained for both STRO-1 and CEMP1. Both stains were negative. Nuclei are stained blue with DAPI.

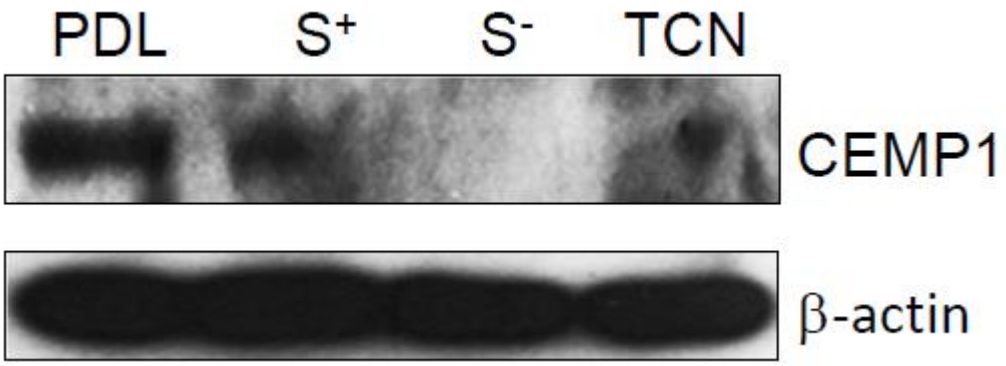



**Figure 7:** Protein expression by Western blot. Parental PDL (PDL), STRO-1 positive ($S^+$), STRO-1 negative ($S^-$), and cementoblastoma (TCN) cells assayed for CEMP1 expression and normalized to Beta-actin. Note bands for parental PDL, STRO-1 positive and cementoblastoma cells and no band visible for STRO-1 negative cells.

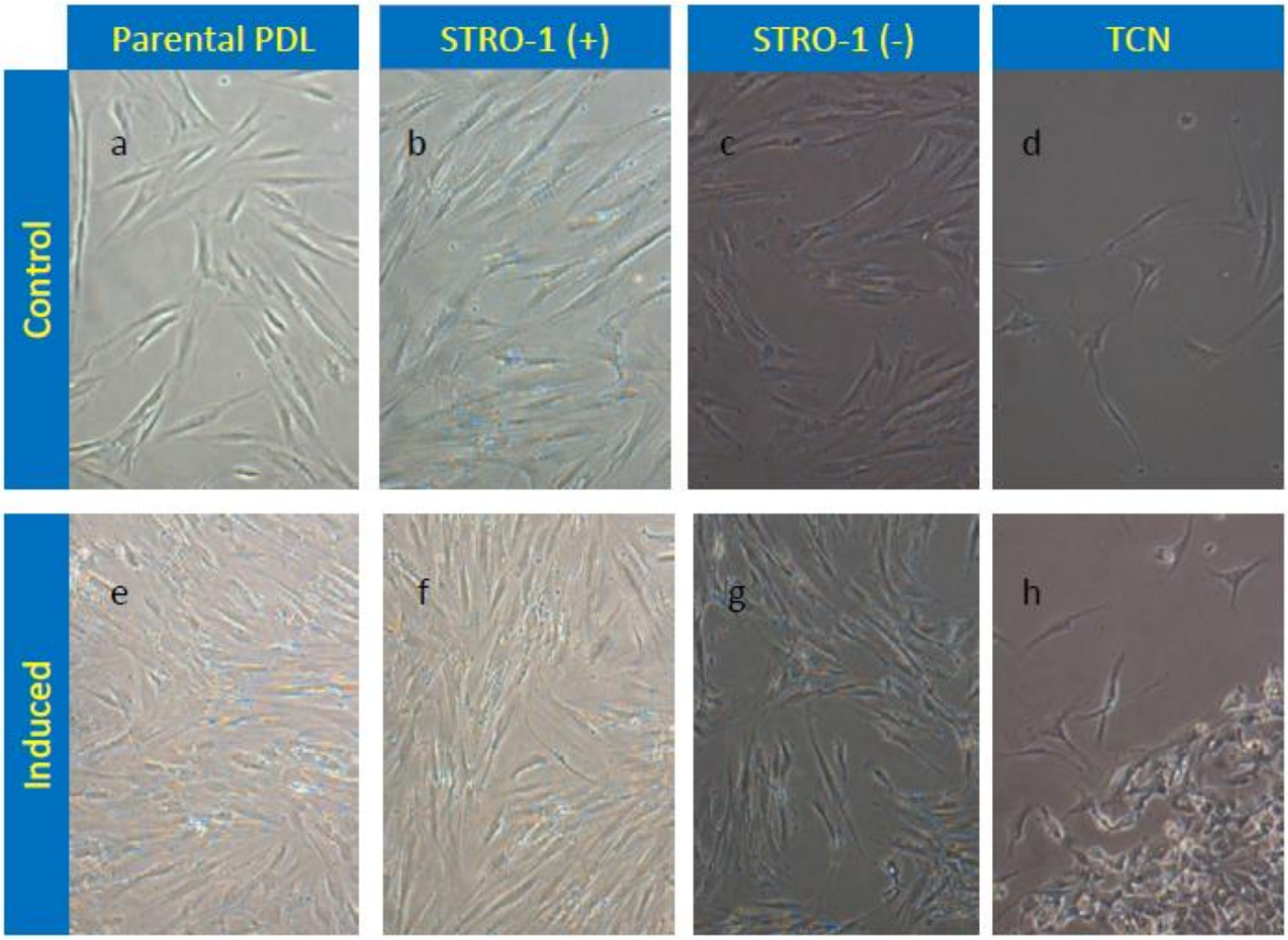



**Figure 8:** Phase contrast images revealing young (P 3-4) cells in both control and osteogenic media. Note images e,f,h depicting small nodule formation. a,e parental PDL cell. b,f STRO-1 (+) Dynabead isolated cells. c,g STRO-1 (-) cells. d,h cementoblastoma cells.

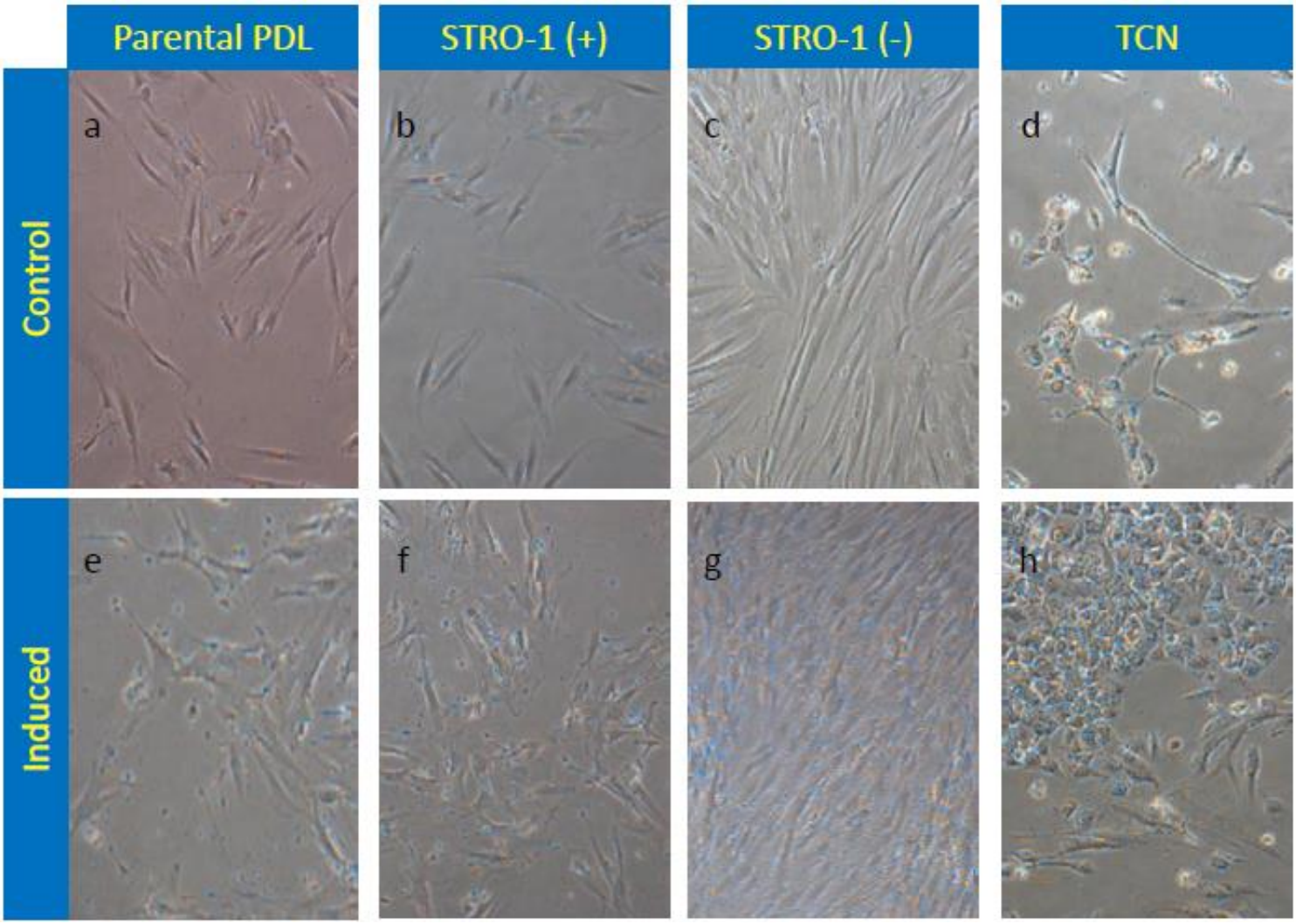



**Figure 9:** Phase contrast images revealing aged (P 11-13) cells in both control and osteogenic media. Note images e, f, and h depicting small nodule formation. a,e parental PDL cell. b,f STRO-1 (+) Dynabead isolated cells. c,g STRO-1 (-) cells. d,h cementoblastoma cells.

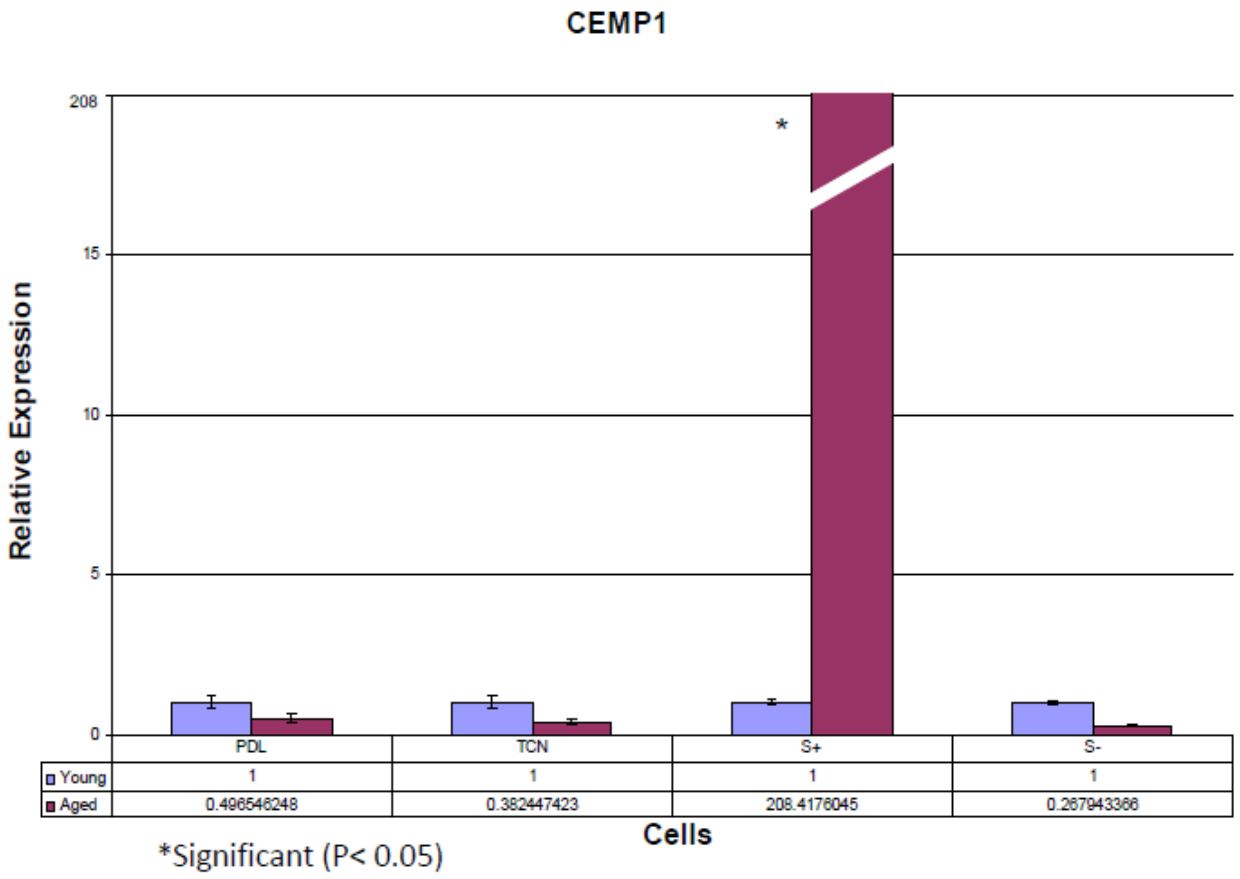



**Figure 10:** Baseline trial of real time PCR showing Cementum Protein 1 (CEMP1) mRNA relative expression comparing young (P 3-4) versus aged (P 11-13) cells in four different cell strains normalized to GAPDH. Note Significant upregulation of CEMP1 expression in "aged" STRO-1 positive cells compared to others (P< 0.05). Also note downregulation of CEMP1 expression in "aged" PDL, TCN and STRO-1 negative cells which was not considered significant (P>0.05).

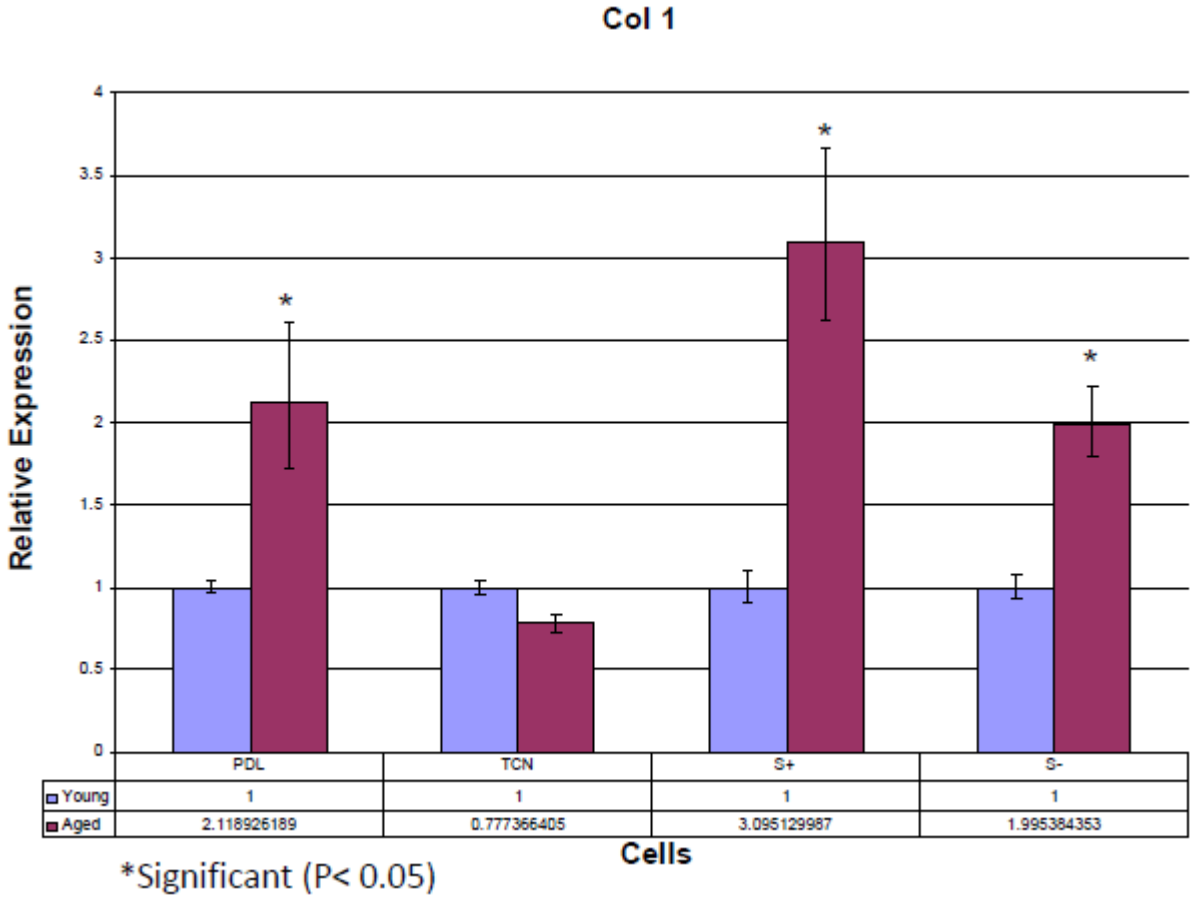



**Figure 11:** Baseline trial of real time PCR showing Collagen 1 (Col 1) mRNA relative expression comparing young (P 3-4) versus aged (P 11-13) cells in four different cell strains normalized to GAPDH. Note significant upregulation of Col 1 expression in "aged" PDL, STRO-1 positive and STRO-1 negative cells compared to others (P< 0.05).

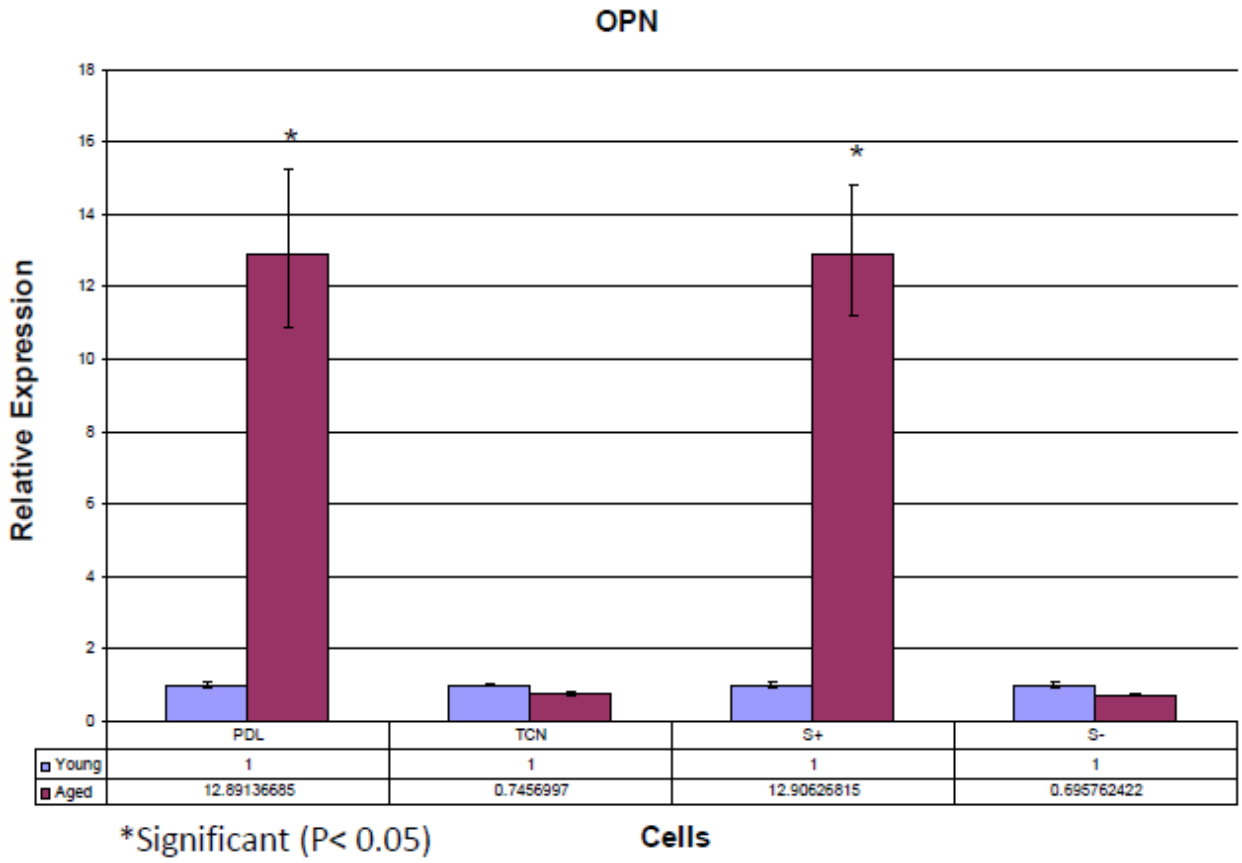



**Figure 12:** Baseline trial of real time PCR showing Osteopontin (OPN) mRNA relative expression comparing young (P 3-4) versus aged (P 11-13) cells in four different cell strains normalized to GAPDH. Note significant upregulation of OPN expression in “aged” PDL and STRO-1 positive cells compared to others (P< 0.05).

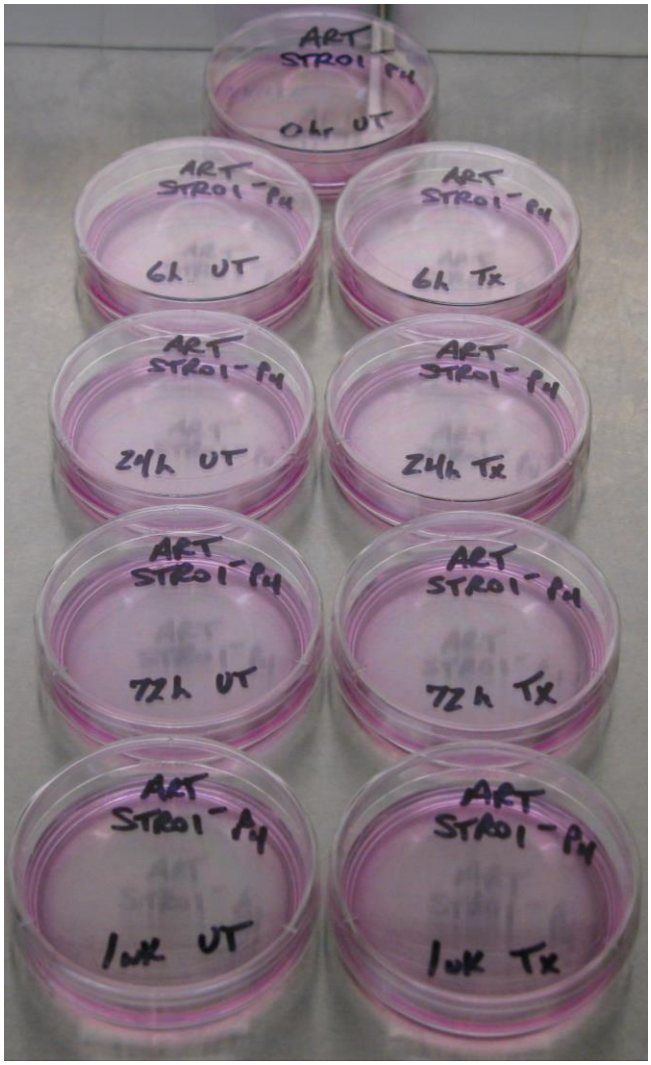



**Figure 13:** Time course experiment image of nine 6cm plates displaying the cell strain (STRO-1 negative), age of cell strain (P4), different time points (0hr-1wk) as well as osteogenic induction (Tx) and untreated control (UT) cells.

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | PDL | 0.0519 | 0.0263 | 1.9749 |
| 6h UT | PDL | 0.0441 | 0.0231 | 1.9062 |
| 6h Tx | PDL | 0.0646 | 0.0337 | 1.9140 |
| 24h UT | PDL | 0.0755 | 0.0385 | 1.9629 |
| 24h Tx | PDL | 0.0688 | 0.0346 | 1.9902 |
| 72h UT | PDL | 0.0688 | 0.0366 | 1.8797 |
| 72h Tx | PDL | 0.0843 | 0.0475 | 1.7754 |
| 1wk UT | PDL | 0.0884 | 0.0466 | 1.8958 |
| 1wk Tx | PDL | 0.1485 | 0.0821 | 1.8081 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | STRO-1 + | 0.0856 | 0.0483 | 1.7745 |
| 6h UT | STRO-1 + | 0.1047 | 0.0600 | 1.7450 |
| 6h Tx | STRO-1 + | 0.0930 | 0.0530 | 1.7549 |
| 24h UT | STRO-1 + | 0.0895 | 0.0488 | 1.8344 |
| 24h Tx | STRO-1 + | 0.1475 | 0.0790 | 1.8668 |
| 72h UT | STRO-1 + | 0.1025 | 0.0549 | 1.8675 |
| 72h Tx | STRO-1 + | 0.1412 | 0.0761 | 1.8550 |
| 1wk UT | STRO-1 + | 0.2304 | 0.1265 | 1.8215 |
| 1wk Tx | STRO-1 + | 0.4878 | 0.2530 | 1.9283 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | STRO-1 - | 0.0852 | 0.0492 | 1.7301 |
| 6h UT | STRO-1 - | 0.1013 | 0.0580 | 1.7444 |
| 6h Tx | STRO-1 - | 0.1125 | 0.0688 | 1.6332 |
| 24h UT | STRO-1 - | 0.1499 | 0.0809 | 1.8520 |
| 24h Tx | STRO-1 - | 0.1427 | 0.0827 | 1.7238 |
| 72h UT | STRO-1 - | 0.1228 | 0.0707 | 1.7362 |
| 72h Tx | STRO-1 - | 0.1024 | 0.0586 | 1.7457 |
| 1wk UT | STRO-1 - | 0.2159 | 0.1181 | 1.8273 |
| 1wk Tx | STRO-1 - | 0.2343 | 0.1370 | 1.7101 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | hMSC | 0.0233 | 0.0137 | 1.6987 |
| 6h UT | hMSC | 0.1314 | 0.1213 | 1.0831 |
| 6h Tx | hMSC | 0.0290 | 0.0173 | 1.6781 |
| 24h UT | hMSC | 0.0352 | 0.0182 | 1.9300 |
| 24h Tx | hMSC | 0.0434 | 0.0248 | 1.7486 |
| 72h UT | hMSC | 0.0334 | 0.0185 | 1.8124 |
| 72h Tx | hMSC | 0.2726 | 0.1525 | 1.7875 |
| 1wk UT | hMSC | 0.0933 | 0.0528 | 1.7689 |
| 1wk Tx | hMSC | 0.2077 | 0.1092 | 1.9027 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | TCN | 0.0856 | 0.0438 | 1.9559 |
| 6h UT | TCN | 0.0296 | 0.0157 | 1.8854 |
| 6h Tx | TCN | 0.0226 | 0.0132 | 1.7053 |
| 24h UT | TCN | 0.0440 | 0.0222 | 1.9783 |
| 24h Tx | TCN | 0.0417 | 0.0226 | 1.8430 |
| 72h UT | TCN | 0.1007 | 0.0540 | 1.8644 |
| 72h Tx | TCN | 0.0552 | 0.0292 | 1.8913 |
| 1wk UT | TCN | 0.0947 | 0.0508 | 1.8661 |
| 1wk Tx | TCN | 0.3233 | 0.1728 | 1.8716 |

**Table 1:** 260/280 absorbance ratio and concentrations of young cell (P 3-4) mRNA samples measured in a Beckman DU-640 spectrophotometer. mRNA was isolated at five different time points from five different cell types. Cells were either untreated (UT) or osteogenically induced (Tx).

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | PDL | 0.0571 | 0.0338 | 1.6874 |
| 6h UT | PDL | 0.0336 | 0.0181 | 1.8553 |
| 6h Tx | PDL | 0.0554 | 0.0335 | 1.6545 |
| 24h UT | PDL | 0.0479 | 0.0270 | 1.7704 |
| 24h Tx | PDL | 0.0746 | 0.0452 | 1.6504 |
| 72h UT | PDL | 0.0446 | 0.0249 | 1.7957 |
| 72h Tx | PDL | 0.0788 | 0.0465 | 1.6953 |
| 1wk UT | PDL | 0.0698 | 0.0392 | 1.7830 |
| 1wk Tx | PDL | 0.0846 | 0.0487 | 1.7365 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | STRO-1 + | 0.0392 | 0.0251 | 1.5598 |
| 6h UT | STRO-1 + | 0.0412 | 0.0237 | 1.7418 |
| 6h Tx | STRO-1 + | 0.0518 | 0.0319 | 1.6248 |
| 24h UT | STRO-1 + | 0.0632 | 0.0367 | 1.7210 |
| 24h Tx | STRO-1 + | 0.0620 | 0.0389 | 1.5939 |
| 72h UT | STRO-1 + | 0.0629 | 0.0360 | 1.7485 |
| 72h Tx | STRO-1 + | 0.0764 | 0.0456 | 1.6741 |
| 1wk UT | STRO-1 + | 0.0824 | 0.0479 | 1.7209 |
| 1wk Tx | STRO-1 + | 0.0753 | 0.0444 | 1.6963 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | STRO-1 - | 0.0738 | 0.0466 | 1.5842 |
| 6h UT | STRO-1 - | 0.0582 | 0.0337 | 1.7292 |
| 6h Tx | STRO-1 - | 0.0644 | 0.0375 | 1.7162 |
| 24h UT | STRO-1 - | 0.0596 | 0.0346 | 1.7230 |
| 24h Tx | STRO-1 - | 0.0642 | 0.0401 | 1.6008 |
| 72h UT | STRO-1 - | 0.0655 | 0.0386 | 1.6951 |
| 72h Tx | STRO-1 - | 0.0389 | 0.0250 | 1.5530 |
| 1wk UT | STRO-1 - | 0.0574 | 0.0342 | 1.6769 |
| 1wk Tx | STRO-1 - | 0.5778 | 0.3166 | 1.8249 |

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | hMSC | 0.0337 | 0.0209 | 1.6161 |
| 6h UT | hMSC | 0.0166 | 0.0081 | 2.0404 |
| 6h Tx | hMSC | 0.0210 | 0.0123 | 1.7009 |
| 24h UT | hMSC | 0.0236 | 0.0126 | 1.8699 |
| 24h Tx | hMSC | 0.0229 | 0.0140 | 1.6355 |
| 72h UT | hMSC | 0.0199 | 0.0101 | 1.9833 |
| 72h Tx | hMSC | 0.0287 | 0.0165 | 1.7406 |
| 1wk UT | hMSC | 0.0205 | 0.0111 | 1.8503 |
| 1wk Tx | hMSC | 0.0374 | 0.0211 | 1.7768 |

**Table 2:** 260/280 absorbance ratio and concentrations of aged cell (P 11-13) mRNA samples measured in a Beckman DU-640 spectrophotometer. mRNA was isolated at five different time points from five different cell types. Cells were either untreated (UT) or osteogenically induced (Tx).

| Sample | Cell Type | 260nm (conc) | 280nm | 260/280 nm |
|---|---|---|---|---|
| 0 hr | TCN | 0.1733 | 0.1021 | 1.6977 |
| 6h UT | TCN | 0.1724 | 0.0960 | 1.7963 |
| 6h Tx | TCN | 0.2009 | 0.1176 | 1.7084 |
| 24h UT | TCN | 0.1883 | 0.1064 | 1.7692 |
| 24h Tx | TCN | 0.2620 | 0.1488 | 1.7609 |
| 72h UT | TCN | 0.1329 | 0.0763 | 1.7412 |
| 72h Tx | TCN | 0.2488 | 0.1478 | 1.6830 |
| 1wk UT | TCN | 0.5127 | 0.2904 | 1.7657 |
| 1wk Tx | TCN | 0.2228 | 0.1335 | 1.6693 |

Table 3

| Gene | Assay ID | Probe Sequence |
|---|---|---|
| GAPDH | Hs02758991_g1 | GACTCATGACCACAGTCCATGCCAT |
| COL1A1 | Hs00164004_m1 | AAGACGAAGACATCCCACCAATCAC |
| SPP1 / OPN | Hs00959010_m1 | TCGCAGACCTGACATCCAGTACCCT |
| CEMP1 | Hs04185363_s1 | TCCCTGCCTGGGAGCCCTGGCAAGA |

**Table 3:** TaqMan® gene expression assay ID and probe sequences for GAPDH, Collagen 1, Osteopontin (OPN), and Cementum Protein 1 (CEMP1).

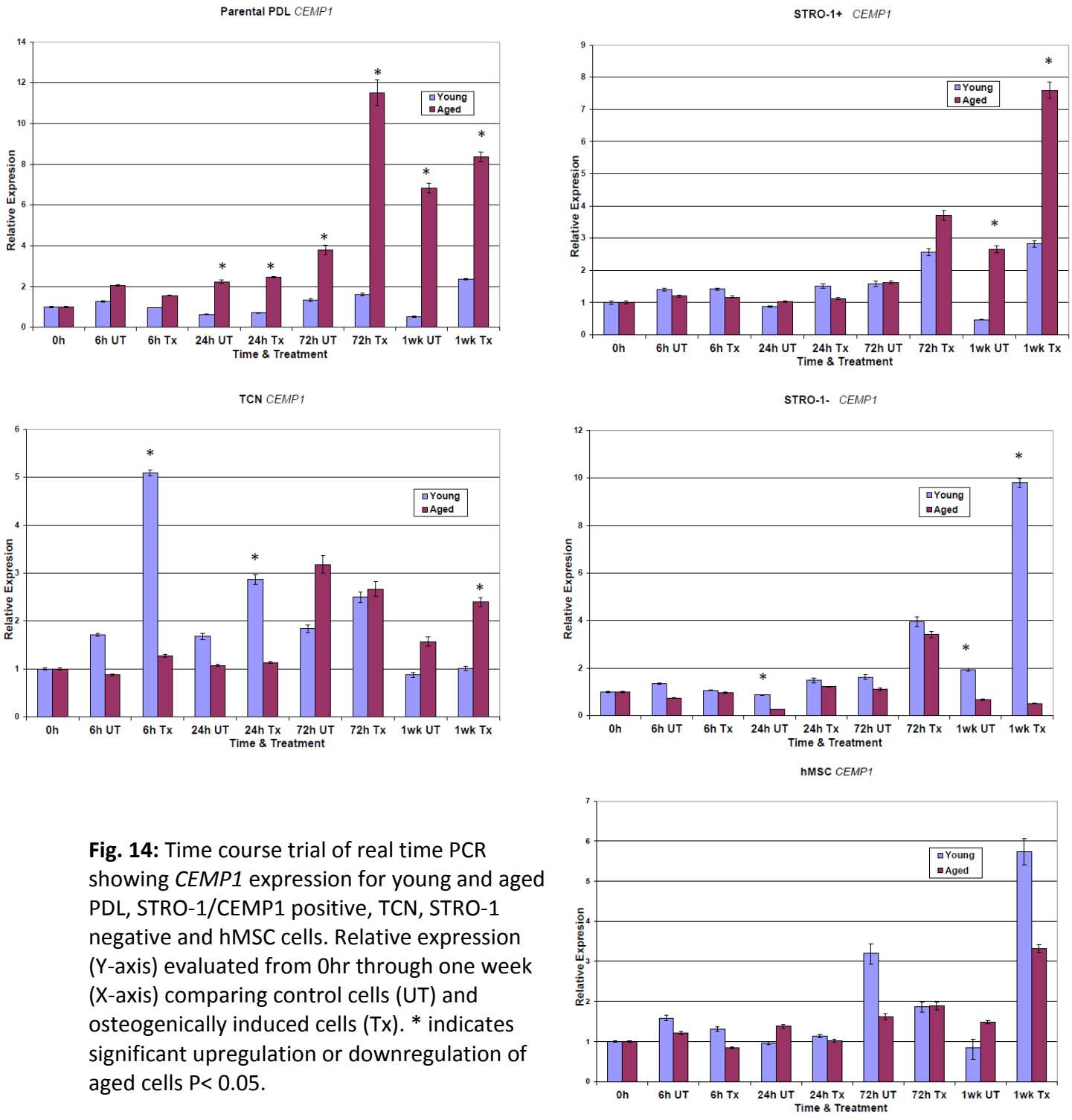



**Fig. 14:** Time course trial of real time PCR showing *CEMP1* expression for young and aged PDL, STRO-1/CEMP1 positive, TCN, STRO-1 negative and hMSC cells. Relative expression (Y-axis) evaluated from 0hr through one week (X-axis) comparing control cells (UT) and osteogenically induced cells (Tx). * indicates significant upregulation or downregulation of aged cells P< 0.05.

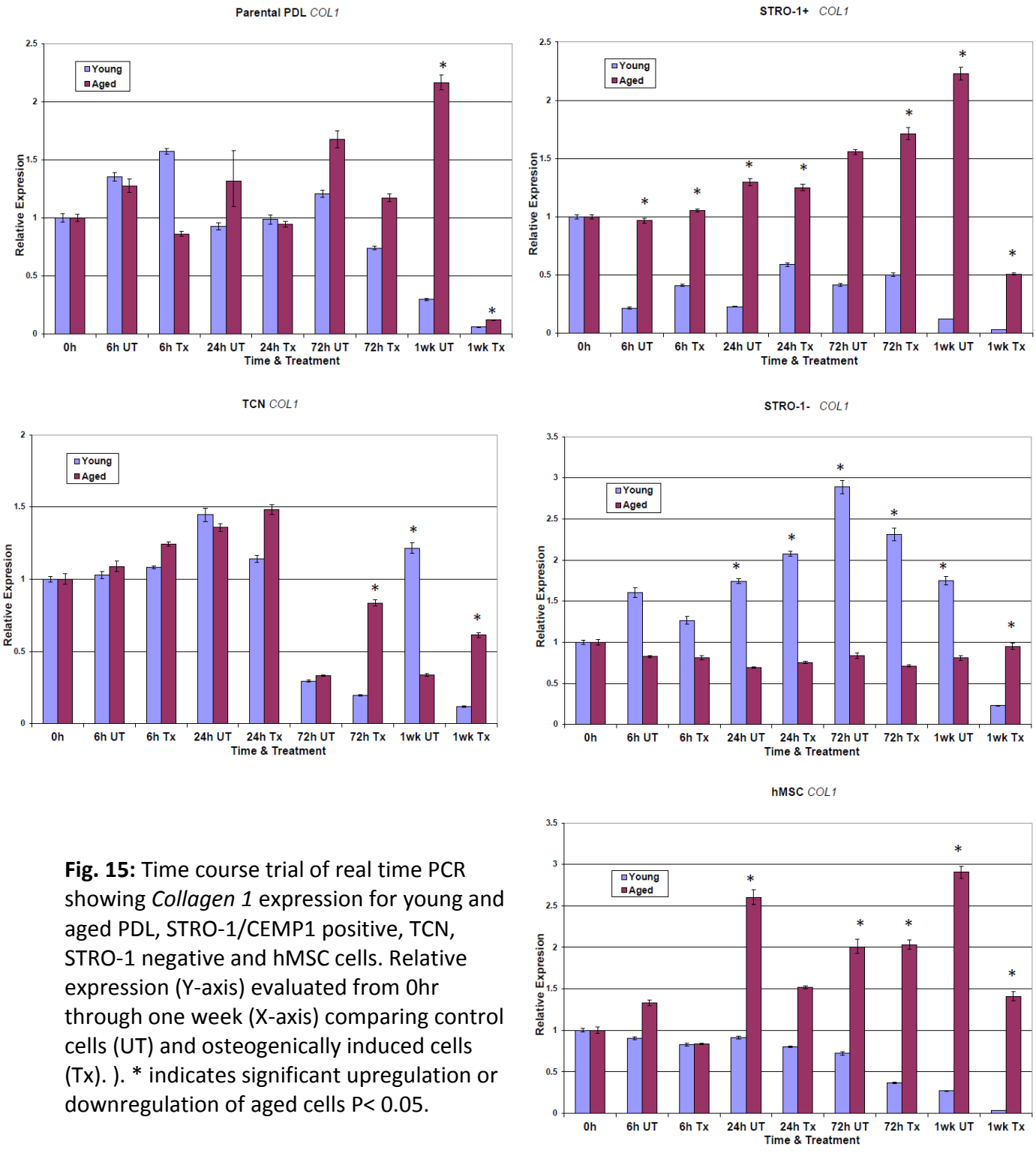
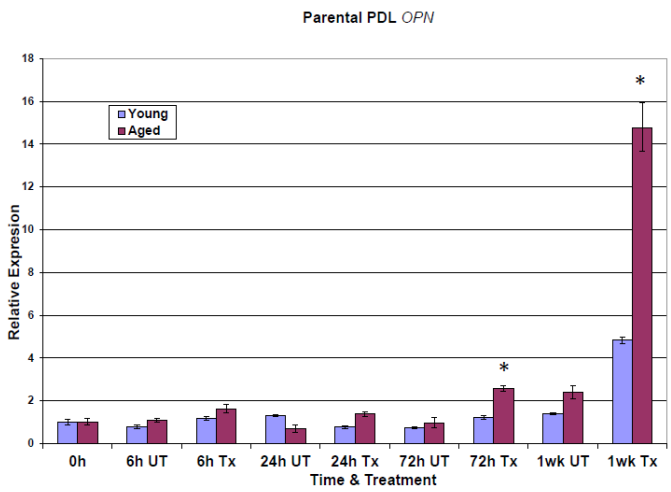
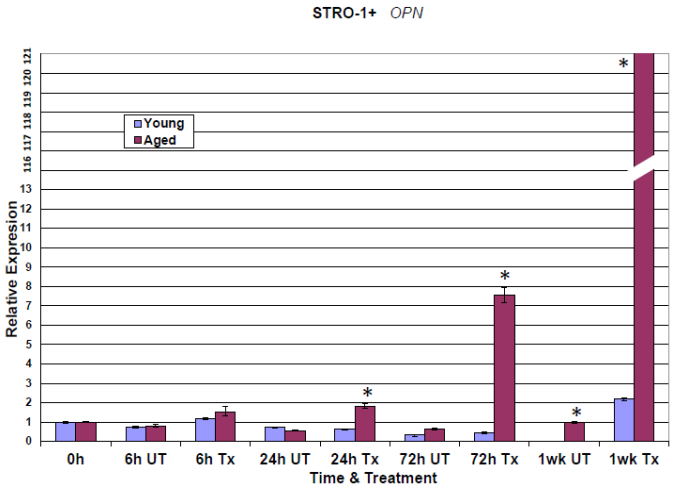
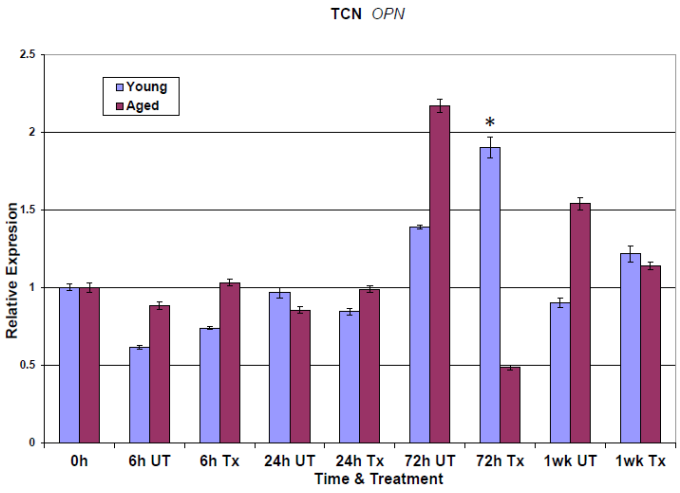
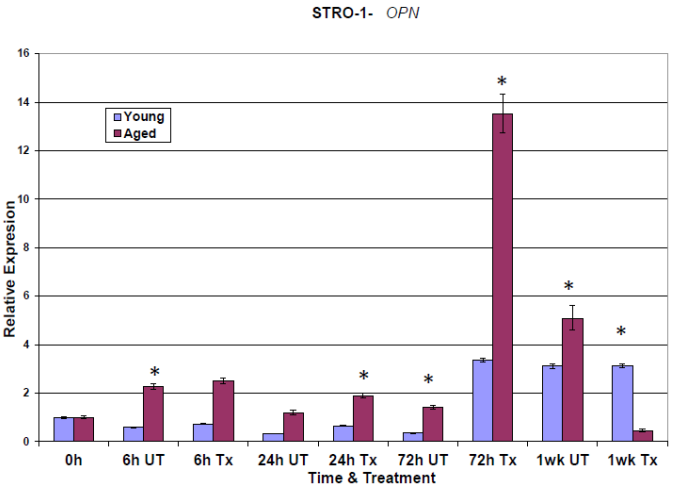



**Fig. 15:** Time course trial of real time PCR showing *Collagen 1* expression for young and aged PDL, STRO-1/CEMP1 positive, TCN, STRO-1 negative and hMSC cells. Relative expression (Y-axis) evaluated from 0hr through one week (X-axis) comparing control cells (UT) and osteogenically induced cells (Tx). ). * indicates significant upregulation or downregulation of aged cells P< 0.05.



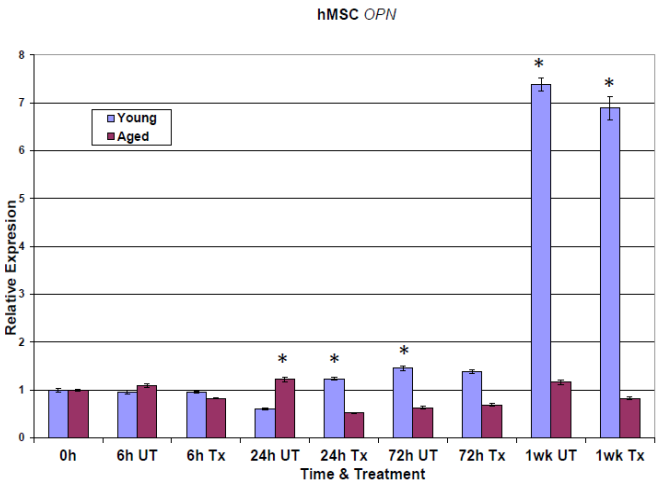




**Fig. 16:** Time course trial of real time PCR showing *Osteopontin* expression for young and aged PDL, STRO-1/CEMP1 positive, TCN, STRO-1 negative and hMSC cells. Relative expression (Y-axis) evaluated from 0hr through one week (X-axis) comparing control cells (UT) and osteogenically induced cells (Tx). ). * indicates significant upregulation or downregulation of aged cells P< 0.05.

| PDL Cell CEMP1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.599 | 0.009 |
| 6hr Tx | 1.623 | 0.003 |
| 24hr UT | 3.581 | 0.00003 |
| 24hr Tx | 3.454 | 0.00001 |
| 72hr UT | 2.841 | 0.0001 |
| 72hr Tx | 7.161 | 0.0000009 |
| 1wk UT | 12.883 | 0.00001 |
| 1wk Tx | 3.546 | 0.00004 |

| S+ Cell CEMP1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.861 | 0.000001 |
| 6hr Tx | 0.827 | 0.00006 |
| 24hr UT | 1.174 | 0.0002 |
| 24hr Tx | 0.742 | 0.00003 |
| 72hr UT | 1.023 | 0.0001 |
| 72hr Tx | 1.442 | 0.059 |
| 1wk UT | 5.707 | 0.0000004 |
| 1wk Tx | 2.693 | 0.000009 |

| TCN CEMP1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.510 | 0.001 |
| 6hr Tx | 0.249 (-4.0) | 0.00001 |
| 24hr UT | 0.638 | 0.02 |
| 24hr Tx | 0.394 (-2.5) | 0.0001 |
| 72hr UT | 1.729 | 0.0002 |
| 72hr Tx | 1.068 | 0.0001 |
| 1wk UT | 1.800 | 0.00003 |
| 1wk Tx | 2.374 | 0.000003 |

| S- Cell CEMP1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.550 | 0.000002 |
| 6hr Tx | 0.902 | 0.0000003 |
| 24hr UT | 0.307 (-3.2) | 0.000003 |
| 24hr Tx | 0.823 | 0.00003 |
| 72hr UT | 0.678 | 0.00002 |
| 72hr Tx | 0.863 | 0.0000002 |
| 1wk UT | 0.348 (-2.8) | 0.00004 |
| 1wk Tx | 0.052 (-19.0) | 0.0000008 |

| hMSC CEMP1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.762 | 0.07 |
| 6hr Tx | 0.644 | 0.03 |
| 24hr UT | 1.437 | 0.00005 |
| 24hr Tx | 0.900 | 0.004 |
| 72hr UT | 0.506 | 0.001 |
| 72hr Tx | 1.012 | 0.00006 |
| 1wk UT | 1.765 | 0.009 |
| 1wk Tx | 0.577 | 0.034 |

**Table 4:** Fold increase/decrease and P value calculations for *CEMP1* time course experiment comparing aged vs young cells. Fold value $\geq$ 2 and P value $\leq$ 0.001 considered significant. Values calibrated and standardized to 0hr UT. Note (-) value indicates decrease or downregulation.

| PDL Cell COL 1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.941 | 0.325 |
| 6hr Tx | 0.547 | 0.000007 |
| 24hr UT | 1.421 | 0.02 |
| 24hr Tx | 0.958 | 0.03 |
| 72hr UT | 1.388 | 0.00004 |
| 72hr Tx | 1.583 | 0.000002 |
| 1wk UT | 7.292 | 0.0000001 |
| 1wk Tx | 2.051 | 0.000002 |

| S+ Cell COL 1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 4.452 | 0.00004 |
| 6hr Tx | 2.567 | 0.00006 |
| 24hr UT | 5.667 | 0.0001 |
| 24hr Tx | 2.121 | 0.00003 |
| 72hr UT | 3.753 | 0.831 |
| 72hr Tx | 3.409 | 0.01 |
| 1wk UT | 18.233 | 0.0000002 |
| 1wk Tx | 15.829 | 0.000002 |

| TCN COL 1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.058 | 0.0003 |
| 6hr Tx | 1.147 | 0.0002 |
| 24hr UT | 0.937 | 0.0007 |
| 24hr Tx | 1.297 | 0.372 |
| 72hr UT | 1.123 | 0.001 |
| 72hr Tx | 4.266 | 0.00001 |
| 1wk UT | 0.278 (-3.5) | 0.00001 |
| 1wk Tx | 5.278 | 0.0001 |

| S- Cell COL 1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 0.512 | 0.000007 |
| 6hr Tx | 0.638 | 0.0000001 |
| 24hr UT | 0.397 (-2.5) | 0.00001 |
| 24hr Tx | 0.362 (-2.7) | 0.00002 |
| 72hr UT | 0.289 (-3.4) | 0.001 |
| 72hr Tx | 0.308 (-3.2) | 0.0003 |
| 1wk UT | 0.462 (-2.1) | 0.00002 |
| 1wk Tx | 4.183 | 0.000001 |

**Table 5:** Fold increase/decrease and P value calculations for *Collagen 1* time course experiment comparing aged vs young cells. Fold value $\geq$ 2 and P value $\leq$ 0.001 considered significant. Values calibrated and standardized to 0hr UT. Note (-) value indicates decrease or downregulation.

| hMSC COL 1 | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.476 | 0.000004 |
| 6hr Tx | 1.017 | 0.000001 |
| 24hr UT | 2.853 | 0.00002 |
| 24hr Tx | 1.887 | 0.0000001 |
| 72hr UT | 2.775 | 0.0003 |
| 72hr Tx | 5.563 | 0.00007 |
| 1wk UT | 10.759 | 0.00003 |
| 1wk Tx | 45.286 | 0.000003 |

| PDL Cell OPN | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.379 | 0.0003 |
| 6hr Tx | 1.366 | 0.00006 |
| 24hr UT | 0.516 | 0.0004 |
| 24hr Tx | 1.794 | 0.00004 |
| 72hr UT | 1.310 | 0.0003 |
| 72hr Tx | 2.135 | 0.0001 |
| 1wk UT | 1.713 | 0.0003 |
| 1wk Tx | 3.052 | 0.00009 |

| S+ Cell OPN | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.052 | 0.00005 |
| 6hr Tx | 1.290 | 0.0006 |
| 24hr UT | 0.785 | 0.00007 |
| 24hr Tx | 2.915 | 0.0008 |
| 72hr UT | 1.934 | 0.003 |
| 72hr Tx | 16.344 | 0.0001 |
| 1wk UT | 62.552 | 0.00001 |
| 1wk Tx | 55.889 | 0.0000005 |

| TCN OPN | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.430 | 0.00003 |
| 6hr Tx | 1.394 | 0.00003 |
| 24hr UT | 0.883 | 0.00003 |
| 24hr Tx | 1.172 | 0.00002 |
| 72hr UT | 1.560 | 0.00004 |
| 72hr Tx | 0.255 (-3.9) | 0.000005 |
| 1wk UT | 1.706 | 0.002 |
| 1wk Tx | 0.934 | 0.00002 |

| S- Cell OPN | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 3.857 | 0.059 |
| 6hr Tx | 3.440 | 0.324 |
| 24hr UT | 3.580 | 0.240 |
| 24hr Tx | 2.927 | 0.034 |
| 72hr UT | 4.046 | 0.003 |
| 72hr Tx | 4.032 | 0.004 |
| 1wk UT | 1.627 | 0.0009 |
| 1wk Tx | 0.146 (-6.8) | 0.00003 |

**Table 6:** Fold increase/decrease and P value calculations for *OPN* time course experiment comparing aged vs young cells. Fold value $\geq 2$ and P value $\leq 0.001$ considered significant. Values calibrated and standardized to 0hr UT. Note (-) value indicates decrease or downregulation.

| hMSC OPN | Fold increase/decrease | P value |
|---|---|---|
| 0hr UT | 1 | |
| 6hr UT | 1.143 | 0.005 |
| 6hr Tx | 0.863 | 0.891 |
| 24hr UT | 2.028 | 0.000001 |
| 24hr Tx | 0.425 (-2.3) | 0.00005 |
| 72hr UT | 0.429 (-2.3) | 0.0001 |
| 72hr Tx | 0.502 | 0.345 |
| 1wk UT | 0.157 (-6.3) | 0.00002 |
| 1wk Tx | 0.120 (-8.2) | 0.00001 |

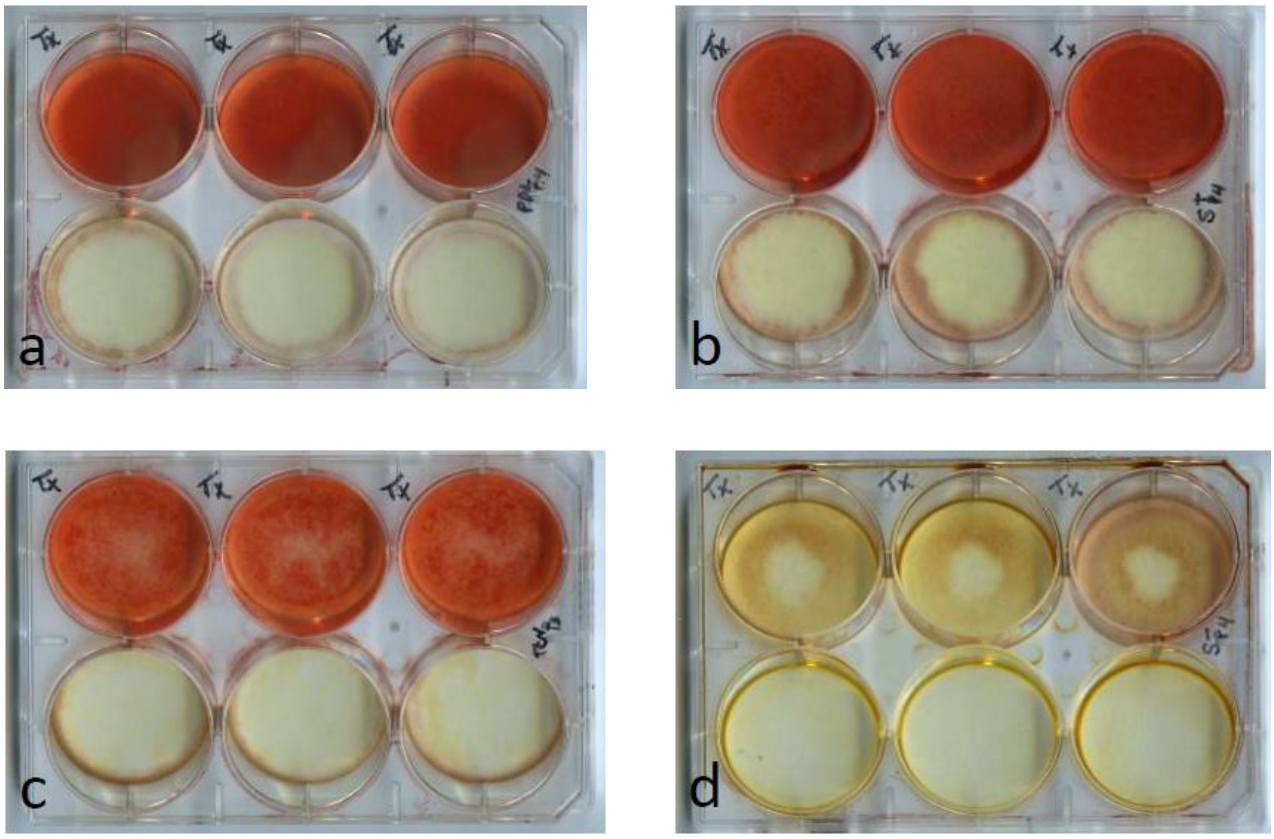



**Fig. 17:** Alizarin red S staining images of four 6-well plates seeded with young (P 3-4) parental PDL (a), STRO-1/CEMP1 positive (b), TCN (c), and STRO-1 negative (d) cells in triplicate. Top row of each plate represents osteoinduced (Tx) cells showing Alizarin red S staining of the PDL, STRO-1/CEMP1 positive and TCN nodules while bottom row of each plate represents cells in control media with no nodule formation. Note little to no staining of STRO-1 negative plate.

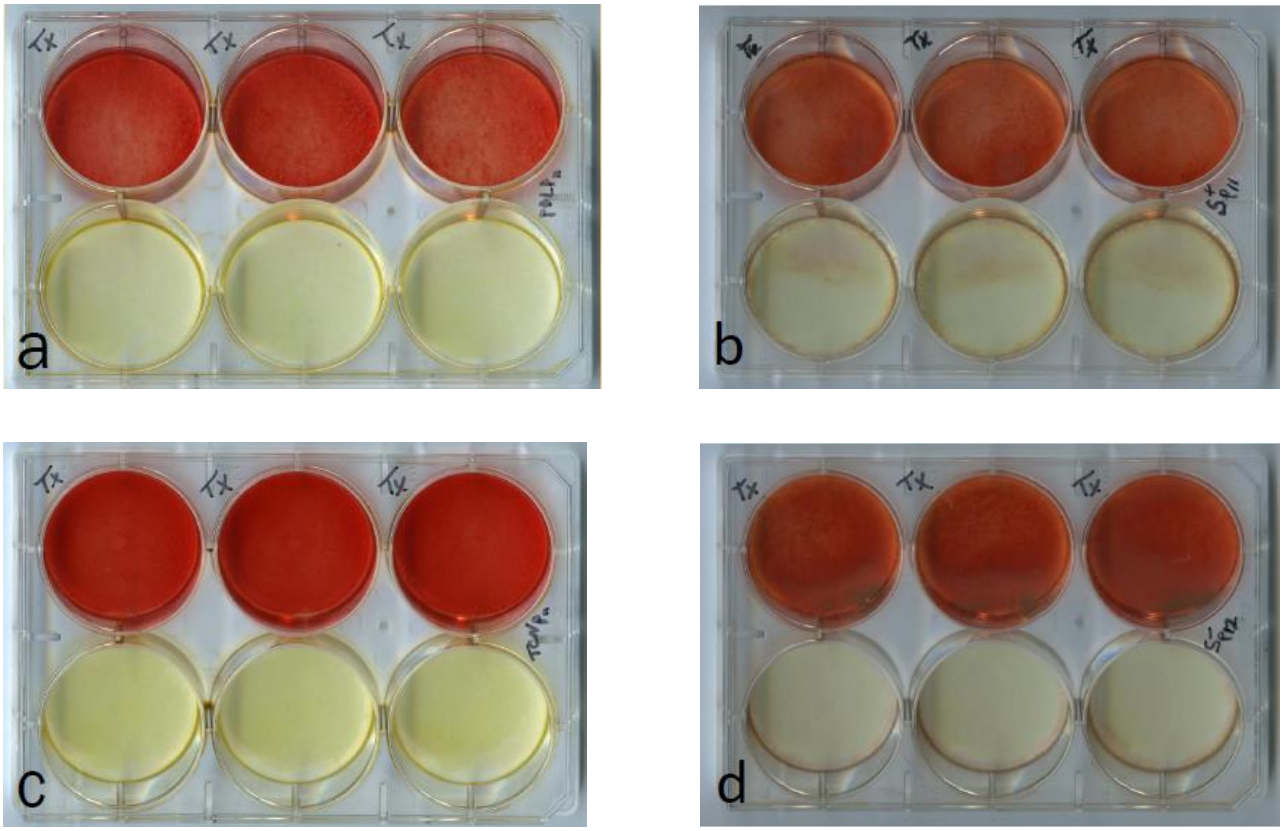



**Fig. 18:** Alizarin red S staining images of four 6-well plates seeded with aged (P 11-13) parental PDL (a), STRO-1/CEMP1 positive (b), TCN (c), and STRO-1 negative (d) cells in triplicate. Top row of each plate represents osteoinduced (Tx) cells showing Alizarin red S staining of the PDL, STRO-1/CEMP1 positive TCN, and STRO-1 negative nodules while bottom row of each plate represents cells in control media with no nodule formation. Note significantly more staining of the aged STRO-1 negative plate compared to young STRO-1 plate in figure 17.

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