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Los Angeles

The role of polycomb group ring finger 1 in dental pulp stem cell differentiation

A thesis submitted in partial satisfaction

of the requirements for the degree of Master of Science

in Oral Biology

By

Kareena Kevork

2015

ABSTRACT OF THE THESIS

The role of polycomb group ring finger 1 in dental pulp stem cell differentiation

By

Kareena Kevork

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Cun Yu Wang, Chair

DPSCs, also known as, dental pulp stem cells, were the first human dental mesenchymal stem cells (MSCs) to be identified from pulp tissues. DPSCs and their classification as mesenchymal stem cells is an attractive target for clinical applications in dentistry. DPSCs have been shown to differentiate into odontoblasts/osteoblasts, chondrocytes, and adipocytes. While studying a rare genetic disorder, Fan et al was able to uncover *BCOR*, a complex in which mutation could have intrinsic effect on the function of MSCs from the root apical papilla. One of the members of the BCOR complex is polycomb group ring finger 1 (PCGF1). PCGF1 is also a member of the polycomb group repressive complex 1 (PRC1). Due to its membership in

repressive complexes, BCOR and PRC1, and its role in development, in this study we decided to investigate the role of PCGF1 in DPSC odontogenic differentiation. In order to evaluate the role of PCGF1 in DPSCs, we used small interfering RNA to silence the PCGF1 gene and observe the changes in our DPSC population. We found that after PCGF1 knockdown and treatment with odontogenic/osteogenic inducing media, mineralized nodule formation and odontogenic potential decreased, suggesting that PCGF1 plays a role in the odontogenic lineage commitment of DPSCs. We also found that mechanistically, PCGF1 blocks inhibition of developmental genes MSX1/MSX2 and DLX2/DLX5 in order to facilitate the odontogenic lineage commitment of DPSCs. Taken together, this study might shed light on the potential therapeutic implications of exploiting this pathway in DPSCs.

The thesis of Kareena Kevork is approved.

Shen Hu

Reuben Kim

Cun Yu Wang, Committee Chair

University of California, Los Angeles

2015

This thesis is dedicated to

my parents, sister, and brother for their unconditional love and support in all of my endeavors.

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INTRODUCTION

Stem cells are immature and unspecialized cells with the ability to renew and divide indefinitely [1]. They are able to differentiate into multiple cell lineages and have properties of "self-renewal". In the last several years, stem cell biology has become a popular field because of the important role they play in the repair of organs and tissues. Stem cells that can be utilized for tissue repair and regeneration are categorized into three main types: i) embryonic stem cells, derived from embryos, ii) adult stem cells, derived from adult tissue, and iii) induced pluripotent stem (iPS) cells that have been produced artificially through genetic manipulation of somatic cells [1]. ES cells and iPS cells are termed pluripotent because of their ability to differentiate into almost all tissues in the human body. Pluripotency is defined as the ability of these cells to produce tissues from all three germ layers including the ectoderm, mesoderm, and endoderm, when transplanted into immune-deficient mice [2]. In terms of regenerative therapy, ES and iPS cells pose multiple complications. Firstly, embryonic stem cells and induced pluripotent stem cells form tumors when they are injected into mice. Secondly, there exists the moral dilemma of harvesting these tissues. Therefore, adult stem cells are the most promising due to their multipotent characteristics and availability in the body.

Adult stem cells are also referred to as somatic stem cells and postnatal stem cells. Postnatal stem cells reside in numerous mesenchymal tissues and are therefore sometimes referred to as mesenchymal stem cells (MSCs) [1].

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells are spindle-shaped cells with the potential for clonogenic proliferation. They were initially reported as fibroblast-like cells that could be isolated from bone marrow via their adherence to plastic in culture and subsequently confirmed as a population

(the colony-forming unit-fibroblast, CFU-F) of bone marrow derived non-hematopoietic cells. MSCs can differentiate into all mesodermal lineages such as, mesodermal, ectodermal and endodermal cell lineages.[3]

The international society for Cellular Therapy (ISCT) proposed the minimal characterization criteria for human MSCs as: propensity for adherence to plastic when maintained under standard culture conditions, the ability to differentiate into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions, and $\geq$ 95% of the MSC population must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II surface molecules. [3,4]. Since the discovery and characterization of bone marrow MSCs, MSC-like populations from other tissues have been characterized based on this criteria. Besides bone marrow, MSCs can be harvested from skeletal muscle, umbilical cord blood, synovia, the liver, adipose tissue, the lungs, amniotic fluid, tendons, placenta, skin, breast milk, and dental tissues. Eight unique populations of dental tissue derived MSCs have been isolated and characterized.

Dental Pulp Stem Cells (DPSCs)

Dental pulp stem cells (DPSCs) were the first human dental MSCs to be identified from pulp tissues. Other dental MSC-like populations include, stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), alveolar bone-derived MSCs (ABMSCs), stem cells from apical papilla (SCAP, tooth germ progenitor cells (TGPCs) and gingival MSCs (GMSCs) [1,3](figure 17).

DPSCs were found to express several surface markers, such as CD73, CD90, CD105, CD 271[5], STRO-1[6], CD140α, CD146[6,7], CD51[7] and CD44[3,8]. These cells have a fast population doubling time, they possess immunosuppressive properties, and they are prone to forming a

dentin-pulp-like complex. DPSCs have odontogenic potential and can develop into adipocytes and neural cells in vitro. Furthermore, DPSCs have been shown to differentiate into osteoblasts, chondrocytes, myocytes, cardio myocytes, active neurons, melanocytes, and hepatocyte like cells[3]. DPSCs participate in the regeneration of non-orofacial tissues as well, such as, hair follicle-, hepatocyte-, neuron-, islet-, myocyte- and cardiomyocyte –like cells[1].

Polycomb Group Proteins

Polycomb Group (PcG) proteins form repressive complexes that mediate epigenetic modifications of histones[9]. PcG proteins are the link between post translational modifications of histones and transcriptional regulation. These proteins were identified in *Drosophila melanogaster* almost 40 years ago as repressors of homeotic (hox) genes during early embryonic development [10]. Most PcG proteins are part of transcriptional-repressive complexes identified as PRC1 and PRC2. In mammals, PRC2 consists of three core PcG compontents: enhancer of zeste 2 (EZH2 or its homolog EZH1), embryonic ectoderm development (EED), and suppressor of zeste 12 (SUZ12). EZH2 and its homolog, EZH1 can catalyze mono-, di- and trimethylation of H3K27 [10]. Trimethyled H3K27 becomes a docking site for the chromobox-domain (CBX) protein subunits of PRC1. This mechanism of action explains how PRC1 and PRC2 are recruited to target genes. The PRC1 complex is composed of a core of CBX proteins along with one member of the PCGF family (PCGF1-PCGF6), RING1 family (RING1a and RING1b) and HPH family (HPH1-HPH3) (figure 18)[10]. The PRC1 complex catalyzes the monoubiquitination of H2A on K119 (H2AK119ub1) through the E3 ligases RING1a and RING1b.

Canonical and non-canonical PRC signaling

The canonical PRC1 complex (c-PRC1) is composed of a Cbx, Pcgf, Ring, and Phc protein [11](8). The Cbx proteins are defined by their ability to bind H3K27me3. In the PRC1

complex the Cbx proteins are responsible for recruiting the PRC1 complex to PRC2 target genes. The Ring1a/b proteins in the canonical PRC1 complex, together with Pcgf proteins then catalyze the monoubiquitination of histone H2A at lysine 119 (H2AK119UB1). This monoubiquitniation is thought to cause chromatin compaction and result in repression of lineage-specific genes.

In recent years, noncanonical PRC1 (nc-PRC1) complexes have been identified. The nc-PRC1 does not require PRC2 activity to mediate the monoubiquitination of histone H2A. The nc-PRC1 complexes are defined by the presence of Rybp or its close homolog, Yaf2 [12] and the lack of a Cbx component [11]. RYBP (Ring 1 and YY1-binding protein) is a newly identified member of the PcG proteins and functions as a transcriptional repressor in mammalian cells by interacting with and repressing the transcriptional activity of several sequence-specific transcription factors, such as YY1 (Yin Yang 1), GABPB1 (GA-binding protein subunit beta-1) and E2F6 (E2F transcription factor 6) [13]. The lack of a Cbx component in the complex accounts for the fact that the PRC1 is recruited to chromatin independently of PRC2 mediated H3K27me3. Studies have found that there are differing yet overlapping functions of the two systems and it still remains unclear why two independent systems exist.

Some members of the PRC complexes are also members of the BCOR complex, which is associated with the following proteins: RING1a or RING1b, PCGF4 (BMI1), PCGF1 (NSPC1), KDM2b (FBXL10), RYBP or YAF2, CK2a, Skp1, CBX5 (HP1γ), CBX8 and the BCL co-repressor, BcoR, which lends its name to the complex [10]. It has been shown that PCGF1 and PCGF4 enhance the catalytic activity of RING1b *in vitro* and *in vivo* [10]. Although some of the characteristics of the BCOR complex have been uncovered, it is not yet clear whether there is a functional interaction with the canonical PRC1 complexes.

OFCD and BCOR

Genetic studies have found that mutations in *BCOR* are responsible for a rare genetic disorder named OFCD[14]. OFCD, or oculofaciocardiodental syndrome, is characterized by teeth with extremely long roots, as well as, craniofacial, ocular and cardiac abnormalities. Since all reported OFCD individuals have been female, it has been suggested that this syndrome is an X-linked dominant trait and it might be embryonic lethal in males[15]. Ocular anomalies include congenital cataracts and microphthalmia. Facial deformities include long and narrow face, high nasal bridge and broad nasal tip with separation of anterior cartilage, and cleft palate. The structural cardiac abnormalities frequently include septal defects and mitral valve prolapse.

The most consistent finding with respect to dental defects in OFCD patients is enlargement of the roots of the canine teeth. The roots consist mainly of dentin, a bone-like mineralized tissue that anchors the tooth in alveolar bone[14]. After tooth eruption, root formation is well-synchronized with alveolar bone growth and stops growing at certain ages. However, in OFCD patients, the roots of the mandibular incisors and canines grow continuously until they reach the lower border of the mandible. The roots of maxillary incisors and canines can extend to the cortical plate of the orbit[14].

OFCD syndrome has frequently been attributed to mutations in *BCOR* [14,15]. Usually, truncation and frameshift mutations in *BCOR* result in premature termination of the protein and deletion of the carboxy-terminal domain. BCOR is identified as the co-repressor of the transcriptional repressor of BCL-6. BCOR has been found to interact with histone deacetylase (HDAC), demethylase and H2A ubiquitin ligase, suggesting that BCOR might mediate repression through chromatin modification [10,14].

As stated earlier, patients with OFCD have been characterized with having enlarged and continuously growing roots as well as a mutation in BCOR. Many studies [14,16] have shown the

expression of BCOR family members such as RING1A/B and PCGF1 in dental mesenchyme. Studies have shown that BCOR member, *RING1A/B* may be responsible for the ability of mesenchymal cells to develop in odontoblasts, however the pathway still remains unclear. This is also true for the other members of the BCOR complex. Here, we have decided to take a closer look at PCGF1 and its regulation of odontogenic differentiation of dental pulp stem cells.

Polycomb Group Ring Finger 1 (PCGF1)

As previously mentioned, Polycomb Group Ring Finger 1 (PCGF1) is a member of the polycomb group proteins. Loss of polycomb protein function in Drosophila leads to abnormal body plan specification[17]. In invertebrates polycomb orthologs are essential for normal embryonic development.

A member of *Drosophila* PRC1, PSC helps to create a compacted chromatin state that is inaccessible to chromatin remodeling enzymes[9]. PSC is also involved in a complex that includes the N-terminal RING finger heterodimer of PSC and dRING1 which act as an ubiquitin ligase (E3) to modify histone H2A. Yet another complex PSC is involved in is the dRAF complex. The dRAF complex includes dKDM2 whose demethylation of dimethylated histone 3 Lys 36 (H3K35me2) is coupled to H2A E3 activity. In humans there are six different PSC orthologs: PCGF1/NSPC1, PCGF2/MEL18, PCGF3, PCGF4/BMI1, PCGF5, and PCGF6/MBLR[9]. The variety of orthologs affords the functional diversification of the complexes they assemble into. PCGF2 and PCGF4 are part of complexes most similar to *Drosophila* PRC1, PCGF1 assembles into a dRAF-like complex that includes KDM2B, an H3K36me2 demethylase and BCOR.

Protein-Protein Interactions

The protein-protein interactions within PcG complexes indicates how larger structural assemblies can emerge. A ubiquitin-like fold domain involved in protein interactions called

RAWUL (Ring finger And WD40 associated Ubiquitin-Like) appears to be key in determining the identity of the larger assembly[9]. The larger assemblies are defined by the identity of the PCGF homolog and its binding partner, RING1B RAWUL. It has been suggested that the RING1B RAWUL ability to form larger complexes stems from its ability to utilize mutually exclusive binding surfaces to associate with short peptides within different proteins that can be diverse in sequence[9]. The RAWUL domains of the PCGF proteins appear to play a similar role given that the RING finger domain of the PCGF homologs plays the role of heterodimerizing with RING1B to facilitate the H2A E3 activity.

The direct binding partner of PCGF1 is BCOR. The binding of PCGF1 to BCOR will lead to assembly of the dRAF-like complex, while PCGF2 and PCGF4 binding to the PcG protein called Polyhomeotic (PHC1, 2, 3) results in formation of the mammalian PRC1 equivalent, or canonical PRC1[9,17]. BCOR 1451-1755 is the previously identified region required for binding PCGF1. Further analysis revealed that BCOR 1634-1748 is both necessary and sufficient for interaction with the PCGF1 RAWUL. This protein interaction domain has been referred to as PUFD or PCGF Ub-like Fold Discriminator[9].

PCGF1 Protein Structure

The PCGF1 RAWUL and BCOR PUFD form an intermolecular β-sheet similar to that observed between RING1B and either RYBP or CBX7[9]. While both RYBP and CBX7 form their β-sheets from ~20 consecutive residues, the BCOR PUFD is unique in that its N-and C-termini come together to form the β-sheet. A key residue at the PCGF1 β-sheet surface is Val 206, which packs against BCOR Phe 1639. In the RING1B RAWUL, a Tyr residue is at a position in the RAWUL equivalent to Val 206. This position may play a key discriminatory role in selecting the binding partner of the RAWUL. In PCGF1 Tyr 191 is required to create a loop

binding surface, which is considered a second binding surface present on PCGF1. A third binding surface is present in PCGF1 and is sometimes refered to as the Leu cage because it completely encases BCOR Leu 1706[9].

Due to the uncertainty of the role of PCGF1 in the PRC1 and BCOR complexes, in this study, we decided to further examine the role of PCGF1 in dental pulp stem cells. We used siRNA to knockdown the gene and observe the resulting phenotype.

In this present study, we examined how PCGF1 regulates DPSC differentiation and proliferation by focusing on the expression of developmental and odontogenic gene markers. The aim of this study is to uncover the mechanism of PCGF1 regulation of DPSC differentiation. We found that mechanistically, PCGF1 up-regulates the expression of developmental markers such as MSX1/MSX2 and DLX2/DLX5. In addition, this study might shed light on the potential therapeutic implications of exploiting this pathway in DPSCs.

MATERIALS AND METHODS

Cell Culture:

Tissues were obtained under approved guidelines set by the University of California Los Angeles Institutional Review Board with informed patient consent (IRB#13-000241-CR-00001). Dental Pulp cells were extracted from the third molar of a 14 year old male patient undergoing routine orthodontic treatment. Extracted teeth were stored in alpha MEM with antibiotics immediately post extraction. The pulp was removed using a dental explorer. Tissue was enzymatically digested with collagenase and dispase. Cells were grown in a humidified 5% CO_2 incubator at 37ºC in alpha modified Eagle's medium supplemented with 10% fetal bovine serum, 100U/mL non-essential amino acids, and 100U/mL penicillin-streptomycin (all reagents from Life Technologies, Carlsbad, CA). Media was changed every 2-3 days and cells were passaged at 80-90% confluency. Dental Pulp Cells used in this study were from passages 4-6.

siRNA Transfection:

Knockdown of specific genes were accomplished by RNA interference using commercially available siRNA duplexes for PCGF1 (GE Healthcare, Piscataway, NJ). At least 4 independent siRNAs were screened for knockdown efficiency against the target, and the best two siRNA duplexes were selected. After the plate reached 80-90% confluence, the cells were detached using trypsin and replated in a 6-well tissue culture dish with antibiotic free medium. 24 hours after plating or 60% confluency, cells were transfected with 10uM PCGF1 siRNA (GE Healthcare, Piscataway, NJ) using Lipofectamine RNAi Max reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Scramble siRNA B (Santa Cruz Biotechnology, Dallas, TX) was used as a negative control. 48 hours after transfection was considered Day 0. The siRNA target sequences were as follows:

PCGF1 si2: CAGAGUGUCUUCAUACUUU 5nmol

PCGF1 si4: GGAACCAGCUCCAGUCAGU 5nmol

Odonogenic/Osteogenic Induction:

48 hours after siRNA transfection (Day 0), cells were treated with odontogenic induction medium (OIM) composed of 90% alpha MEM 10% FBS 50 microg/mL ascorbic acid, 5mM beta-glycerphosphate and 100nM dexamethasone. OIM was replaced every 2-3 days. Time points used to evaluate osteogenic induction were 4hr, 12hr, Day3, Day7, Day 10, and Day 14.

ALP staining and quantification:

Seven days after odonotogenic induction, cells were fixed with 4% paraformaldehyde and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.1M Tris buffer (pH 9.3). ALP activity assay was performed using an ALP kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturers instructions and normalized based on protein concentration.

Alizarin red staining and quantification:

For detecting mineralization, we induced DPSCs with odontogenic medium for 2 weeks, fixed the cells with 4% paraformaldehyde and stained them with 2% Alizarin Red solution (Sigma-Aldrich). To quantify the calcium mineral deposition, Alizarin Red was destained with 10% cetylprydiniumcholoride in 10mM sodium phosphate for 30 minutes at room temperature. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader using a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentrations prepared from a duplicate plate.

Western Blot:

Cells (7 x 10^5) were plated in 6-cm tissue culture dishes the day before siRNA transfection. Whole cell extracts were collected 48 hours after transfection. Cells were lysed in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1: 100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, 50 mM sodium fluoride). Lysates were separated on a 10% SDS polyacrylamide gel and transferred to membranes by a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 5% milk for one hour at room temperature and then incubated with primary antibodies at 4ºC overnight. After rinsing, the immunocomplexes were incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI) and membranes were visualized with SuperSignal Chemiluminiscent substrate (Pierce, Rockford, IL). Primary antibodies were purchased from the following commercial sources: anti-PCGF1 (1:1000; ab84108; Abcam) and anti-α-tubulin (1:10000; 75168; Sigma-Aldrich).

Real time-RT PCR:

Total RNA was isolated from DPSCs using Trizol reagent (Life Technologies, Carlsbad, CA, USA). Two microgram aliquots of RNAs were synthesized using random hexamers and reverse transcriptase according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). The real-time PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and the Icycler iQ Multi-color Real-time PCR Detection System. The following primers were used: GAPDH forward 5'-TCATTGACCTCAACTACATG- 3'; reverse 5'-GCTCCTGGAAGATGGTGAT-3', DLX2 forward 5'-ACACCTCCTACGCTCCCTATG- 3'; reverse 5'-TCACTATCCGAATTTCAGGCTCA-3', DLX5 were: forward 5'-GCTCTCAACCCCTACCAGTAT-3'; reverse, 5'-CTTTGGTTTGCCATTCACCATTC-3', OCN forward 5'-AGCAAAGGTGCAGCCTTTGT-3';

reverse 5'-GCGCCTGGGTCTCTTCACT-3', ON forward 5'-ACCCCTGCCTGAAGGTAAAAT-3'; reverse 5'-GGCTTGCACTTGACCAAATTC-3', MSX1 forward 5'-CCAGAAGCAGTACCTGTCCA-3'; reverse 5'-GCGGTTCTGGAACCATATCT-3', MSX2 forward 5'-ATGGCTTCTCCGTCCAAAGG- 3'; reverse 5'-CGGCTTCTTGTCGGACATGA-3', COL1A1 forward 5'-CTGGAATGAAGGGACACAGA-3'; reverse 5'-CTCACCCTTAGGACCAGCAG-3'.

Fluorescent Activated Cell Sorting (FACS):

DPSCs were harvested using trypsin in 0.25% EDTA. After neutralization, single-cell suspensions were washed with PBS, containing 2% FBS and 0.01% NaN_3 (FACS buffer). 1×10^5 cells were incubated with direct conjugated antibodies for 20 minutes on ice in the dark. After washing, stained cells were sorted on a FACS Aria II cell sorter (BD Biosciences, USA). The following conjugated anti-human antibodies were used for cell sorting: Phycoerythrin (PE)-CD271 (Miltenyi Biotec, Auburn, CA) PE- CD146 (Biolegend Inc, San Diego, CA), and PE-CD51 (Biolegend Inc, San Diego, CA), and allophycocyanin (APC)-CD140α (BD Biosciences, USA). PE-IgG was used as a negative control. FACs-sorted DPSCs were routinely passaged or plated into 12-well plates (Corning, New York, USA) to determine their differentiation potential.

MTT Cell Proliferation Assay:

MTT assay was used to measure cell viability and the cell proliferation rate after PCGF1 knockdown. 2000-4000 cells per well were plated in a 96 well plate. The cells were treated with 10uL of 12mM stock MTT solution. The plate was incubated at 37^0C for 2-4 hours until the purple formazan that forms a precipitate was visible. After aspirating the media the plate was shook for 10 minutes in the dark. DMSO was used as a control. The data was quantified using a

spectrophotometer at 520-600nm. The assay was terminated days 1, 3, 5, 7 after PCGF1 knockdown.

Statistical Analysis:

Unpaired Student's *t* test was used for statistical analysis. A p value of <0.05 was considered to be statistically significant. Data were expressed as the mean ± SD from at least three independent experiments.

RESULTS

FASC revealed MSC marker expression in DPSCs

Due to the heterogeneous nature of DPSCs we wanted to confirm that our cell population had MSC characteristics. We performed FACS analysis using four MSC positive markers and two markers that MSCs are negative for. The MSC positive markers were as follows: CD51+, CD140α+, CD146+, and CD271+ (figure 1A-D). The MSC negative markers were CD45- and CD34- (figure 1E and 1F). Cells positive for the stain are depicted in red and the negative control is depicted as the black line on the graph. Almost 100% of DPSCs were positive for CD51+, CD146, and CD140α+.

PCGF1 knockdown by siRNA

In order to investigate the role of PCGF1 in DPSC differentiation, we first needed to confirm the knockdown of our gene of interest to see if this was a viable model to use. At least four independent siRNAs were screened for knockdown efficiency against our target and the best two siRNA duplexes were selected. Knockdown was confirmed by western blot (figure 2) and real time-RT PCR (figure 2). siRNA knockdown showed decrease in PCGF1 mRNA levels as well as protein levels in DPSCs (figure 3). PCGF1 siRNA 1 showed the least amount of knockdown in mRNA expression as well as protein levels. MTT assays were performed to determine whether the siRNAs had adverse effects on cell growth and proliferation (figure 4). After analyzing the data it was decided that PCGF1 siRNA 2 and siRNA 4 would be best suited for the following experiments.

PCGF1 knockdown decreases ALP activity in DPSCs

Alkaline phosphatase (ALP) activity is an early marker for odontogenic differentiation of DPSCs[18]. To determine whether PCGF1 affected DPSC differentiation, we first examined whether PCGF1 knockdown affected ALP activities in DPSCs**.** 48 hours after PCGF1 knockdown we replaced the media with odonogenic/osteogenic inducing media. Seven days after treatment cells were fixed and stained. ALP activity showed a marked decrease in both PCGF1 siRNA groups compared to scramble control after seven days of odontogenic induction (figure 5).

ALP quantification further confirmed these findings. ALP quantification showed a significant ($*p < 0.05$; $**p < 0.001$) threefold decrease in the PCGF1 siRNA 4 knockdown group compared to scramble control (figure 6). PCGF1 siRNA 2 also showed a significant decrease in ALP activity. These data indicate a decrease in odontogenic potential in DPSC after PCGF1 depletion and odontogenic induction.

We continued to investigate the osteoblast formation capacity of DPSCs after 14 days of treatment with odontogenic inducing media. ALP activity confirmed that after PCGF1 knockdown there was a marked decrease in the osteoblast forming potential of DPSCs. Both experimental groups showed a significant decrease in staining after 14 days of treatment (figure 7).

The results from our ALP quantification experiments were in line with the ALP activity results. ALP quantification showed a significant decrease in PCGF1 siRNA 2 and 4 compared to scramble control indicating a decrease in the odontogenic potential of DPSCs after depletion of PCGF1 (figure 8). These data suggest that PCGF1 may play a role in osteogenic differentiation of DPSCs.

PCGF1 knockdown decreases mineralization matrix formation of DPSCs

In order to further examine the role of PCGF1 in DPSC differentiation, we performed an Alizarin Red stain to assess the mineralization matrix formation of our cells after knockdown. Alizarin Red staining showed a significant decrease in mineralization matrix formation in our PCGF1 siRNA knockdown groups compared to scramble control (figure 9). PCGF1 siRNA 2 and 4 showed a decrease in mineralized nodule formation after 14 days in culture with bone differentiation-inducing medium.

Alizarin Red quantification further confirmed these results. ARS quantification showed a significant reduction in the mineralization matrix formation of DPSCs after PCGF1 knockdown compared to scramble control (figure 10).

PCGF1 knockdown decreases expression of developmental gene markers

Due to the role that polycomb group proteins play in development, we decided to examine the expression of Msx1 and Msx2 in DPSCs after knockdown of PCGF1 and osteogenic induction. Msx1 and Msx2 are expressed in a number of vertebrate tissues including neural crest cells, bone and teeth[19]. For this reason we decided to look at the expression level of these markers in our cell population. After three and ten days of treatment with bone differentiation-inducing medium, both PCGF1 knockdown groups showed decreased mRNA expression of Msx1 and Msx2 (figure 11).

Furthermore, Dlx2 and Dlx5 have been implicated as important homeobox transcription factors for osteoblast growth and development[20]. We used real time RT PCR to determine the mRNA expression levels of these transcription factors in our PCGF1 depleted DPSCs. After four hours and twelve hours of treatment with osteogenic inducing media, both experimental

groups showed a marked decrease in the expression of Dlx2 mRNA (figure 12). After four hours and three days of treatment with osteogenic inducing media, Dlx5 mRNA expression also decreased in both experimental groups compared to scramble control (figure 13).

Effects of PCGF1 knockdown on collagen expression

To confirm whether DPSCs can modulate the expression of collagen after PCGF1 depletion and osteogenic induction, we evaluated the mRNA expression of Col1a1 via real time RT PCR. After three days of treatment with bone differentiation inducing media we found a drastic decrease in the mRNA expression levels of Col1a1 in both experimental groups compared to scramble control (figure 14).

Effects of PCGF1 knockdown on odontogenic mRNA expression

Osteonectin (ON), a well-known osteogenic/odontogenic marker implicated in mineral deposition[21], showed a decrease in mRNA expression after seven days of osteogenic/odontogenic induction (figure 15). Osteocalcin (OCN), another differentiated osteoblast marker[19], also showed a decrease in mRNA expression after treatment with osteogenic inducing media for seven and fourteen days (figure 16). Both PCGF1 siRNA 2 and 4 showed a decrease in expression of ON and OCN compared to scramble control.

DISCUSSION

Previous studies have shown the benefits of using mesenchymal stem cells in therapeutic applications. In the present study, we aim to elucidate the pathway responsible for the phenotype we observed in our PCGF1 depleted DPSCs. Furthermore, little is known about the role of PCGF1 in differentiation and development. Here, we attempt to understand how PCGF1 regulates DPSC differentiation. In our study, we found that knockdown of PCGF1 in DPSCs significantly decreased their odontogenic potential as evidenced by ALP staining. Furthermore, decreased mineralized nodule formation was observed after PCGF1 knockdown and treatment with odontogenic inducing media. These data indicate that PCGF1 may play an important role in the odontogenic lineage commitment of DPSCs. PCGF1 is a member of transcriptional repressive complexes such as, PRC1[17] and BCOR. With removal of this repressive function, genes responsible for osteogenesis may not have the opportunity to get activated resulting in a decrease in expression of odontogenic markers.

In this study we used human dental pulp tissues, DPSCs shown to express odontogenic markers and respond to induction of osteogenic/odontogenic differentiation. Compared to bone marrow mesenchymal cells (BMMSCs), DPSCs have the ability to produce more colony-forming units (CFUs)[22]. DPSCs also have a higher proliferation rate and longer survival time compared to BMMSCs[23]. As such, DPSCs offer an attractive cell source for tissue and bone regeneration. DPSCs are a heterogenous cell population in nature. In order to ensure that the population we were working with had mesenchymal stem cell properties, we checked the expression of known MSC markers by FASC (figure 1). FASC confirmed the expression of MSC markers such as CD51+, CD146+, CD140α+ and CD271+ (figure 1A-D) in our DPSC

population. We then investigated the expression of developmental and odontogenic genes to see how PCGF1 regulates odontogenesis.

MSX1 and MSX2 are expressed in a number of vertebrate tissues including the neural crest cells, bone and teeth[19,24]. They are also associated with epithelial-mesenchymal interactions and intramembranous ossification in the skull. The function of Msx2 in osteoblasts is still controversial. One study suggests that Msx2 promotes osteoblast differentiation and proliferation[25]. This is in line with our findings, which show that after PCGF1 knockdown there is a decrease in MSX2 expression (figure 11) with a subsequent decrease in ALP activity (figure 5 and 7) indicating a decrease in odontogenic potential. Therefore, we can conclude that PCGF1 is responsible for regulating MSX1/2 expression thereby promoting odontogenic differentiation. It would be interesting to see the effects of PCGF1 knockdown on the mono-ubiquitylation of H2A to determine if this is the mode of action in odontogenic differentiation in DPSCs.

The vertebrate Dlx genes encode a family of transcription factors that contain the homeobox domain[26]. The homeobox genes in Drosophila are responsible for patterning of the body. Dlx5 overexpression studies have shown that Dlx5 can induce osteoblastic differentiation in osteoblastic cell culture. Many studies have also shown that Dlx5 knock-out mice suffer craniofacial defects, suggesting that this gene plays a pivotal role in craniofacial development [25,26]. One study has shown that the expression of Dlx2 and Dlx5 increased as calvarial osteoblast cultures matured[26]. This finding is in line with our experimental results, in that, after PCGF1 knockdown we exhibited a decrease in expression of DLX2 and DLX5 (figure 12 and 13) explaining the decrease in odontogenic potential of our cultures. Additionally, we can conclude that PCGF1 regulates the expression of DLX2/5 by allowing their expression and thereby facilitating odontogenic development in DPSCs.

Type I collagen, or COL1A1, is the main structural protein of the extracellular matrix of bone, skin, and tendons. It consists of two pro-α-1 chain and one pro-α-2 chain that interweave forming a rigid triple helix[27]. COL1A1 also plays an important role in mineralization[28]. Additionally, Type I collagen protein is produced by osteoblasts and works in concert with osteocalcin to combine extracellularly to form osteoid, the organic substrate upon which mineralization occurs[29]. These findings are consistent with our observations in DPSCs after PCGF1 knockdown. We found that after knockdown of PCGF1 and treatment with osteogenic inducing media, DPSCs showed a decreased propensity for mineralization formation (figure 9). This was further confirmed by the decreased expression of type I collagen mRNA levels (figure 14).

Osteocalcin, OCN, is secreted solely by osteoblasts and it is generally used as a marker for the late stages of osteoblast differentiation[30]. Osteonectin, ON, on the other hand, is an extracellular matrix protein[31]. The mRNA expression levels of both markers involved in osteoblast differentiation are down regulated in DPSCs after PCGF1 knockdown (figure 14 and 15). This is yet another confirmation that PCGF1 plays a role in odontogenic lineage commitment in DPSCs.

Ross et al. studied the effects of Runx1 on differentiation of hematopoietic cells. However, to understand the effects on self-renewal they investigated gene-expression profiles of Pcgf1- depleted Lin^- mouse cells. They found that Pcgf1 knockdown significantly increased the expression of the posterior HoxA cluster genes *HoxA7, HoxA9*, and *HoxA10*[32], suggesting that Pcgf1 functions in suppressing self-renewal by down regulating HoxA cluster genes. Although there are many differences between this study and the one Ross et al conducted, there are similar themes. This study supports our findings in that, PCGF1 may play a role in self-renewal in

DPSCs. Even though the developmental and homeobox genes we examined (DLX5, DLX2, MSX1, MSX2) decreased in expression after PCGF1 knockdown, this could be explained by the fact that these markers are related to bone development. It would be interesting to check the expression of more HOX genes to see if they increased in expression to corroborate this theory.

Osteoblastogenesis is a complex and intricate process regulated by temporal and spatial expression of transcription factors, cytokines, growth factors, and hormones in a stage specific manner[28]. Subtle differences in any of these factors can affect the coordinated effort towards lineage commitment to lineage maturation. With our study we were able to shed some light on this sophisticated system. We were able to uncover the functional role of one of the BCOR members, PCGF1, and in this way understand more about the function of the complex in DPSCs. Furthermore, we were able to understand that PCGF1 regulates the differentiation of DPSC into odontogenic lineages by up-regulating the expression of developmental genes, MSX1/2 and DLX2/5.

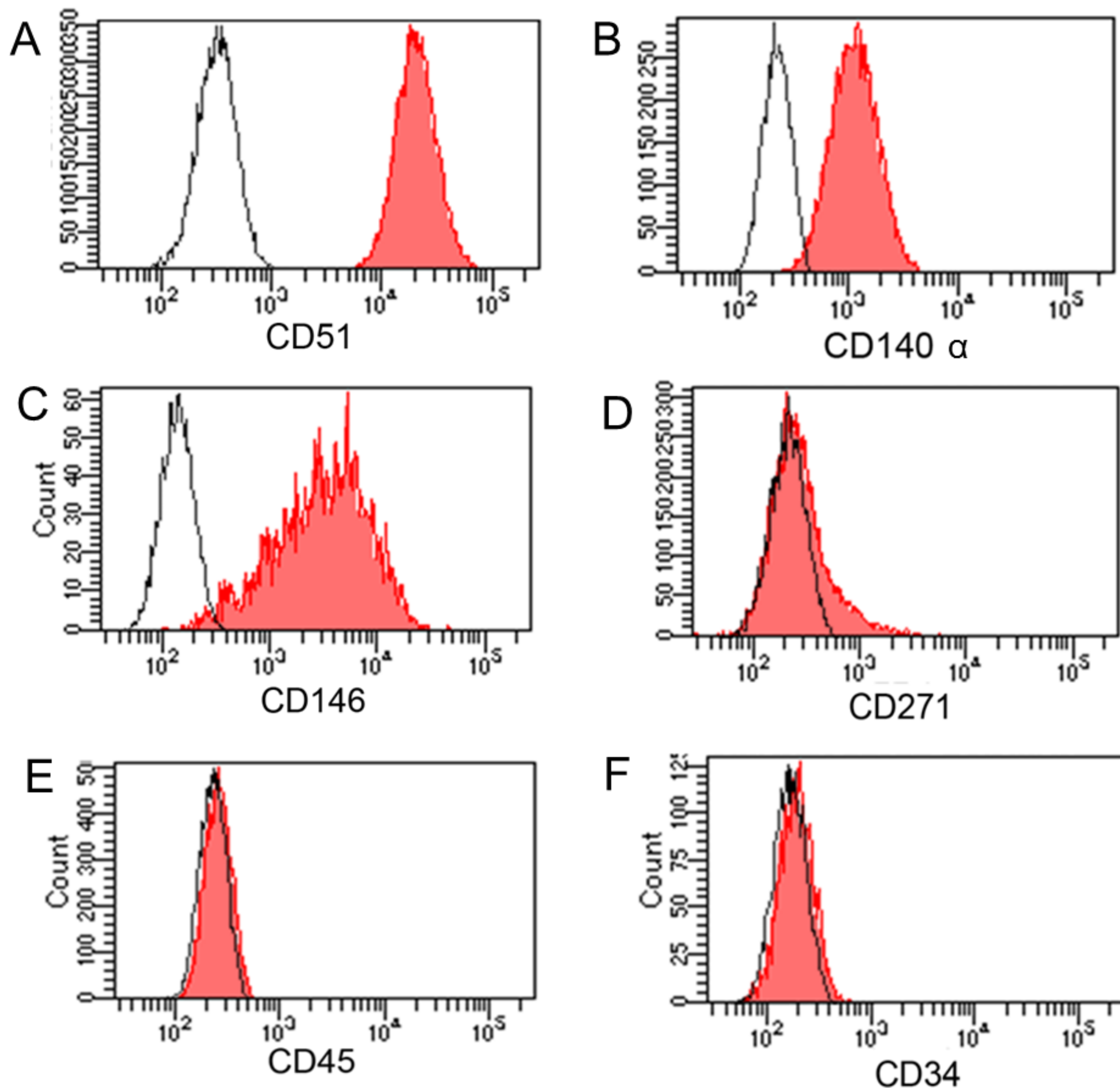


Figure 1. Flow cytometry acquisition of mesenchymal stem cell markers in DPSCs. Figures A-D are positive for the following MSC markers: CD51+, CD140α+, CD146+, and CD271+. Figures E and F are negative for the following MSC markers: CD45- and CD34-. IgE was used as a negative control.

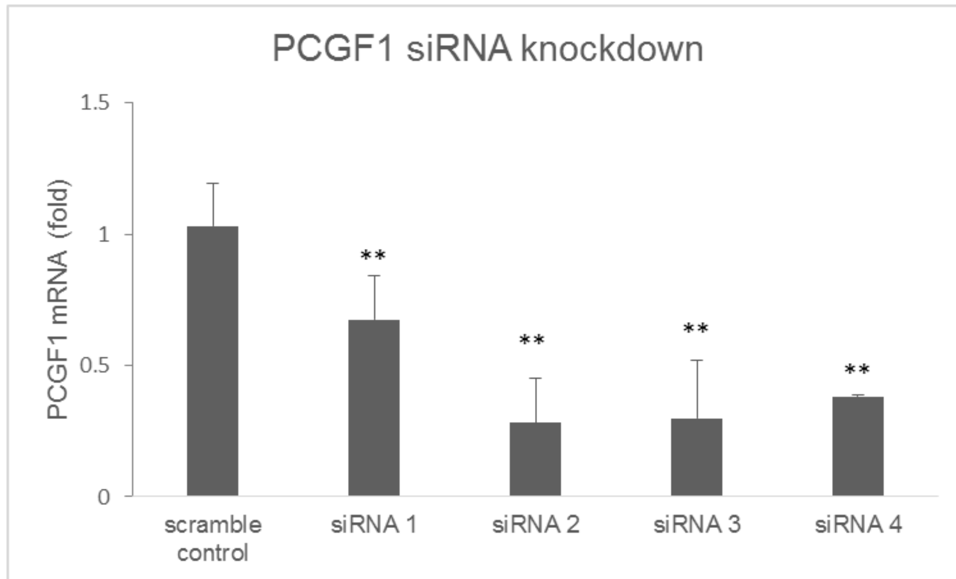


Figure 2. Confirming knockdown of PCGF1 mRNA in Dental Pulp cells using siRNA transfection. PCGF1 mRNA levels show a decrease compared to scramble control indicating that siRNA transfection is an effective method to use to observe changes in DPSCs after knockdown. Pcgf1 siRNA 1 shows the least amount of knockdown. $*p < 0.05$ $**p < 0.001$

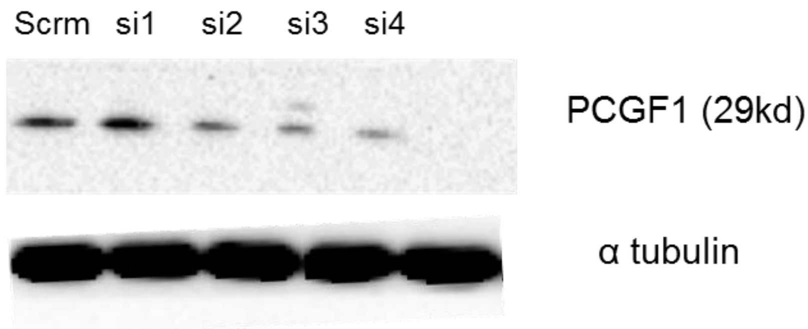


Figure 3. Confirming knockdown of PCGF1 protein in Dental Pulp cells after siRNA knockdown by Western blot. All PCGF1 siRNA show a decrease in protein expression compared to scramble control except PCGF1 siRNA 1.

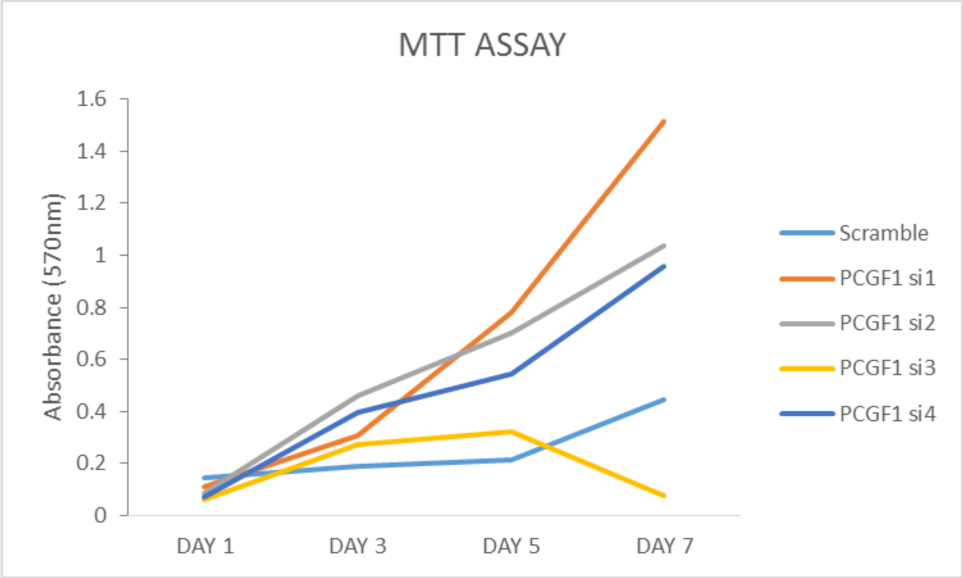


Figure 4. MTT Cell proliferation Assay was performed on PCGF1 knockdown cell. The assay was terminated at days 1, 3, 5 and 7 after PCGF1 knockdown. PCGF1 siRNA 2 and 4 showed the greatest amount of viable cells.

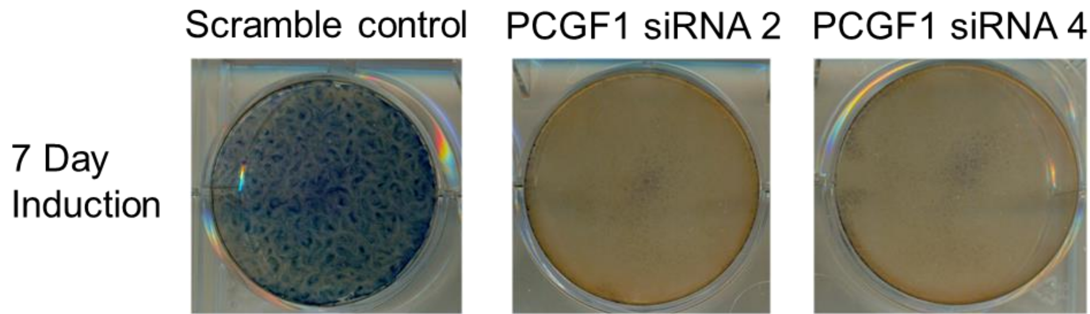


Figure 5. Alkaline Phosphatase (ALP) activity assay showed that depletion of PCGF1 in dental pulp cells inhibited ALP activity after 7 days of osteogenic/odontogenic induction compared to scramble control.

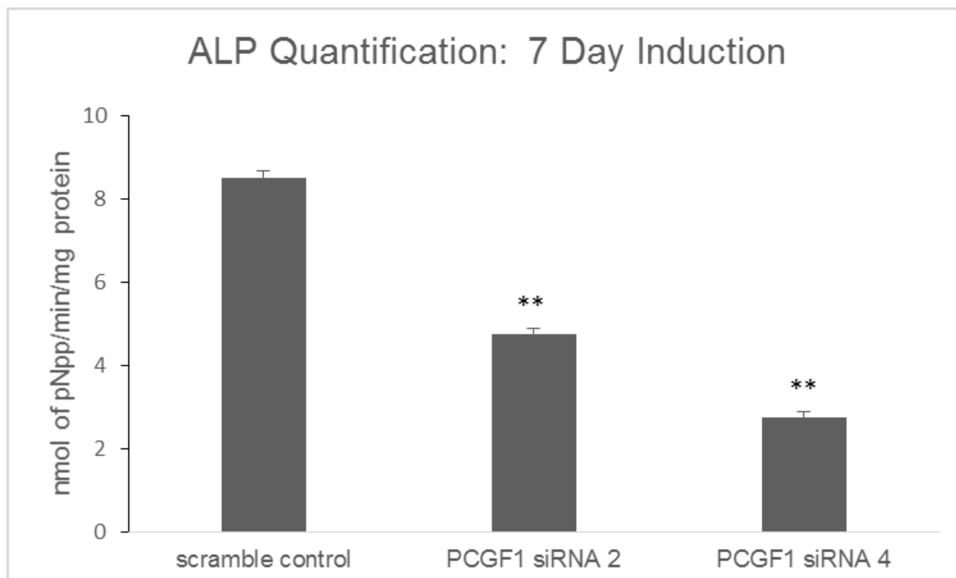


Figure 6. ALP quantitative assay further confirmed the decrease in odontogenic potential in dental pulp cells after PCGF1 siRNA transfection and induction with osteogenic/odontogenic media. PCGF1 siRNA 2 and 4 shows a significant decrease in alkaline phosphatase activity. $*p < 0.05$; $**p < 0.001$.

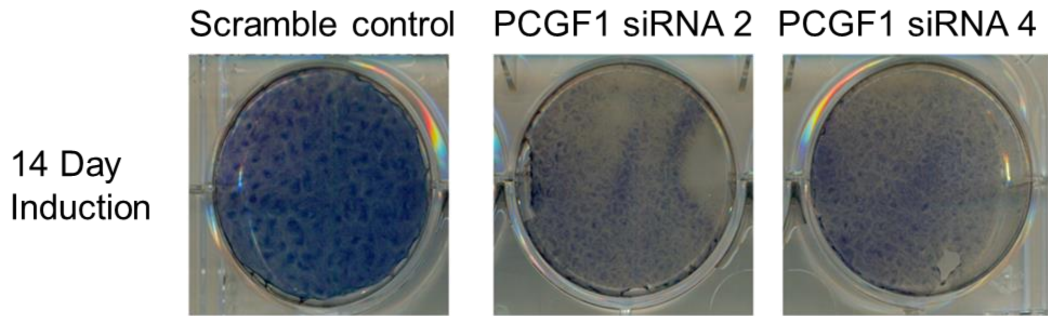


Figure 7. ALP activity assay showed that depletion of PCGF1 in dental pulp cells inhibited ALP activity after 14 days of osteogenic/odontogenic induction compared to scramble control.

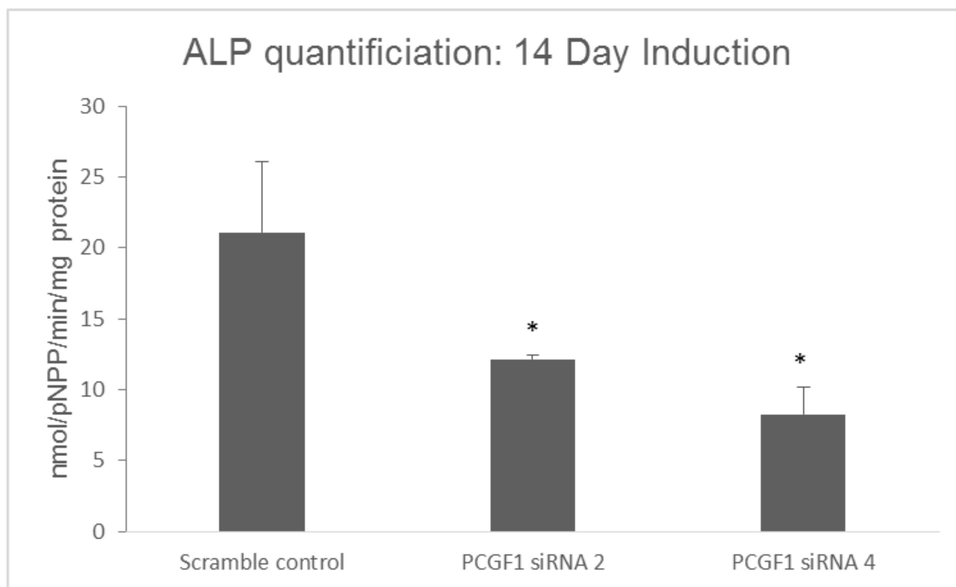


Figure 8. ALP quantification further confirmed the decrease in odontogenic potential in dental pulp cells after PCGF1 siRNA knockdown and induction with osteogenic/odontogenic media for 14 days. PCGF1 siRNA 2 and 4 show a significant decrease in alkaline phosphatase activity. *p < 0.05 **p; < 0.001.

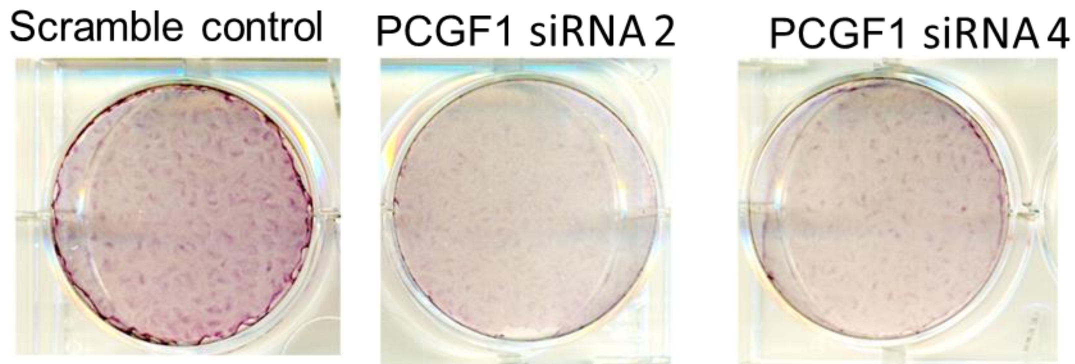


Figure 9. Alizarin Red Stain showed a decrease in mineralization nodules in PCGF1 depleted dental pulp cells 14 days after treatment with osteogenic/odontogenic induction media compared to scramble control.

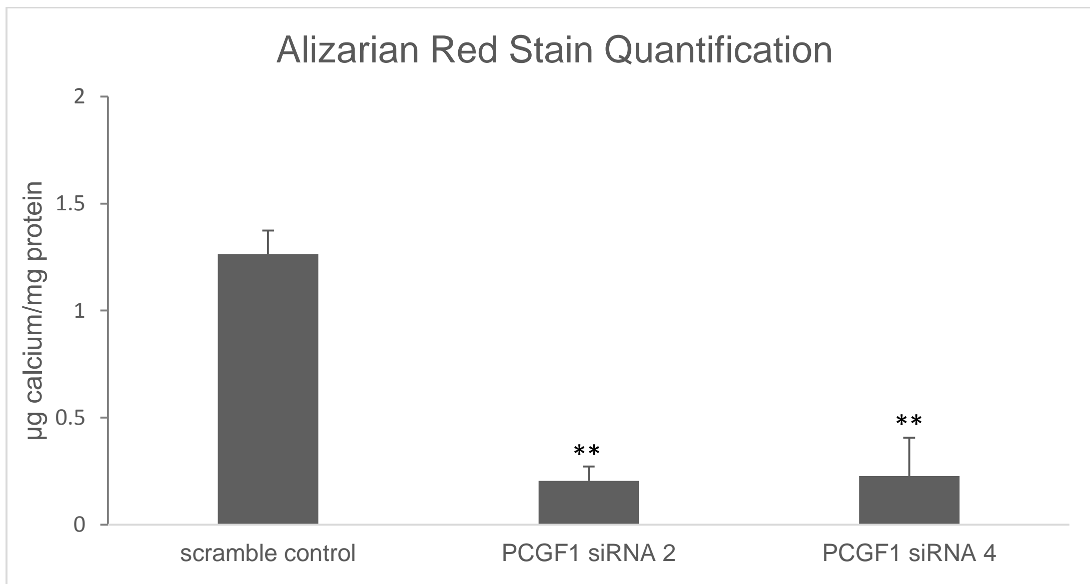
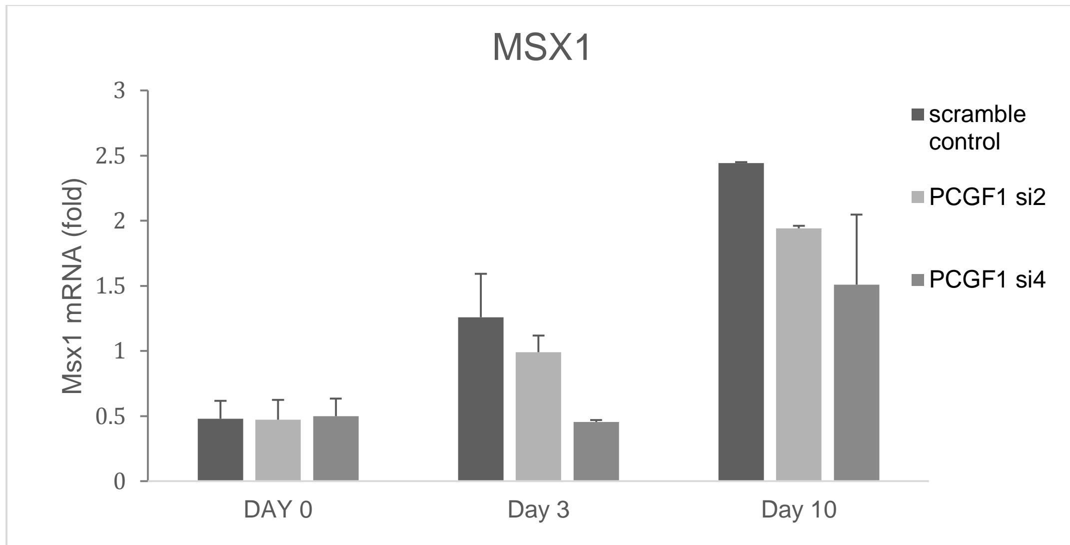


Figure 10. Alizarin Red Stain Quantification confirmed that there is a decrease in odontogenic potential in PCGF1 depleted dental pulp cells compared to scramble control. Cells were treated with osteogenic/odontogenic inducing media for 14 days. $*p < 0.05$;$**p < 0.001$.



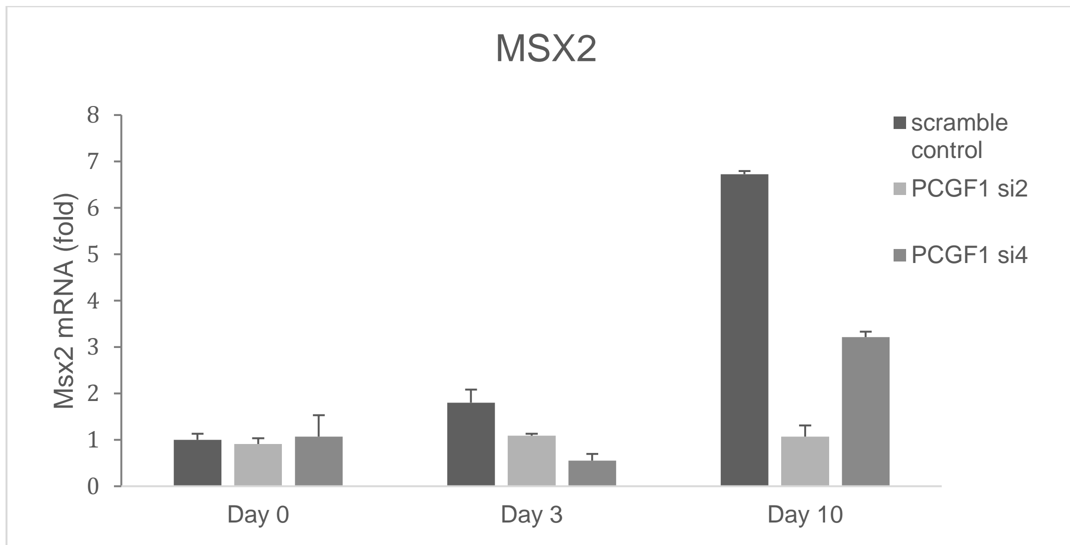


Figure 11. Real time PCR analysis for determining the relative mRNA levels of MSX1 and MSX2 in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 3 and 10 days. $*p < 0.05$; $**p < 0.001$.

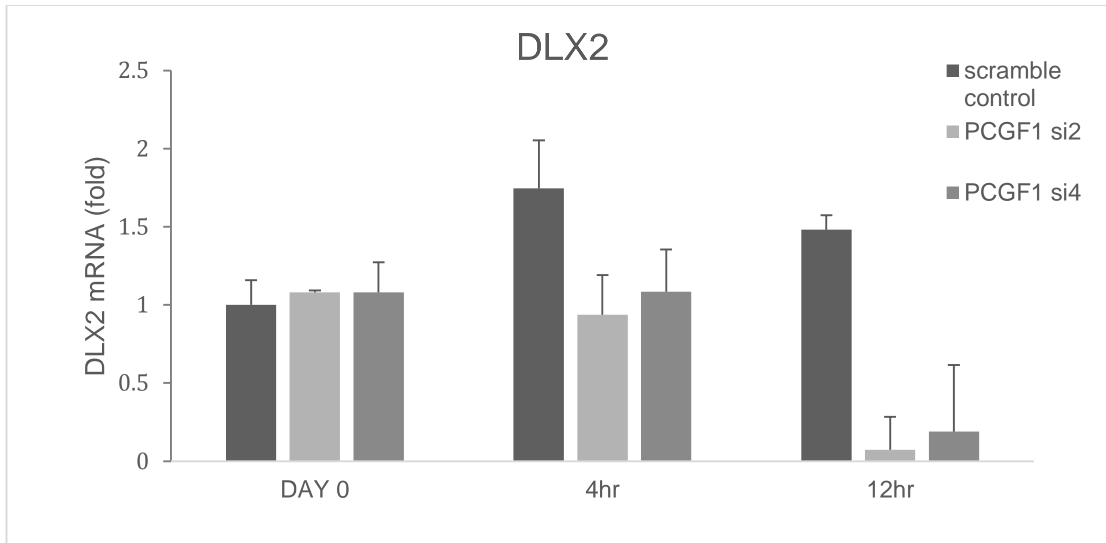


Figure 12. Real time PCR analysis for determining the relative mRNA levels of DLX2 in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 4 and 12 hours. *p < 0.05; **p < 0.001.

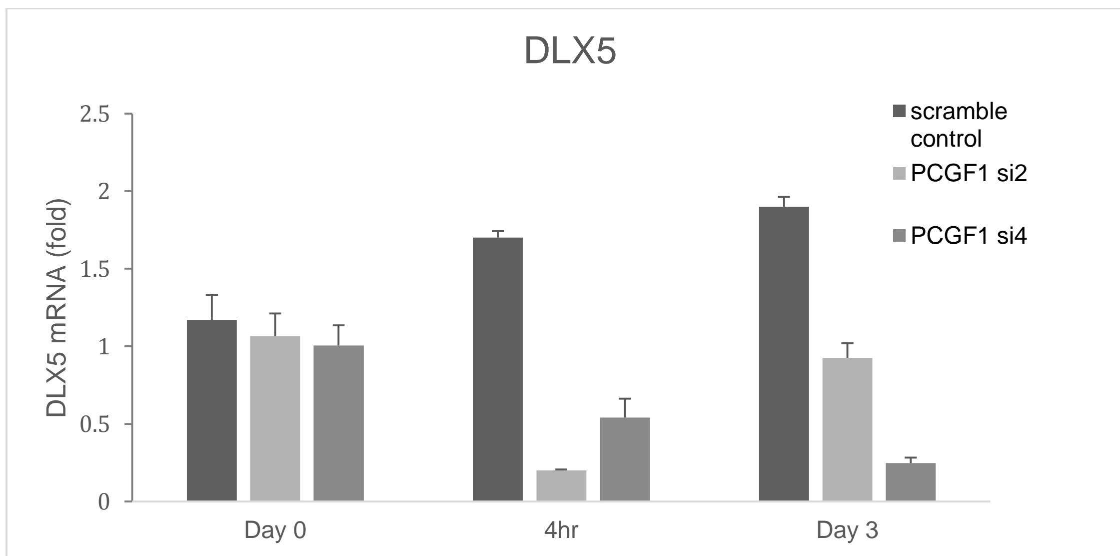


Figure 13. Real time PCR analysis for determining the relative mRNA levels of DLX5 in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 4 hours and 3 days. *p < 0.05; **p < 0.001

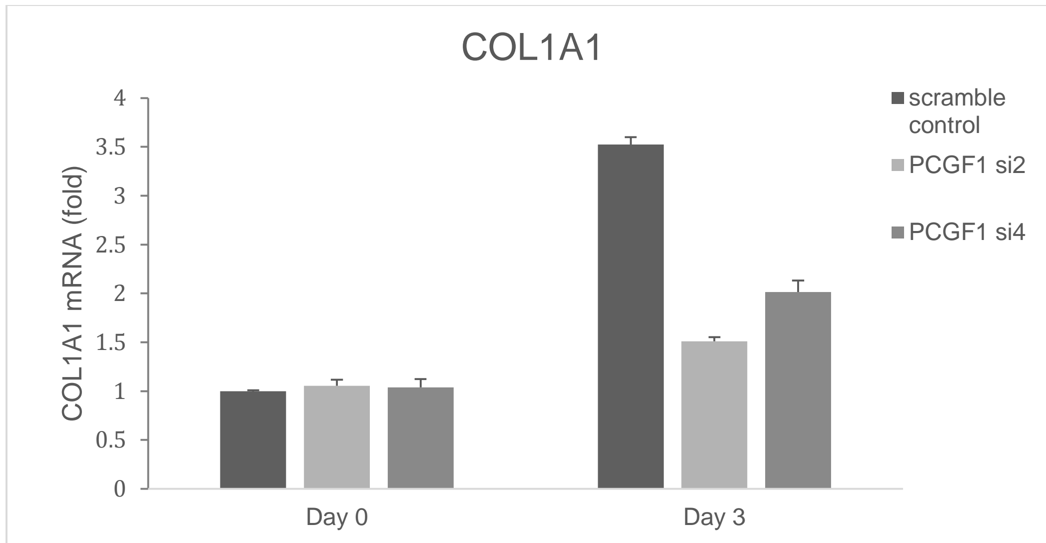


Figure 14. Real time PCR analysis for determining the relative mRNA levels of Col1a1 in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 3 days. *$p < 0.05$; **$p < 0.001$.

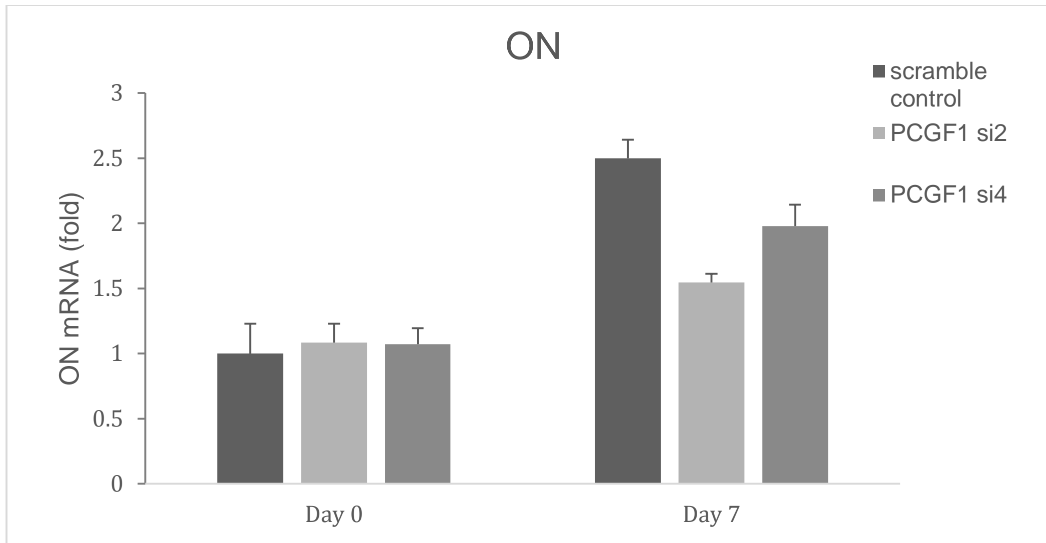


Figure 15. Real time PCR analysis for determining the relative mRNA levels of ON in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 7 days. *p < 0.05; **p < 0.001.

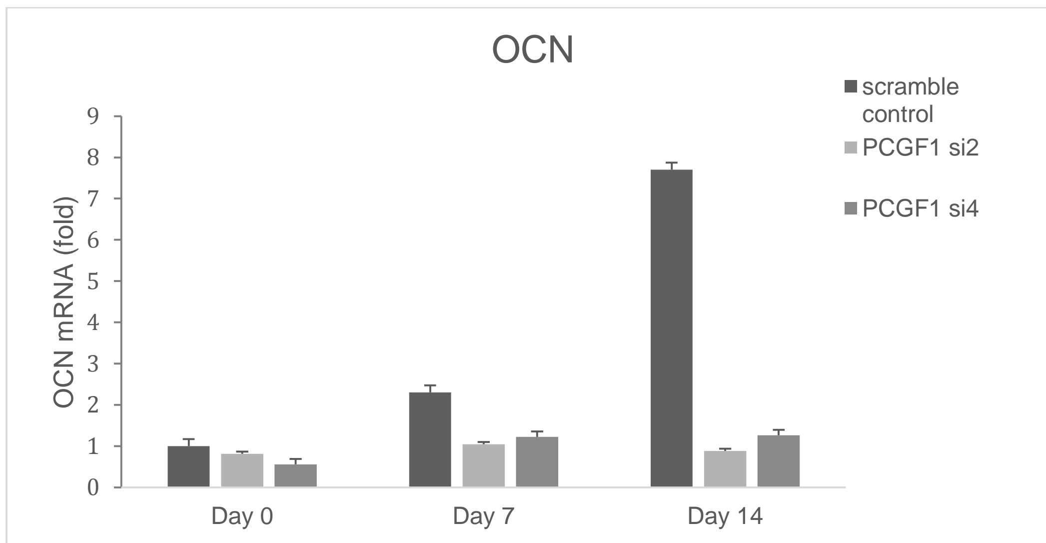


Figure 16. Real time PCR analysis for determining the relative mRNA levels of OCN in PCGF1-depleted dental pulp cells with scramble control and osteogenic/odontogenic induction for 7 and 14 days. *p < 0.05; **p < 0.001.

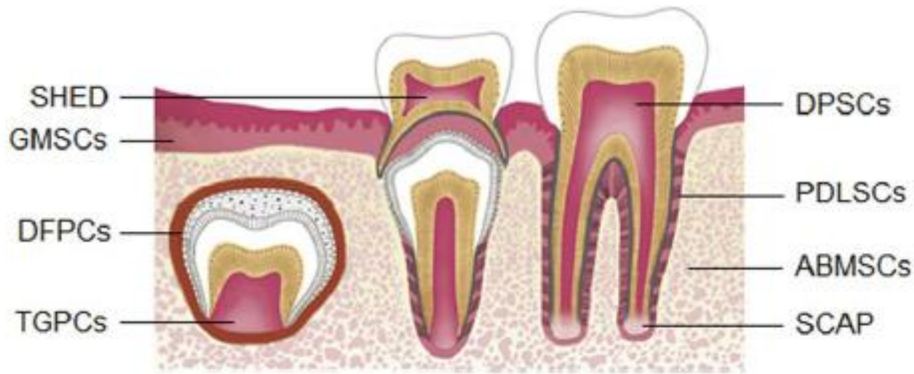


Figure 17. Schematic drawing illustrating sources of human dental tissue-derived MSCs. DPSCs: dental pulp stem cells; SHED: stem cells from exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFPCs: dental follicle progenitor cells; ABMSCs: alveolar bone-derived mesenchymal stem cells; SCAP: stem cells from the apical papilla; TGPCs: tooth germ progenitor cells; GMSCs: gingiva-derived MSCs.[3]

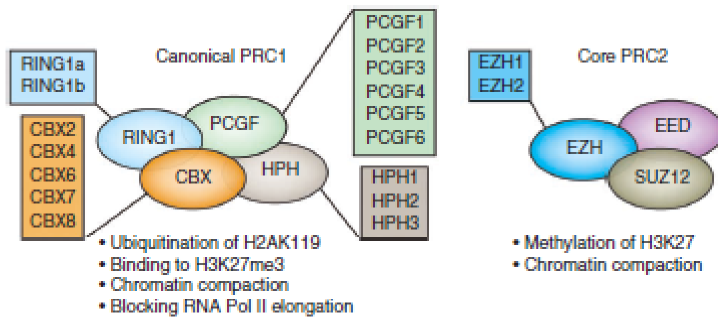


Figure 18. Composition and function of the main Polycomb complexes. Core components of mammalian PRC1 and PRC2 are shown. The diversity of the Polycomb complexes is achieved by the incorporation of the homologous proteins. Here, we show the canonical PRC1 complex but not the recently reported variations of it. Additional protein components of PRC1 and PRC2 that are present depending on cell type and isolation procedure are not depicted.[10]

REFERENCE

1. Tatullo, M., Marrelli, M. & Paduano, F. The Regenerative Medicine in Oral and Maxillofacial Surgery: The Most Important Innovations in the Clinical Application of Mesenchymal Stem Cells. *International journal of medical sciences* **12**, 72-77 (2015).
2. Ding, D.C., Chang, Y.H., Shyu, W.C. & Lin, S.Z. Human umbilical cord mesenchymal stem cells: a new era for stem cell therapy. *Cell transplantation* (2015).
3. Liu, J.*, et al.* Characteristics and Potential Applications of Human Dental Tissue-Derived Mesenchymal Stem Cells. *Stem cells* (2014).
4. Berardis, S., Dwisthi Sattwika, P., Najimi, M. & Sokal, E.M. Use of mesenchymal stem cells to treat liver fibrosis: Current situation and future prospects. *World journal of gastroenterology : WJG* **21**, 742-758 (2015).
5. Alvarez-Viejo, M.*, et al.* Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. *Transplantation proceedings* **45**, 434-439 (2013).
6. Park, S.Y.*, et al.* Ex vivo bone morphogenetic protein 2 gene delivery using periodontal ligament stem cells for enhanced re-osseointegration in the regenerative treatment of peri-implantitis. *Journal of biomedical materials research. Part A* **103**, 38-47 (2015).
7. Pinho, S.*, et al.* PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *The Journal of experimental medicine* **210**, 1351-1367 (2013).
8. Hwang, H.I., Lee, T.H. & Jang, Y.J. Cell proliferation-inducing protein 52/Mitofilin is a surface antigen on undifferentiated human dental pulp stem cells. *Stem cells and development* (2015).

9. Junco, S.E., *et al.* Structure of the polycomb group protein PCGF1 in complex with BCOR reveals basis for binding selectivity of PCGF homologs. *Structure* **21**, 665-671 (2013).

10. Di Croce, L. & Helin, K. Transcriptional regulation by Polycomb group proteins. *Nature structural & molecular biology* **20**, 1147-1155 (2013).

11. Turner, S.A. & Bracken, A.P. A "complex" issue: deciphering the role of variant PRC1 in ESCs. *Cell stem cell* **12**, 145-146 (2013).

12. Basu, A., Wilkinson, F.H., Colavita, K., Fennelly, C. & Atchison, M.L. YY1 DNA binding and interaction with YAF2 is essential for Polycomb recruitment. *Nucleic acids research* **42**, 2208-2223 (2014).

13. Wang, W., *et al.* RYBP expression is associated with better survival of patients with hepatocellular carcinoma (HCC) and responsiveness to chemotherapy of HCC cells in vitro and in vivo. *Oncotarget* **5**, 11604-11619 (2014).

14. Fan, Z., *et al.* BCOR regulates mesenchymal stem cell function by epigenetic mechanisms. *Nature cell biology* **11**, 1002-1009 (2009).

15. Di Stefano, C., *et al.* Oculo-facio-cardio-dental (OFCD) syndrome: The first Italian case of BCOR and co-occurring OTC gene deletion. *Gene* (2015).

16. Lapthanasupkul, P., *et al.* Ring1a/b polycomb proteins regulate the mesenchymal stem cell niche in continuously growing incisors. *Developmental biology* **367**, 140-153 (2012).

17. Blackledge, N.P., *et al.* Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2 recruitment and polycomb domain formation. *Cell* **157**, 1445-1459 (2014).

18. Tang, P., Xiong, Q., Ge, W. & Zhang, L. The role of MicroRNAs in Osteoclasts and Osteoporosis. *RNA biology* **11**, 1355-1363 (2014).

19. Franz-Odendaal, T.A. Induction and patterning of intramembranous bone. *Frontiers in bioscience* **16**, 2734-2746 (2011).

20. Harris, S.E., Guo, D., Harris, M.A., Krishnaswamy, A. & Lichtler, A. Transcriptional regulation of BMP-2 activated genes in osteoblasts using gene expression microarray analysis: role of Dlx2 and Dlx5 transcription factors. *Frontiers in bioscience : a journal and virtual library* **8**, s1249-1265 (2003).

21. Mezzabotta, F.*, et al.* Spontaneous calcification process in primary renal cells from a medullary sponge kidney patient harbouring a GDNF mutation. *Journal of cellular and molecular medicine* (2015).

22. Kugimiya, F.*, et al.* Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. *The Journal of biological chemistry* **280**, 35704-35712 (2005).

23. Gronthos, S., Mankani, M., Brahim, J., Robey, P.G. & Shi, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13625-13630 (2000).

24. Gouti, M., Briscoe, J. & Gavalas, A. Anterior Hox genes interact with components of the neural crest specification network to induce neural crest fates. *Stem cells* **29**, 858-870 (2011).

25. Komori, T. Regulation of osteoblast differentiation by transcription factors. *Journal of cellular biochemistry* **99**, 1233-1239 (2006).

26. Li, H.*, et al.* Expression and function of Dlx genes in the osteoblast lineage. *Developmental biology* **316**, 458-470 (2008).

27. Valadares, E.R., Carneiro, T.B., Santos, P.M., Oliveira, A.C. & Zabel, B. What is new in genetics and osteogenesis imperfecta classification? *Jornal de pediatria* **90**, 536-541 (2014).

28. Saeed, H. & Iqtedar, M. Aberrant gene expression profiles, during in vitro osteoblast differentiation, of telomerase deficient mouse bone marrow stromal stem cells (mBMSCs). *Journal of biomedical science* **22**, 11 (2015).

29. Lanza, D.*, et al.* Impact of the uremic milieu on the osteogenic potential of mesenchymal stem cells. *PloS one* **10**, e0116468 (2015).

30. Schwarting, T.*, et al.* Bone Morphogenetic Protein 7 (BMP-7) Influences Tendon-Bone Integration In Vitro. *PloS one* **10**, e0116833 (2015).

31. Rachmiel, A. & Leiser, Y. The Molecular and Cellular Events That Take Place during Craniofacial Distraction Osteogenesis. *Plastic and reconstructive surgery. Global open* **2**, e98 (2014).

32. Ross, K.*, et al.* Polycomb group ring finger 1 cooperates with Runx1 in regulating differentiation and self-renewal of hematopoietic cells. *Blood* **119**, 4152-4161 (2012).