Differentiation 124 (2022) 17–27

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

BMP-2 and asporin expression regulate 5-aza-dC-mediated osteoblast/cementoblast differentiation of periodontal dental ligament mesenchymal progenitor cells

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ARTICLE INFO

Keywords:
Osteoblasts
Epigenetics
Transcription factors
Cell differentiation
Bone regeneration

ABSTRACT

Periodontal dental ligament (PDL) is composed of heterogeneous population of mesenchymal progenitor cells. The mechanisms that regulate the differentiation of these cells towards osteoblast/cementoblast phenotype are not fully understood. Some studies have demonstrated that is possible to change the pattern of cell differentiation *via* epigenetic mechanisms. The proposal of this study was to investigate whether 5-aza-2'-deoxycytidine (5-aza-dC) treatment would stimulate the osteoblast/cementoblast differentiation of periodontal ligament mesenchymal progenitor cells (PDL-CD105$^+$ enriched cells), characterized as low osteoblast potential, through bone morphogenetic protein-2 (BMP-2) modulation. PDL-CD105$^+$ cells from a single donor were cloned and characterized in two populations as high osteoblast/cementoblast potential (HOP) and low osteoblast/cementoblast potential (LOP) by mineralization *in vitro* and expression of osteogenic gene markers, such as runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALP*), osteocalcin (*OCN*), bone morphogenetic protein 2 (*BMP-2*) and asporin (*ASPN*). Next, two LOP clones (L1 and L2) were pretreated with 5-aza-dC (10 μM) for 48 h, cultured under osteogenic condition and evaluated for mineralized matrix *in vitro*, transcription modulation of osteogenic gene markers, methylated and hydroxymethylated DNA levels of *BMP-2* and *ASPN* and intracellular/extracellular expression of BMP-2 protein. LOP clones showed high expression of *ASPN* transcripts associated with low mRNA levels of *BMP-2*, *RUNX2*, *ALP*, and *OCN*. 5-aza-dC treatment raised hydroxymethylated DNA levels of *BMP-2* and increased the expression of *BMP-2* transcripts in both LOP clones. However, BMP-2 protein (intracellular and secreted forms) was detected only in L1 cell clones, in which it was observed an increased expression of osteoblast/cementoblast markers (*RUNX2*, *ALP*, *OCN*) associated with higher mineralization *in vitro*. In L2 cell clones, 5-aza-dC increased gene expression of *ASPN*, with no great change in for osteoblast/cementoblast differentiation potential. These data show that 5-aza-dC improves osteoblast/cementoblast differentiation of PDL-CD105$^+$ cells *via* BMP-2 secretion, and this effect depends on low levels of *ASPN* expression.

1. Introduction

Nowadays, even with numerous clinical therapies and available technologies, periodontal regeneration is still a challenge to achieve. Since the periodontium is a complex structure, the migration, differentiation and proliferation of remnant undifferentiated mesenchymal cells that exist in the periodontal ligament (PDL) may guarantee the success of remodeling oral tissues through neoformation of root cementum and

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https://doi.org/10.1016/j.diff.2022.02.003
Received 26 August 2021; Received in revised form 3 February 2022; Accepted 4 February 2022
Available online 5 February 2022

alveolar bone (Bartold et al., 2000; Kramer et al., 2004; Ripamonti and Petit, 2009). However, preview studies performed aiming to evaluate the regenerative potential and mineralization of human periodontal ligament mesenchymal stem cells (hPDLSCs) have shown that these cell populations are highly heterogeneous and a great amount of them present low potential to renovate the hard tissue damaged by periodontal disease (Seo et al., 2004; Singhatanadgit et al., 2009; Silvério et al., 2010; Sununliganon and Singhatanadgit, 2012; Saito et al., 2014).

The main difference between the differentiation potential of these cells is linked to regulation of osteoblast/cementoblast modulators. The upregulation of runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALP*) and osteocalcin (*OCN*) are required during the process of acquiring osteoblast/cementoblast phenotype (Hayrapetyan et al., 2015), while overexpression of asporin (*ASPN*) seems to prevent the mineralization of bone marrow-derived mesenchymal stem cells (BMSCs) (Sun et al., 2014) and PDL cells (Yamada et al., 2006, 2007; Ueda et al., 2016).

In this context, the activation of osteogenic signaling pathways is a promising solution to modulate gene expression and change the phenotype of hPDLSCs that are less compromised with hard tissue regeneration (Franceschi et al., 2003; Galli et al., 2012; Gao et al., 2013; Jin et al., 2015; Cho et al., 2016). Among these pathways, bone morphogenetic proteins pathway is one of the main signaling cascades accountable for osteogenesis (Arvidson et al., 2011; Chen et al., 2012). From the BMPs family members, BMP-2 is a great osteoinductor, playing an important role on upregulating mRNA levels of *RUNX*2 (Hyun et al., 2011), a transcript osteogenic factor involved in the regulation of other osteogenic-related genes, such as *OCN*, bone sialoprotein (*BSP*), osteopontin (*OPN*), and collagen type I (*COL1*) (Banerjee et al., 1997; Komori et al., 1997).

In fact, human recombinant BMP-2 protein is commercially available for clinical procedures (Bishop and Einhorn, 2007). However, studies that evaluated its application on periodontal regeneration showed as disadvantage the presence of alkalosis due high required dosage (Wikesjö et al., 2003; Sorensen et al., 2004; Takahashi et al., 2005). Therefore, a possible alternative would be enhancing endogenous BMP-2 through modulation of its gene expression inside the body (Hayrapetyan et al., 2015). In this way, epigenetic tools may help to tissue regeneration and improve clinical approaches through of *BMP-2* expression modulation.

Epigenetic modulators have been used to promote DNA changes and thus regulating signaling pathways and expression of genes involved in the differentiation of multipotent mesenchymal cells. In particular, CpG island methylation represents an important epigenetic target associated with gene silencing (Cedar and Bergman, 2009). This mechanism is catalyzed by DNA methyltransferases (DNMTs). DNMT1 plays a role of ensuring the conservation of methylated DNA pattern after cell replication. DNMT1 cognizes hemi-methylated DNA after replication and methylates new DNA strands (Cedar and Bergman, 2009). The epigenetic modulator 5-Aza-2′-deoxycytidine (5-aza-dC) is a cytidine analog and acts preventing the DNMT1 function of disseminating DNA methylation code during cell division. The particles of 5-aza-dC bind to DNMT1 by covalent connection in the CpG sites of DNA, inactivating DNMT1 action and leading to DNA demethylation, and consequently, increase gene expression (Christman, 2002).

In fact, *in vitro* studies using different cell populations have shown a positive effect of 5-aza-dC on increasing *BMP-2* transcripts (Azechi et al., 2014; Wolf et al., 2014; Mitsui et al., 2015). Further, a transdifferentiation from gingival fibroblasts to osteoblasts was achieved using this modulator (Cho et al., 2017). However, until now, the possibility of changing the osteoblast/cementoblast capacity of hPDLSC through epigenetic modulation of the *BMP-2* gene has not been evaluated.

Thus, the aim of this study was to investigate whether 5-aza-2'-deoxycytidine (5-aza-dC) treatment would stimulate the osteoblast/cementoblast differentiation of periodontal ligament mesenchymal progenitor cells (PDL-CD105$^+$ enriched cells), characterized as low osteoblast potential, through *BMP-2* modulation.

2. Material and methods

2.1. Cell culture and clone isolation

This study was approved by the Institutional Review Board of Piracicaba Dental School–University of Campinas (#030/2017), São Paulo, Brazil. One population of mesenchymal progenitor cells (CD105$^+$ CD34$^-$ CD45$^-$ cells) from PDL of permanent teeth were obtained and characterized in a previous study (Silvério et al., 2010). Briefly, CD105$^+$ enriched cell subsets from PDL were isolated by magnetic cell sorting and characterized by flow cytometry and immunostaining. This cell population showed multipotential capabilities to differentiate *in vitro* to osteoblast/cementoblast-like cells, and adipocyte-like cells (Silvério et al., 2010). The population was cultured in Dulbecco™ modified Eagle medium (Gibco™), supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (standard medium), frozen in Recovery Cell Culture Freezing Medium (Gibco™), and kept in liquid nitrogen for subsequent experiments. Only one cryovial from this cell population was used for cloning isolation. The Ring-cloning Technique was performed as described in a previous study (Saito et al., 2014). Briefly, two hundred fifty cells (passage 2) were seeded into 100-mm dishes and incubated at 37C, 5% CO_2, in standard medium. Individual clones were allowed to develop for 14–21 days until they reached approximately 50 cells per colony, and then 8-mm-diameter cylinder polystyrene rings were placed around the colonies. The cells were detached with 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA transferred to 24-well plates, and re-cultured as above.

2.2. Osteoblast/cementoblast differentiation of PDL-CD105$^+$ cell clones

To evaluate the capacity to produce mineralized matrix *in vitro*, PDL-CD105$^+$ cell clones were seeded (1×10^5 cells/well) in 24-well plates and incubated for 24 h, at which time osteogenic medium was added (10% fetal bovine serum-DMEM and osteogenic supplements containing 50 mg/ml ascorbic acid, 10 mM b-glycerophosphate, and 10^{-5} M dexamethasone), and the cultures were re-incubated for 28 days. The medium was changed every other day. On designated days up to day 28, the ability of cells to promote mineral nodule formation was determined by Alizarin Red Staining (AR-S) (Gregory et al., 2004). PDL-CD105$^+$ cell clones were classified as high osteoblast/cementoblast potential (HOP) and low osteoblast/cementoblast potential (LOP) after the discoloration procedure of mineral nodules and quantitative analysis (562 nm) using VersaMax micro-plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3. RNA extraction and RT-qPCR

For RNA isolation, HOP and LOP cell clone were seeded (2×10^5 cells/well) in 60 cm^2 plates with standard medium and incubated for 24 h. After this period, the medium was removed, and cells were incubated in osteogenic medium for 14 days. The medium was changed every other day. After 14 days, cells were lyzed for total RNA extraction using TRIzol reagent (Invitrogen™, Waltham, Massachusetts, USA), as previously described (Saito et al., 2014), followed by a phenol/chloroform extraction, and isopropanol precipitation. RNA samples were treated with Turbo DNA-free to remove genomic DNA (DNA-free™, Ambion Inc., Austin, TX, USA). Single-stranded complementary DNA (cDNA) was synthesized from 2 μg total DNA-free RNA using First-strand cDNA synthesis (Roche Diagnostic Co., Indianapolis, IN, USA) following manufacturer's recommendations for final volume of 20 μl.

The primers for *ACBT*, *ALP, ASPN, BMP-2, GAPDH, RUNX2* and *OCN* (Table 1) were designed using Primer3web version 4.1.0 (Untergasser et al., 2012) and sequences confirmed by UCSC PCR *in silico* (Kent et al., 2002). For subsequent analysis of secondary structures and confirmation

Table 1
Primer sequences used for RT-qPCR.

Gene	Primer (5′ - 3'sequence)	Annealing temperature	Product Size
ACTB	F: CCAACCGCGAGAAGATGA R: CCAGAGGCGTACAGGGATAG	61 °C	538 bp
ALP	F: CGGGCACCATGAAGGAAA R: GGCCAGACCAAAGATAGAGTT	55 °C	184 pb
ASPN	F: CCAACCGCGAGAAGATGA R: CCAAGCAAGGTCTTCCAAAG	60 °C	223 pb
BMP-2	F: CTTCCACCCCTCTTTCTTCC R: GTCTCCCGGAAACACTTGAA	58 °C	221 pb
GAPDH	F: ACATCATCCCTGCCTCTAC R: CCACCTTCTTGATGTCATCATATTTG	51 °C	171 pb
OCN	F: AGCTCAATCCGGACTGT R: GGAAGAGGAAAGAAGGGTGC	55 °C	150 pb
RUNX2	F: CCGTCCATCCACTCTACCAC R: ATGAAATGCTTGGGAACTGC	55 °C	139 pb

ACTB: β-actin; ALP: alkaline phosphatase; ASPN: asporin; BMP-2: Bone morphogenetic.
Protein 2; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; OCN: osteocalcin.
RUNX2: runt-related transcription factor 2.

of annealing temperature, the Beacon Designer free edition was used (Premier Biosoft International, Palo Alto, CA, USA). The efficiency of reactions for each "primer" was optimized prior to the start of the RT-qPCR reactions themselves. The real-time PCR reactions was performed with the LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) using the "LightCycler 480 SYBR Green I Master" kit (Roche Diagnostics GmbH, Mannheim, Germany.).

The reaction profile was determined by formula suggested by the equipment manufacturer. For each run, the water was used as a negative control, and the reaction product was quantified using the LightCycler Relative Quantification Software (Roche Diagnostics GmbH, Mannheim, Germany). *GAPDH* and *ACBT* were used as the reference genes ("housekeeping") for the normalization of values.

2.4. 5-aza-2′-deoxycytidine treatment and mineralization assay in vitro

Two low osteoblast/cementoblast potential cell clones (L1 and L2) were seeded (1×10^5 cells/well) in 24-well plate and cultured for 24 h under the following conditions: 1) Control - cultured in standard medium (DMEM 10% FBS), 2) OM - cultured in osteogenic medium and 3) 5AZA - pre-treatment with 5-aza-2′-deoxycytidine (5-aza-dC) + osteogenic induction. Cells in 5AZA group were treated for 48 h (Alvarez-Garcia et al., 2016) with 10 μM (Cho et al., 2017) 5-aza-dC (SigmaAldrich, St. Louis, MO, USA) diluted in 50 μM DMSO (SigmaAldrich, St. Louis, MO, USA), being the culture medium with 5-aza-dC replace after 24 h.

The osteogenic induction started at the distinct time point according to the groups described above. The cells were maintained under osteogenic condition for 28 days, and the osteogenic medium was changed every 2 days. In order to observe the dynamics of mineral nodules formation on days 14, 21 and 28, the samples were incubated with Xylenol Orange (SigmaAldrich, St. Louis, MO, USA) at a concentration of 20 μM for 4 h. After this period, the samples were visualized by Fluorescence Microscopy (Leica TCS SP5AOBS, Leica Microsystems, Mannheim, Germany), using the TRITC red filter (Chroma Technology, Rockingham, VT, USA) for qualitative analysis of mineral nodule deposition. At 14 and 21 days after data collection, the medium containing Xylenol Orange Staining (XO-S) were removed, cells were washed with phosphate buffered saline (PBS) and incubated again with culture medium, according to each experimental group. For quantitative analysis of mineralized matrix production, AR-S assay (Gregory et al., 2004) was performed as described in 2.2 section.

2.5. RT-qPCR and DNA extraction

To verify the effect of 5-aza-dC treatment on gene expression and methylated and hydroxymethylated DNA levels, L1 and L2 cell clones were seeded (1×10^6) in 150 cm-plates and cultured for 14 days under the three conditions described above. Then for each plate, 2 samples of RNA and 3 samples of DNA was collected. For that, culture media was removed, cells washed with PBS and incubated with trypsin solution for 5 min at 37 °C. Then the trypsin was neutralized, cells were collected and resuspended in TRIzol reagent (Invitrogen™, Waltham, Massachusetts, USA) for RNA isolation, and in 10 μl of proteinase K for DNA isolation. RNA extraction was performed as described above (2.3 section), as well as qRT-PCR technique, primers, conditions and analysis. For DNA extraction, the samples were left overnight in a 55 °C water bath and the next day, Genomic DNA (DNAg) was performed. DNA concentration was evaluated in NanoDrop (2000) spectrophotometer (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.6. Real-time PCR assay for methylated and hydroxymethylated DNA levels analysis

To distinguish methylated (mC) and hydroxymethylated (hmC) DNA levels, DNAg samples were treated with 1X NE buffer, 40 mM UDP glucose, T4-β-glucosyltransferase (T4-BGT) (New England Biolabs, Beverly, MA), in a final volume of 20 μL, and incubated at 37 °C for 1 h, followed by 10 min at 65 °C. T4-BGT adds glucose moiety to 5hmC, generating glucosylated hydroxymethylated cytosine (ghmC). Each sample was then divided into three groups: 1) samples digested using the restriction enzymes MspI (New England Biolabs, Beverly, MA, USA), which is blocked by ghmC, but not by mC or hmC, 2) samples digested using the methylation sensitive restriction enzymes HpaII (New England Biolabs, Beverly, MA, USA), blocked by all three modifications (mC, hmC and ghmC and 3) Samples not digested (Control – H_20), in a final volume of 25 μL at 37 °C for 1 h. Tubes containing HpaII were submitted to an additional incubation of 10 min at 65 °C to promote the enzyme inactivation. Afterwards, 40 ng of DNAg was subjected to real-time PCR amplification in triplicate with primers designed to flank regions of interest containing CCGG. Real-time PCR reactions was performed with the LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) using the "LightCycler 480 SYBR Green I Master" kit (Roche Diagnostics GmbH, Mannheim, Germany). The method of comparison of cycle threshold (Ct) was used to detect epigenetic patterns. Samples were normalized by the sample called control (H_2O). This normalization gives the approximate percentage of methylated alleles (HpaII-MspI/Control) and hydroxymethylated alleles (MspI/Control). The primers flanking CCGG sites in regions of interest of *ASPN* and *BMP-2* genes (Table 2) were designed as described in 2.3 section.

Table 2
Primer sequences for methylated/hydroxymethylated DNA analysis.

Gene	Primer (5′ - 3'sequence)	Annealing temperature	Product Size	Restriction enzymes sites position and number of CCGG analyzed
ASPN	F: TTAAAGGCAGGGAGGGTCTC R: GGCAGGGAGCAGAACAGTAG	58 °C	164 bp	chr 9: 1 CCGG site 92,479,252
BMP-2	F: CCCAGCGTGAAAAGAGAGAC R: GAGACCGCAGTCCGTCTAAG	58 °C	168 pb	chr 20: 2 CCGG sites 6,768729/ 6,768736

ASPN: asporin; BMP-2: bone morphogenetic protein-2.

2.7. Western blot analysis for intracellular BMP-2

L1 and L2 cells were seeded (2×10^5 cells/well) in six-well culture plates and incubated overnight in standard medium. Next, cells were cultured under conditions described in 2.4 section. For protein quantification, cells were harvested, washed with PBS and lysed with 50 mM Tris–HCl pH 7.4 buffer containing 1% Triton X-100, 150 mM NaCl, 0.5 mM EGTA and 0.5 mM EDTA, as well as an anti-protease cocktail (Complete Protease inhibitor Cocktail Tablets, Roche, France). The total protein content in the clear supernatant was measured using a Bradford assay (Bradford reagent, SigmaAldrich, St. Louis, MO, USA) with BSA as a standard and quantification by spectrophotometry (Genesys TM 2, Thermo Spectronic US, Rochester, NY, USA). Fresh protein extracts from three replicates (20 μg each one) were pooled and separated by SDS-PAGE and transferred to Hybond-C Extra nitrocellulose membranes (GE Healthcare Systems, Chicago, Illinois, USA).

The membrane was blocked with 4% milk for 1 h, then incubated overnight with polyclonal anti-BMP-2 IgG (1:1000 dilution; clone ab14933, Abcam, Cambridge, UK). After four washes, the membrane was incubated with anti-rabbit IgG conjugated (1:10,000 dilution; GE Healthcare Systems, Chicago, Illinois, USA) for 2 h. Signals were detected using Enhanced Chemiluminescence (ECL) reagents (PerkinElmer, Inc., Waltham, Massachusetts, USA).

2.8. ELISA for extracellular BMP-2

L1 and L2 cells were seeded (2×10^5 cells/well) in six-well culture plates and incubated overnight in standard medium. Next, cells were cultured under conditions described in 2.4 section. Conditioned cell culture media was collected. After centrifugation, microtiter plate wells were coated with 100 μl of the conditioned-media for 2 h at room temperature. The wells were then washed 3 times with 400 μl of 1% Tween 20 in PBS and nonspecific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, polyclonal rabbit antibody anti-BMP-2 (clone ab14933, Abcam, Cambridge, UK), diluted 1:100 in PBS, was added to the wells and incubated for 2 h. After another washing step, peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, USA) diluted 1:1000 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenylenediamine (SigmaAldrich, St. Louis, MO, USA) in 0.5 M citric buffer pH 5.5 containing 0.01% H2O2 for 20 min. After terminating the reaction with 50 μl of 2 N H2SO4, absorbance was read at 450 nm with λ correction at 570 nm (Genesys TM 2, Thermo Spectronic US, Rochester, NY, USA). BMP-2 levels, as represented by absorbance values, were calculated by diving these values by a standard curve of BMP-2 concentration.

2.9. Mineralization assay for BMP-2 agonist and antagonist

L1 and L2 were seeded (1×10^5 cells/well) in 24-well plate and cultured for 24 h under the same conditions described in 2.4 section. To verify the effects of Noggin (BMP antagonist) and BMP2 (agonist of BMP signaling pathway), two groups were added: 1) 5AZA + NOGGIN – L1 cells pre-treated with 5-aza-dC followed by osteogenic induction in the presence of 0.1 μg/ml rhNOGGIN (R&D Systems, Minneapolis, Minnesota, USA), and 2) 5AZA + BMP2 – L2 cells pre-treated with 5-aza-dC followed by osteogenic induction in the presence of 0.1 μg/ml rhBMP-2 (R&D Systems, Minneapolis, Minnesota, USA). Cells were cultivated for 28 days and the culture medium were replaced every other day for each group. For quantitative analysis of mineralized matrix production, AR-S assay (Gregory et al., 2004) was performed as described in 2.2 section.

2.10. Statistical analysis

All experiments were performed in triplicate and repeated on at least three separate occasions. Data were initially examined for normality by Kolmogorov-Smirnov test and expressed as mean or relative comparison ± standard deviation (SD). After normal distribution was confirmed, Student's T-test was used to analyze the difference between two groups. One-way analysis of variance (ANOVA) followed by pairwise multiple-comparison test (Tukey) was used to identify the difference among three or more groups. A linear regression model was employed to test the correlation between *BMP-2* and *ASPN* hydroxymethylated DNA and gene expression levels, with hydroxymethylated DNA levels as the predictor and gene expression level as the outcome. A linear regression model was also employed to test the correlation between *BMP-2* and *ASPN* gene expression levels, with *BMP-2* expression level as the predictor and *ASPN* gene expression level as the outcome (GraphPad Prism 8 – GraphPad Software Inc., San Diego, CA, USA). P values of <0.05 were considered significant.

3. Results

3.1. PDL-CD105$^+$ cell clones present different deposition of mineralized matrix and expression of osteogenic markers

A total of sixteen PDL-CD105$^+$ cell clones were obtained from a mesenchymal progenitor population by cloning ring technique. In culture, all cell clones could produce single-derived colonies and retained their fibroblastic spindle shape (data not shown). After 28 days under osteogenic induction, Alizarin Red Staining (AR-S) quantification results revealed that 5 cell clones were able to produce great amount of mineralized matrix and were classified as high osteoblast/cementoblast potential (HOP), while 11 cell clones did not present the same capacity and were classified as low osteoblast/cementoblast potential (LOP) (Fig. 1A).

To better characterized the osteoblast/cementoblast potential, a pool of HOP clones and a pool of LOP clones were performed for the investigation of transcript expression of known osteogenic-related genes (*RUNX2*, *ALP* and *OCN*). The results of RT-qPCR revealed a significantly low expression of *RUNX2* and *ALP* genes in LOP cells compared to HOP cells cultured under control condition (Fig. 1B and C). On the other hand, LOP cells showed a higher level of *OCN* transcripts compared to HOP cells (Fig. 1D). After 14 days under osteogenic induction, the three osteogenic markers were upregulated in HOP cells in comparison to control condition. In LOP cells, it was observed an upregulation only of *ALP* gene, which was significantly lower when compared to HOP cells (Fig. 1D). Furthermore, the transcripts levels of *RUNX2 e OCN* were downregulated in LOP cells under osteogenic condition (Fig. 1B, D). In this way, these results confirm a higher osteoblast/cementoblast potential of HOP cells compared to LOP cells.

3.2. The modulation of BMP-2 gene expression is similar in HOP and LOP cell clones after osteogenic induction but different for ASPN gene

Having demonstrated that LOP cells express lower levels of transcripts for osteogenic gene markers compared to HOP cell clones, and that osteogenic induction did not significantly alter this profile of expression, the next step was to investigate whether there were differences in the expression of the osteogenic inductor *BMP-2* and its potential blocker *ASPN*.

After being cultivated for 14 days under osteogenic conditions, both HOP and LOP presented a decrease in their *BMP-2* transcripts levels, however it was much lower in LOP (22%) than in HOP (88%) (Fig. 1D). Regards to *ASPN* gene, LOP cells showed a significantly increase in its transcript level under osteogenic induction (2593%), while in HOP cells the expression of these gene decreased to 17% under this same condition (Fig. 1E).

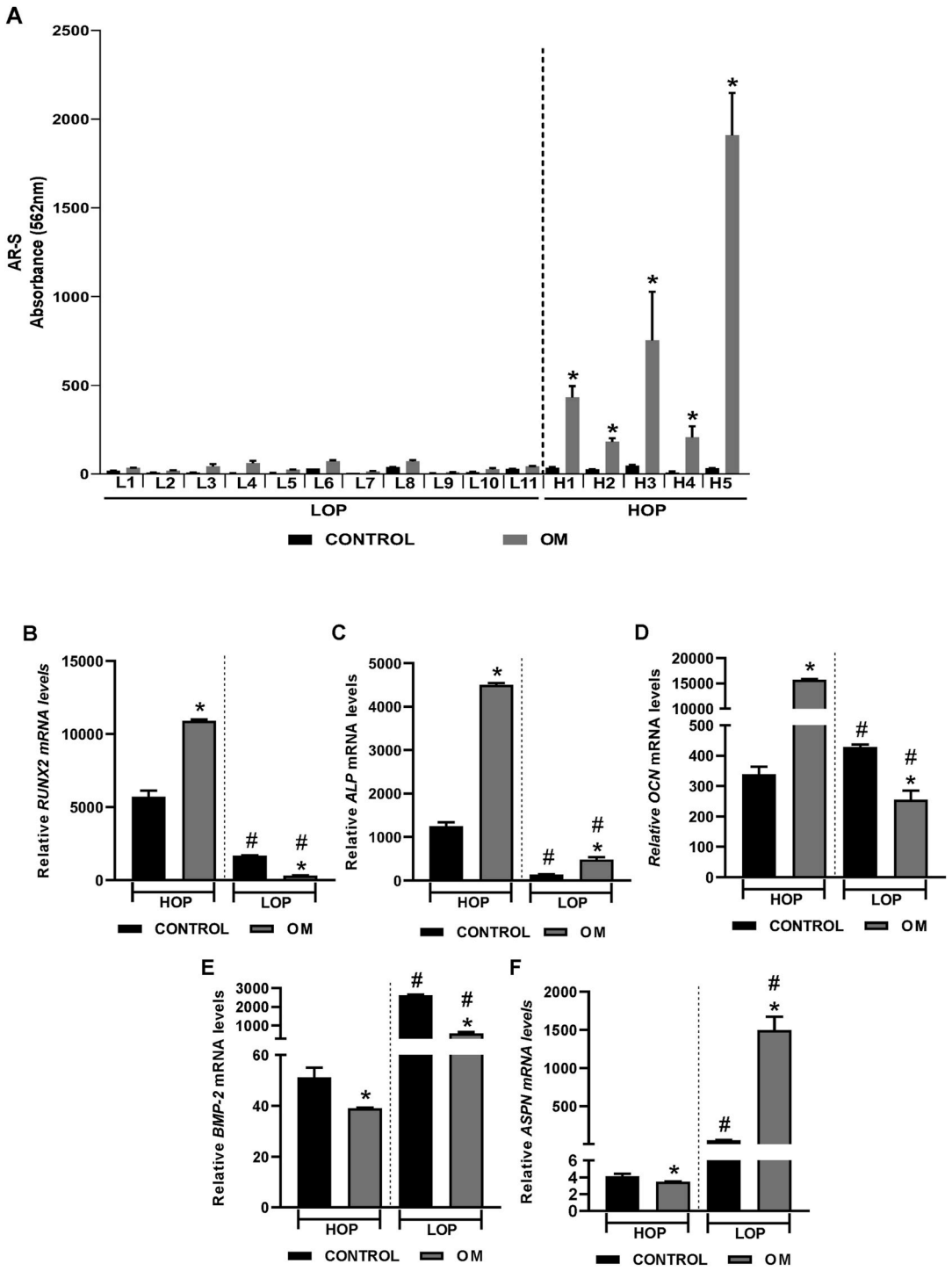

Fig. 1. *PDL-CD105^{+} cell clones present distinct osteoblast/cementoblast differentiation potential.* Quantification of Alizarin Red Staining (AR-S) of mineralized matrix deposition of sixteen PDL-CD105^{+} cell clones cultured in standard medium (CONTROL) and osteogenic medium (OM) for 28 days (A). Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) for *RUNX2* (B), *ALP* (C), *OCN* (D), BMP-2 (E), and *ASPN* (F) in LOP and HOP cell clones cultured in control and osteogenic medium for 14 days. Bars in A - F represents mean ± SD where intragroup (control *versus* OM) analysis statistical differences are indicated by * ($p < 0.05$) and intergroup (HOP *versus* LOP under the same culture condition) analysis statistical differences are indicated by # ($p < 0.05$). Results correspond to mRNA levels ratio normalized to the *GAPDH* and *ACTB* levels.

3.3. 5-aza-dC increases osteoblast/cementoblast differentiation potential of two LOP cell clones, but with distinct amount of mineralized matrix and modulation of osteogenic gene expression

Given that osteogenic medium did not induce the differentiation of LOP cell clones, the next step was to assess whether 5-aza-dC, an epigenetic modulator, is able to change this process. Among the LOP cells, two clones (L1 and L2) were selected, due their similar cell viability when exposed to 10 μM of 5-aza-dC for 48 h (data not shown).

The results of Xylenol Orange Staining (XO-S) revealed microscopic mineral nodule deposition in L1 and L2 cell clones cultured under osteogenic induction, which was enhanced after 5-aza-dC pre-treatment. Further, by this assay, it was possible to verify that mineral nodule deposition started between the 14th and 21st day of osteogenic

induction since at 14 d none of the cell clones presented nodules stained by XO-S, which was identified at 21 d (Fig. 2 A, B).

Additionally, L1 and L2 cell clones showed a distinct ability to produce mineralized matrix *in vitro*, which was confirmed by AR-S assay. After 28 d in culture, AR-S revealed that osteogenic medium did not induce a significant mineral nodule deposition in L1 and L2 cells (Fig. 2 C, D). On the other hand, 5-aza-dC pre-treatment increased mineralization compared to osteogenic medium alone in both clones (Fig. 2 C, D). Comparing the behavior of the two LOP cell clones, L1 cell clones has greater ability to produce mineralized matrix compared to L2 cell clones independent of culture condition (Fig. 2E).

3.4. 5-aza-dC modulates osteogenic-related genes' expression in a distinct manner in L1 and L2 cells clones

The results of mineral nodule deposition were also confirmed by expression of osteogenic-related genes. Here, OM group was used as 100% for relative comparison. In L1 cell clones, the pre-treatment with 5-aza-dC upregulated the expression of transcripts for *RUNX2*, *ALP* and *OCN*, with an increase of more than 1000% in the transcript levels for each analyzed gene compared to osteogenic medium only (Fig. 2 F, G, H). On the other hand, 5-aza-dC had an opposite effect on L2 cell clones, leading to a reduction on mRNA levels for all these genes compared to OM group (Fig. 2 F, G, H).

These findings suggest that 5-aza-dC pre-treatment stimulated LOP cell clones' differentiation towards the osteoblast/cementoblast

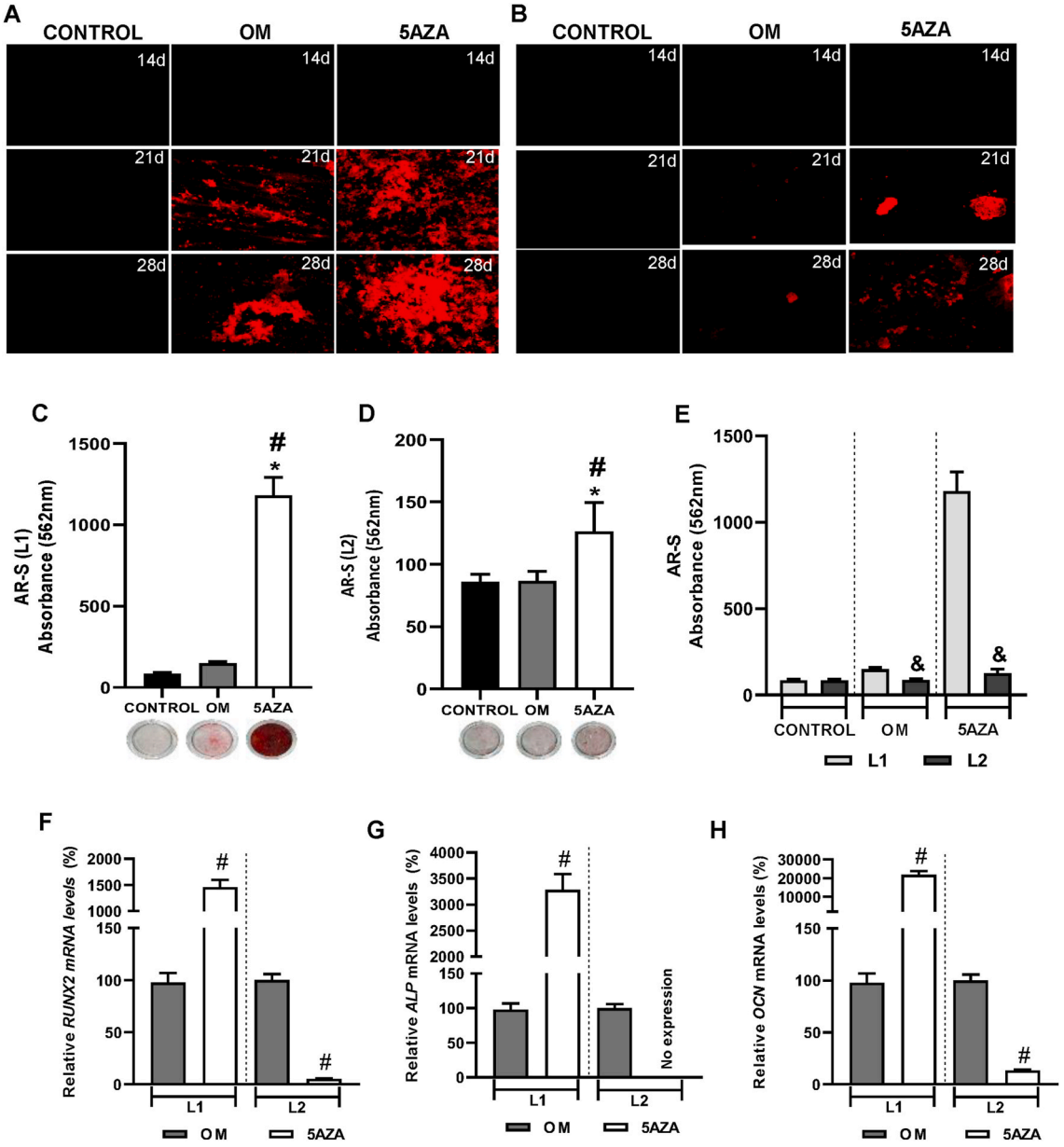

Fig. 2. *Osteoblast/cementoblast differentiation of LOP cell clones after 5-aza-dC treatment.* Microscopic aspect of Xylenol Orange Staining Mineralization of L1 (A) and L2 (B) at 14, 21, and 28 days. Quantification of Alizarin Red Staining (AR-S) in L1 (C) and L2 (D) at 28 days. Comparison of matrix mineralized deposition between L1 and L2 cells (E). Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) for *RUNX2* (F), *ALP* (G), and *OCN* (H) in L1 and L2 cell clones after 14 days cultured under osteogenic condition associated or not with 5-aza-dC pre-treatment. Bars in C–H represents mean ± SD where statistical differences compared with CONTROL are indicated by * ($p < 0.05$), statistical differences between 5AZA *versus* OM are indicated by # ($p < 0.05$), and statistical differences between L1 *versus* L2 are indicated by & ($p < 0.05$). Results correspond to mRNA levels ratio normalized to the *GAPDH* and *ACTB* levels.

phenotype, however, this effect seems to be more effective in L1 cell clones than in L2, concerning to the amount of mineral deposited and the upregulation of osteogenic-related genes.

3.5. 5-aza-dC increases hydroxymethylated DNA levels and expression of *BMP-2* gene in two LOP cell clones

To evaluate the DNA modulation promoted by 5-aza-dC on *BMP-2* gene, CONTROL group was used as 100% for relative comparison of methylated and hydroxymethylated DNA levels of this gene. First, for methylated DNA, 5-aza-dC increased methylation levels in both L1 (≈106%) and L2 (≈107%) compared their respectively OM group (L1≈104% and L2≈101%) (Fig. 3 A, B). The modulation of methylated DNA between OM and 5AZA was 2% for L1 and 6% for L2. On the other hand, hydroxymethylated DNA results showed a more significant modulation. In osteogenic environment (OM), clone L1 did not show a significant modulation in hydroxymethylated DNA levels compared to CONTROL (≈95%), while L2 revealed a significant decreased (≈92%) (Fig. 3 A, B). After 5-aza-dC treatment, hydroxymethylated DNA levels were increased in both clones (L1≈117% and L2≈110%) compared to CONTROL and OM (Fig. 3 A, B). The variation of hydroxymethylated DNA levels between OM and 5AZA was ≈22% for L1 and ≈18% for L2 (Fig. 3 A, B). These results show that 5-aza-dC has greater hydroxymethylated DNA modulation effect in both clone L1 and clone L2, where hydroxymethylated DNA levels were greater than methylated DNA levels. The augment of *BMP-2* hydroxymethylated DNA in 5AZA group resulted in upregulation of *BMP-2* transcripts in L1 (17755%) and L2 (1502%) cell clones compared to OM group (100%) (Fig. 3 C). Finally, a positive correlation between hydroxymethylated DNA levels and gene expression of *BMP-2* in L1 ($r = 0.9146$) and L2 ($r = 0.9688$) cells confirmed a cause-effect result of 5-aza-dC on *BMP-2* gene (Fig. 3 D, E).

3.6. *ASPN* gene expression is modulated in 5AZA group only in L2 cell clones

The results of RT-qPCR showed that after 14 days in osteogenic medium, there was no difference between OM (100%) and 5AZA (99%) regarding to mRNA expression of *ASPN* in L1 cell clones (Fig. 4A). However, in L2 cell clones, 5-aza-dC enhanced the expression of *ASPN* to 486% when compared to OM group (100%) (Fig. 4 A).

In order to understand whether 5-aza-dC has an epigenetic effect on *ASPN*, it was also investigated the modulation of methylated and hydroxymethylated DNA levels of this gene. The data showed that, in L1 cell clones, methylated DNA levels of *ASPN* gene increased to 107% in 5AZA group compared to CONTROL (100%) and OM (101%), while hydroxymethylated DNA levels in 5AZA group (104%) were also enhanced compared to CONTROL (100%) but revealed no significant difference compared to OM (106%) (Fig. 4 B). In clone L2, there was an increase in methylated DNA in the group treated with 5-aza-dC (107%) compared to the other groups, while there was no modulation in hydroxymethylated DNA between groups (Fig. 4 C).

To verify whether 5-aza-dC treatment was responsible for the increase of *ASPN* gene expression in L2 cell clones, a linear regression model was employed to test the correlation between *ASPN* hydroxymethylated DNA levels and *ASPN* gene expression levels. The findings showed no correlation between hydroxymethylated DNA and expression of *ASPN* gene in either L1 ($r = 0.6191$) or L2 cell clones ($r = 0.07811$) (Fig. 4 D, E).

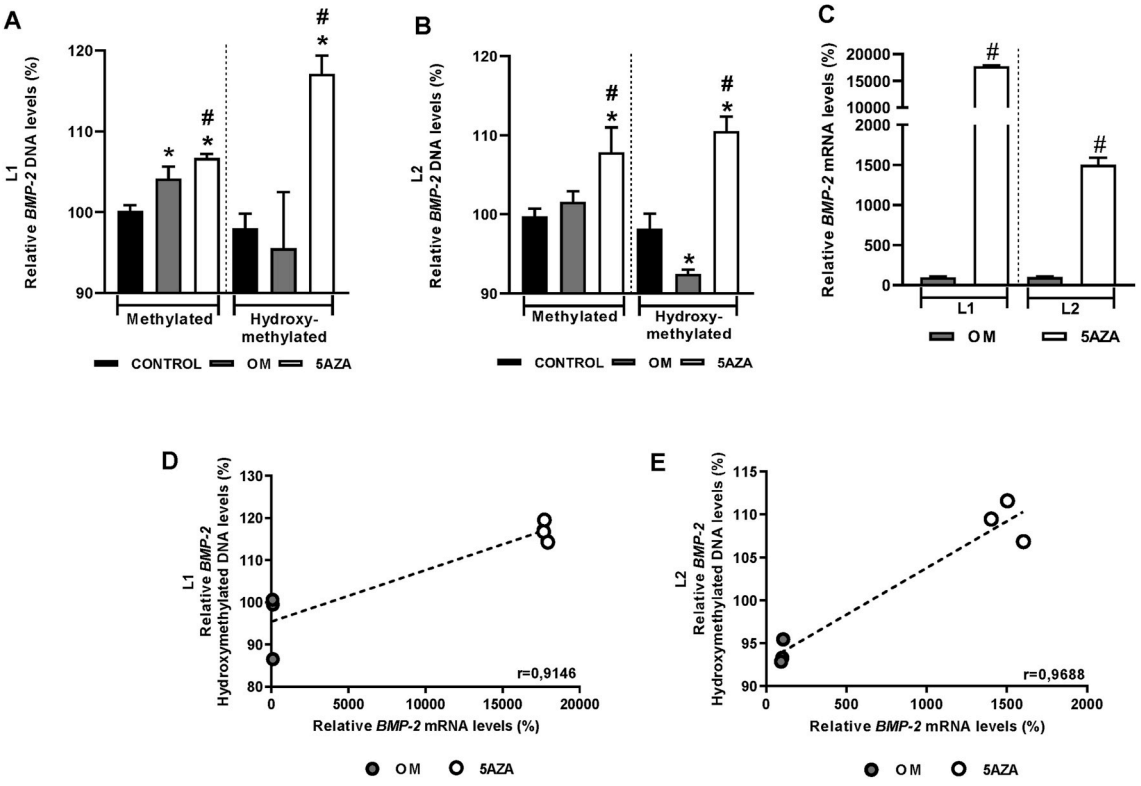

Fig. 3. *5-aza-dC promotes epigenetic modulation of BMP-2 in LOP cell clones.* (A, B) Methylated/hydroxymethylated DNA analysis of *BMP-2* after 14 days of osteogenic induction associated or not with 5-aza-dC pre-treatment in L1 and L2 cell clones, respectively. (C) Real-Time PCR analysis showed an increase of mRNA levels of *BMP-2* in L1 and L2 cell clones after 5-aza-dC pre-treatment associated to osteogenic induction for 14 days. (D, E) Positive correlation between hydroxymethylated DNA levels of *BMP-2* and its transcripts expression levels in L1 and L2 cell clones. Bars in A-C represent mean ± SD where statistical differences compared with CONTROL are indicated by * ($p < 0.05$), and statistical differences between 5AZA *versus* OM are indicated by # ($p < 0.05$).

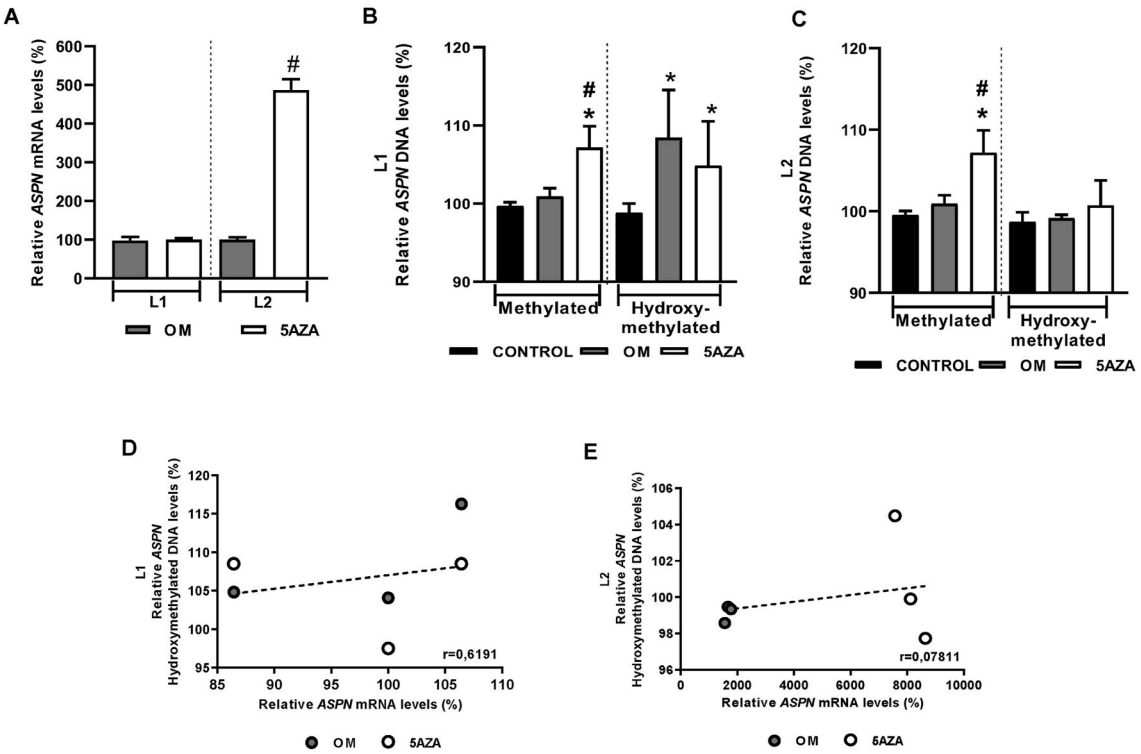

Fig. 4. *Effect of 5-aza-dC on ASPN gene expression.* (A, B) Real-Time PCR analysis showed an increase of mRNA levels of *ASPN* only in L2 cell clones after 5-aza-dC pre-treatment associated to osteogenic induction for 14 days. (C, D) hydroxymethylated DNA levels of *ASPN* after 14 days of osteogenic induction associated or not with 5-aza-dC pre-treatment in L1 and L2 cell clones, respectively. (E, F) No correlation between hydroxymethylated DNA levels of *ASPN* and its transcripts expression levels in L1 and L2 cell clones. Bars in A-C represent mean ± SD where statistical differences compared with CONTROL are indicated by * ($p < 0.05$), and statistical differences between 5AZA *versus* OM are indicated by # ($p < 0.05$).

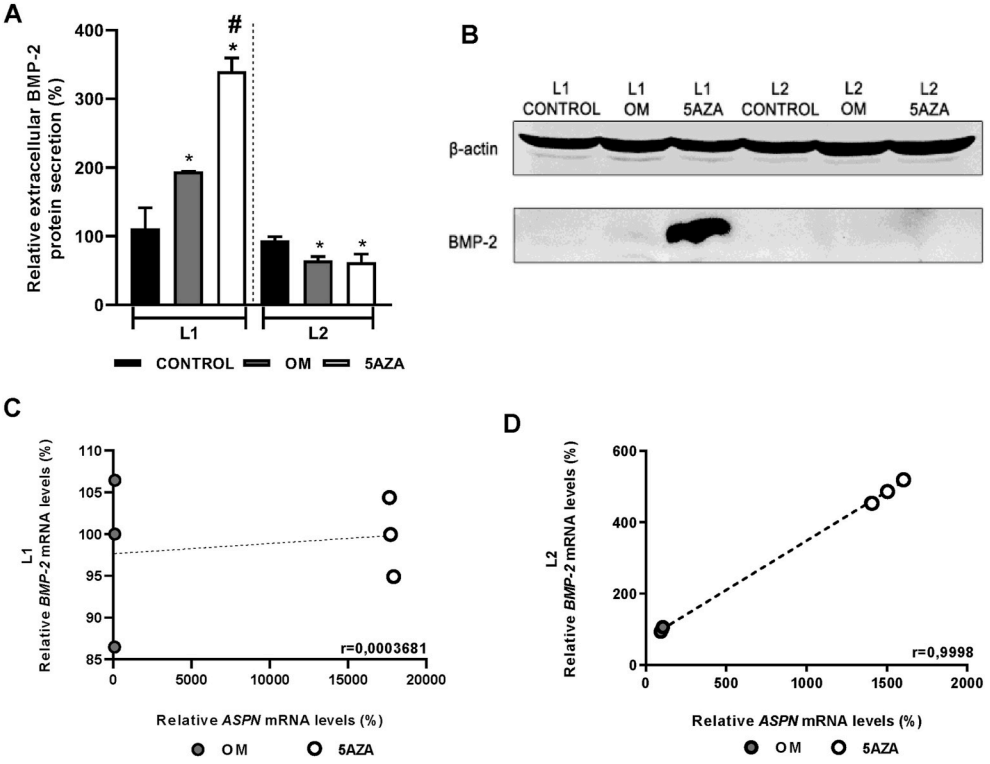

Fig. 5. *BMP-2 protein expression in LOP cell clones.* (A) Quantification of BMP-2 secreted in supernatant of L1 and L2 cell clones after 14 days of osteogenic induction associated or not with 5-aza-dC pre-treatment. (B) Western blot analysis confirmed intracellular BMP-2 expression only in L1 cell clones after 5-aza-dC pre-treatment. (C, D) Positive correlation between *ASPN* and *BMP*-2 mRNA levels in L2 cell clones. Bars in A represent mean ± SD where statistical differences compared to CONTROL are indicated by * ($p < 0.05$), and statistical differences between 5AZA *versus* OM are indicated by # ($p < 0.05$).

3.7. Intracellular and extracellular expression of BMP-2 protein are different in two LOP cell clones after 5-aza-dC pre-treatment

To understand the distinct response of L1 and L2 cell clones after 5-aza-dC treatment, it was analyzed the presence of BMP-2 in extracellular and intracellular environment in both clones after 14 days of osteogenic induction.

The results of the ELISA assay showed significantly higher levels of BMP-2 in supernatant of L1 cells after osteogenic induction (194%) compared to CONTROL group (100%) (Fig. 5 A). Furthermore, 5-aza-dC pre-treatment increased BMP-2 secretion to 340% compared to OM group (Fig. 5 A). On the other hand, in L2 cell clones, BMP-2 levels decreased in OM group (64%), and even after administration of 5-aza-dC, the levels of secreted protein remained lower (61%) than CONTROL group (100%) (Fig. 5 A).

The Western Blot assay was performed to analyze the expression of intracellular protein, and the data revealed the presence of BMP-2 only in L1 cell clones treated with 5-aza-dC (Fig. 5 B). These results suggest that even with the increase of the *BMP-2* gene expression, L1 and L2 present different behavior regarding to the expression of the BMP-2 protein.

Since some studies have reported that *ASPN* acts as a negative regulator of mineralization, a linear regression model was also employed to test the correlation between *BMP-2* and *ASPN* gene expression levels. It was found a positive correlation between the expression of the *BMP-2* gene and *ASPN* gene expression in L2 ($r = 0.9998$), but not in L1 ($r = 0{,}0003681$) after 5-aza-dC treatment (Fig. 5 C, D). These data suggest that *ASPN* initiates a negative *BMP-2* feedback loop which may be important in controlling the BMP-2 protein expression and consequently, the differentiation of L2 cell clones towards osteoblast/cementoblast phenotype.

3.8. NOGGIN and BMP-2 recombinant protein modulate the effect of 5-aza-dC pre-treatment on the matrix mineralized deposition

To verify that BMP-2 signal was responsible for the osteogenic phenotype induced by 5-aza-dC pre-treatment, L1 cells were cultured in osteogenic medium supplemented with rhNoggin, an antagonist of BMP signaling pathway. After 28 days, it was observed a significative reduction of mineralized matrix deposition in 5AZA + NOGGIN group compared to 5AZA only ($p < 0.05$) (Fig. 6A). On the other hand, the treatment of L2 cells with 5-aza-dC followed by exposure to rhBMP2 promoted a significant increase in the mineral nodule deposition compared to 5-aza-dC alone ($p < 0.05$) (Fig. 6B). These findings show that 5-aza-dC-mediated osteoblast differentiation of L1 cells occurs through BMP2 signaling pathway. At the same time, the lack of BMP-2 protein expression in L2 cells pretreated with 5-aza-dC contributed to the low production of mineralized matrix production.

4. Discussion

Periodontal dental ligament harbors a heterogenous mesenchymal progenitor cells, and a great part of them present low potential to differentiate into mineralized-tissue producing cells, including osteoblasts and cementoblast (Seo et al., 2004; Singhatanadgit et al., 2009; Silvério et al., 2010; Sununliganon and Singhatanadgit, 2012; Saito et al., 2014). In this present study, sixteen cell clones were isolated from a CD105-enriched PDL progenitor cell population using the cloning ring technique, based on their ability to form single cell–derived colonies. CD105 was chosen for sorting progenitor cells from PDL heterogeneous population because it is the most frequently reported positive surface marker in hPDLSCs (Silvério et al., 2010). Furthermore, the expression of CD105 is one of the minimal criteria to define human MSCs according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici et al., 2006). Because the formation of mineralized tissues (alveolar bone and cementum) is critical for periodontal regeneration, the currently study investigated whether 5-aza-dC, an epigenetic modulator, could stimulate the osteoblast/cementoblast differentiation of periodontal ligament mesenchymal progenitor cells, characterized as low osteoblast potential, through *BMP-2* modulation.

Under osteogenic condition, the sixteen PDL-CD105^{+} clones showed distinct potential to differentiate toward osteoblast/cementoblast phenotype. Among these, only five cell clones (33%) presented ability to produce high quantity of mineralized matrix *in vitro*, while the others 11 clones were identified as low osteoblast/cementoblast potential. This finding is in agreement with previous studies that reported a range of 10–50% of PDL mesenchymal progenitor cells with potential to give rise to osteoblast/cementoblast-like cells (Singhatanadgit et al., 2009; Sununliganon and Singhatanadgit, 2012; Saito et al., 2014). The high osteoblast/cementoblast potential of HOP cells was confirmed by the upregulation of osteoblast genes markers, such as *RUNX2, OCN* and *ALP* after 14 days under osteogenic condition. On the other hand, in low osteoblast/cementoblast potential cells (LOP) it was observed a down-regulation of *RUNX2* and *OCN* mRNA levels. Considering that *RUNX2* is a transcription factor that drives the expression of other genes linked to osteogenesis (Banerjee et al., 1997; Komori et al., 1997), and osteoblast/cementoblast maturation seems to occur in the presence of *OCN* expression (Ducy and Karsenty, 1995), it is possible to suggest that LOP cells are not committed to differentiation to osteoblast/cementoblast phenotype.

This is further supported by the pattern of gene expression of *BMP-2* and *ASPN* during osteogenic induction. *BMP-2*, a potent inductor of osteoblast/cementoblast differentiation, was downregulated in HOP and LOP cells at 14 days of osteogenic induction. However, the reduction of transcripts levels was significantly higher in LOP cells (80%) compared to HOP cells (18%). Further, the pronounced suppression of *BMP-2* transcripts in LOP cells was accompanied by the increase of *ASPN* gene, suggesting that asporin may play a role on blocking the differentiation of LOP cells toward osteoblast/cementoblast phenotype as observed by previous studies in BMSCs (Sun et al., 2014) and PDL cells (Yamada et al., 2006; Yamanda et al., 2007; Ueda et al., 2016).

As described above, the periodontal ligament tissue contains a low proportion of HOP cells, which have capacity to regenerate alveolar

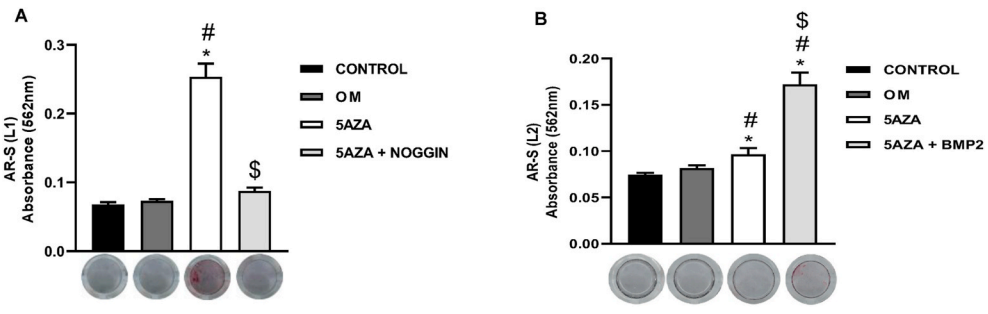

Fig. 6. *Effect of Noggin and BMP2 on mineral deposition after 5-aza-dC treatment.* Quantification of Alizarin Red Staining (AR-S) in L1 (A) and L2 (B) cells after 5-aza-dC pre-treatment and cultured in osteogenic medium supplemented with Noggin or BMP2 at 28 days. Bars represent mean ± SD where statistical differences compared with CONTROL are indicated by * ($p < 0.05$), statistical differences compared with OM are indicated by # ($p < 0.05$), and statistical differences between 5AZA *versus* 5AZA + NOGGIN (A) or 5AZA + BMP2 (B) by $ ($p < 0.05$).

bone e cementum. In this context, the enhancement of LOP cells to differentiate into osteoblast- and cementoblast-like cells may be an approach to improve predictability of periodontal tissue regeneration. 5-aza-2'-deoxycytidine is a drug that can be incorporate into DNA during cell division and interacts with *DNMT1,* leading to demethylation of DNA (Christman, 2002). This drug is approved to use in the treatment of myelodysplastic syndrome (Mund et al., 2005), and some studies have already reported an improvement in the osteogenic potential of different cells types treated with this epigenetic modulator (Cho et al., 2017; Kadler et al., 2020). Here, 5-aza-dC was effective to increase the levels of *BMP-2* hydroxymethylation, an identified intermediate step during DNA demethylation (Richa and Sinha, 2014), and consequently, increased *BMP-2* gene expression in LOP cells. This effect of 5-aza-dC on *BMP-2* gene were also observed in rabdhomyosarcoma (RMS) cells (Wolf et al., 2014) and renal cell carcinoma (RCC) (Mitsui et al., 2015).

However, the positive modulation of *BMP-2* gene promote by 5-aza-dC did not result in similar expression of the osteoblast-related genes when compared two LOP cell clones (L1 and L2 cells). The results of RT-qPCR revealed an increase of *RUNX2*, *ALP* and *OCN* transcripts in L1 cells, while L2 showed a downregulation of these genes. As a result of this expression profile, L1 cells produced a significantly higher amount of mineralized matrix compared to L2 cells as observed in AR-S and XO-S results.

These findings can be explained by the fact that intracellular and secreted form of BMP-2 protein were found only in L1 cells. BMP-2 signaling molecules are require to activate phosphorylation and nuclear translocation of SMAD proteins, which interact with *RUNX2* resulting in upregulation of *ALP* and *OCN* transcripts, and consequently, in osteoblast differentiation (Chen et al., 2004; Hyun et al., 2011; Silvério et al., 2012). Thus, it is possible to suggest that even with the increase of *BMP-2* gene expression in L2 cells pretreated with 5-aza-dC, BMP-2 signaling pathway has not been activated, because these cells did not secreted BMP-2 protein necessary to mediate the signals by type I and II BMP receptors (Hayrapetyan et al., 2015). In fact, when L2 cells were exposure to rhBMP2 after 5-aza-dC pre-treatment, the deposition of mineralized matrix significantly increase, whereas the treatment of L1 cells with Noggin reduced the mineral deposition, since Noggin inhibits BMP2 signaling pathway by blocking the type I and II BMP receptors (Chen et al., 2004).

Additionally, it is plausible to suggest that the increased expression of *ASPN* gene in L2 cells is also related to the limited osteoblast/cementoblast differentiation induced by 5-aza-dC. Asporin was identify as a negative regulator of PDL cells differentiation and mineralization suppressing BMP-2-induced osteoblast differentiation (Yamada et al., 2007). Moreover, the levels of *ASPN* transcripts seems to be regulated by the increase of *BMP-2* expression in PDL cells, suggesting a crosstalk between these two genes (Yamada et al., 2007). Here, the upregulation of *BMP*-2 mRNA levels promoted by 5-aza-dC was also accompanied by an increase of *ASPN* gene expression in L2 cell clones, which may have inhibited BMP-2 protein synthesis by a mechanism that still needs to be better understood.

The reason why a modulation of *ASPN* gene expression did not occur in L1 cell clones is uncertain. A plausible explanation could be the fact that even between LOP cells, there is still a great heterogeneity among $CD105^+$ populations of periodontal ligament. And, L1 cells may also be more committed to osteoblast/cementoblast phenotype, requiring only a signaling to differentiate and produce mineralized matrix (Silvério et al., 2010; Saito et al., 2014). Also, it is possible to suggest that inhibitory mechanism for osteoblast/cementoblast differentiation exist within the PDL to prevent the mineralization of this tissue (Yamada et al., 2006). Nevertheless, further studies are required to better understand the crosstalk between BMP-2 and ASPN signaling pathways, and to elucidate how this interaction can drive the osteoblast/cementoblast differentiation.

In conclusion, this current study showed that 5-aza-dC improves osteoblast/cementoblast differentiation of PDL-$CD105^+$ cells through BMP-2 secretion, and this effect depends on low levels of *ASPN* expression. These findings not only increase the comprehension about the processes governing periodontal development, but also give insight into the pathways that are of value to modulate periodontal tissue regeneration.

5. Conclusions

This study showed that 5-aza-dC improves osteoblast/cementoblast differentiation of PDL-$CD105^+$ cells through BMP-2 secretion, and this effect depends on low levels of *ASPN* expression. These findings not only increase the comprehension about the processes governing periodontal development, but also give insight into the pathways that are of value to modulate periodontal tissue regeneration.

Authors contribution

C.M.S. methodology, formal analysis, investigation, data curation, writing the original draft. R.I.F.A. methodology, validation, investigation. M.T.S. validation, investigation. R.D.C. methodology, investigation, tesources. M.R.D. validation, investigation. E.A.S. resources, writing the original draft. F.H.N Jr. conceptualization, methodology, writing the original draft. R.C.V.C. formal analysis, writing the original draft. D.C.A. conceptualization, methodology, resources, formal analysis, writing the original draft. K.G.S. conceptualization, methodology, writing the original draft, supervision, project administration, funding acquision.

Ethical issues

This study was approved by the Institutional Review Board of Piracicaba Dental School–University of Campinas (#030/2017), São Paulo, Brazil.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported in part by the Fundação de Amparo à Pesquisa do Estado de São Paulo, São Paulo, Brazil (Grant 2017/19697-2), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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