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DOI:
10.1089/dna.2020.6239

Document Version
Peer reviewed version

Citation for published version (Harvard):
Assis, RIF, Schmidt, AG, Racca, F, da Silva, RA, Zambuzzi, WF, Silvério, KG, Nociti Júnior, FH, Pecorari, VG, Wiench, M & Andia, DC 2021, 'DNMT1 inhibitor restores RUNX2 expression and mineralization in periodontal ligament cells', *DNA and Cell Biology*, vol. 40, no. 5, pp. 662-674. https://doi.org/10.1089/dna.2020.6239

DNMT1 inhibitor restores RUNX2 expression and mineralization in periodontal ligament cells

Short title: DNMT inhibitor restores osteogenic potential.

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ABSTRACT
Periodontal ligament cells (PDLCs) have well documented osteogenic potential; however, this commitment can be highly heterogenous, limiting their applications in tissue regeneration. Here we use PDLC populations characterized by high and low osteogenic potential (h-PDLCs and l-PDLCs, respectively) to identify possible sources of such heterogeneity and to investigate whether the osteogenic differentiation can be enhanced by epigenetic modulation. In h-PDLCs, low basal expression levels of pluripotency markers (*NANOG, OCT4*), DNA methyltransferases (*DNMT1*, *DNMT3B*) and enzymes involved in active DNA demethylation (*TET1*, *TET3*) were prerequisite to high osteogenic potential. Furthermore, these genes were downregulated upon early osteogenesis, possibly allowing for the increase in expression of the key osteogenic transcription factors, *RUNX2* and *SP7*, and ultimately, mineral nodule formation. l-PDLCs appeared locked in the multipotent state and this was further enhanced upon early osteogenic stimulation, correlating with low *RUNX2* expression and impaired mineralization. Further upregulation of *DNMTs* was also evident while pre-treatment with RG108, the DNMTs' inhibitor, enhanced the osteogenic program in l-PDLCs through downregulation of *DNMTs*, increased RUNX2 expression and nuclear localization, accelerated expression of osteogenic markers and increased mineralization. These findings point towards the role of DNMTs and TETs in osteogenic commitment and support application of epigenetic approaches to modulate biomineralization in PDLCs.

Key words: Periodontal ligament stem cells, osteogenic potential, RUNX2, DNA methyltransferases, RG108

INTRODUCTION
Mesenchymal stem cells (MSCs) are widely distributed across adult connective tissues and are known for their self-renewing ability and the capacity to differentiate into osteogenic, chondrogenic and adipogenic cell lineages (Dominici et al., 2006; Huang et al., 2009). Periodontal ligament cells (PDLCs) obtained from mature periodontal ligament have been shown to display stem cell properties similar to other MSCs (Seo et al., 2004). As such, PDLCs express genes responsible for maintenance of pluripotency and undifferentiated state, such as POU5F1-POU-class-5-homeobox-1 *(OCT4)* and NANOG-Homeobox *(NANOG)* (Kawanabe et al., 2010). Both were demonstrated to be involved in inhibiting spontaneous differentiation in MSCs (Tsai et al., 2012). When cultivated under osteogenic conditions, PDLCs differentiate into osteoblast-like cells *in vitro* (Gay et al., 2007; J. Liu et al., 2020; Seo et al., 2004). This is associated with up-regulation of osteoblast-related genes, such as Runt-related transcription factor 2 (*RUNX2*) which encodes for an essential and initiating transcription factor required for osteoblastogenesis (Enomoto et al., 2003; Komori et al., 1997; Li et al., 2011). However, the differentiation potential can vary greatly for PDLCs isolated from different donors (Silvério et al., 2010). Our group has recently characterized clones of PDLCs presenting high (h-PDLCs) and low (l-PDLCs) capacity for osteoblastic differentiation (Saito et al., 2014), however, the mechanisms responsible for such distinct phenotypes remain to be identified.
Changes in cell phenotype during osteoblastogenesis involve suppression of genes required for stem cell maintenance and concurrent activation of bone related genes. These changes

are often controlled by epigenetic mechanisms and require chromatin modification at the genes undergoing regulation (Barrand & Collas, 2010; G. Fu et al., 2016; Yeo et al., 2007). In addition to histone modifications and chromatin remodelling, DNA methylation (5mC) contributes to the control of the transcription factors' accessibility to regulatory elements (Eslaminejad et al., 2013; Vincent & Van Seuningen, 2009; Wiench et al., 2011). 5mC deposition is catalysed by DNA methyltransferases (DNMTs); in addition, Ten Eleven Translocation (TET) enzymes promote the oxidation of 5mC, converting it to 5-hydroxymethylcytosine (5hmC), an intermediary in the process of active DNA demethylation (Tahiliani et al., 2009).
Compounds, such as DNA methylation inhibitors have been used to experimentally manipulate epigenetic patterns and cell fate specifications (Zhai et al., 2018). RG108 specifically inhibits the enzymatic activity of DNMTs (Brueckner et al., 2005), leading to global DNA demethylation, increase in TFs accessibility to chromatin and gene activation. Here, we use two populations of primary PDLCs with different osteogenic potential to investigate how epigenetic regulation contributes to osteoblastic differentiation and whether it could be enhanced by the demethylating drug RG108.

MATERIAL AND METHODS

Cell acquisition and culture: PDLCs were collected from extracted third molars from subjects aging between 20 and 22 years, after signing an informed consent approved by the Ethics Committee of Piracicaba Dental School, University of Campinas (CAAE55588816.4.0000.5418). PDL tissue isolation and culture of PDL-derived cells were performed as previously described (Silvério et al., 2010) and detailed in Supplementary Information. PDLCs were characterized to confirm their ability to differentiate towards osteogenic lineage (Alizarin Red staining) and to assess population doubling times .

Osteogenic stimulation

To investigate early changes following osteogenic differentiation, two PDLC populations presenting either high (h-PDLCs) or low (l-PDLCs) capacity to form mineral nodules *in vitro* were plated in osteogenic induction medium (OM) (Lonza, Walkersville, MD, USA) in 150 mm dishes for 72h (OM group), after which the cells were collected for DNA, RNA or protein extraction. The results were compared to cells maintained in DMEM media for 72h (DMEM group). To investigate later stages of osteogenic differentiation the cells were cultured for 21 days with media change every three days.

Assessment of osteogenic potential after RG108 and DMSO pre-treatments

In order to investigate the effects of RG108 and DMSO on PDLCs osteogenic potential, the cells were pre-treated with 50 µM RG108 (DMSO as vehicle) for 3 days and either collected at this stage (RG108) or subsequently cultured in OM (OM/RG108) for either 3 days or 21 days [22]. For control purposes, PDLCs were pre-treated with an equivalent concentration of DMSO (0.025%) with (OM/DMSO) or without (DMSO) the subsequent OM stimulation. After 14 days of osteogenic induction, PDLCs were subjected to xylenol orange staining (Supplementary Information). Photomicrographs and Alizarin Red staining were performed at 21 days.

To evaluate cytotoxicity of treatments, viability and apoptosis assays were performed using MTT (Invitrogen™, Thermo Fisher, UK) and PE-7AAD/FITC-Annexin V (BD Pharmingen™, San Jose, CA, USA), respectively (Supplementary Information).

DNA, RNA and protein extraction

PDLCs were seeded at 2.23 x 10^6 cells in 150 mm dishes in DMEM, treated according to the experimental groups and collected for DNA, RNA and protein extraction (details in Supplementary Information).

mRNA expression analysis

cDNA synthesis was performed using 1 µg RNA as described previously (Assis et al., 2018). qPCR reactions were carried out using LightCycler 480 Real Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) and FastStart Essential DNA Green Master (Roche Diagnostic Co., Indianapolis, IN, USA) according to the manufacturer's instructions and in technical triplicates. The primers' sequences and reaction details are shown in Supplementary Table 2. The results of three biological replicates were analysed by $\Delta\Delta$Ct method (Schmittgen & Livak, 2008) and are presented as relative amounts of the target gene using *ACTB* or *18S* as inner reference genes.

Gene-specific DNA methylation and hydroxymethylation analysis

To distinguish between methylated (mC) and hydroxymethylated (hmC) CpGs the genomic DNA was initially treated with T4-β-glucosyltransferase (T4-BGT) and UDP glucose (New England Biolabs, Beverly, MA, USA), adding glucose moiety to 5hmC and generating ghmC. Subsequently, samples were digested with *Msp*I which is blocked by ghmC but not mC or hmC, while *Hpa*II which is blocked by all three modifications. The reactions' details are described in the Supplementary and all reagents were purchased from New England Biolabs, Beverly, MA, USA. 40 ng of digested gDNA was subjected to 40 amplification cycles using gene-specific primers (Supplementary Table 3) as described previously (Assis et al., 2018). The PCR product following *MspI* digestion identifies 5hmC, while total modification (5mC and 5hmC) are detected after *HpaII* digestion. See Supplementary for explanation regarding amplicon locations, primers design, control reactions and percentage calculation of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) at CCGG sites.

Western Blotting analysis

The nuclear/cytoplasmic localization assay was performed as previously described (Ferreira et al., 2004) with details of cytoplasmic (CE) and nuclear (NE) fractions preparation shown in Supplementary Information. A total of 75 mg of either CE or NE was resolved by SDS-PAGE and blotted using primary antibodies (see Supplementary). Densitometric analysis of blots was performed using the ImageJ software and the graphical representation using the GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, EUA).

Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked by the Brown-Forsythe and Shapiro-Wilks test. *NANOG*, *OCT4*, *DNMT3A, TET1* data met the presuppositions of parametric analysis (two-way ANOVA); *RUNX2*, *DNMT1*, *DNMT3B, TET2* data were transformed to log10 to satisfy the assumptions of the two-way ANOVA; for *TET3* data the Kruskal-Wallis and Dunn tests were performed. To compare the OM between

PDLCs, the data were analyzed by t-test for homogeneous (*OCT4*, *DNMT3A*, *DNMT3B, TET1*, *TET2, TET3*, *RUNX2, SP7*) or heterogeneous variances (*NANOG, DNMT1*). The analyses had a 5% significance level and were performed by Prisma GraphPad Software (Version 5, San Diego, CA, USA) or in the GLM procedure in the SAS/STAT® software (Version 9.3, SAS Institute Inc., Cary, NC, USA).

RESULTS

Basal expression pattern of pluripotency genes, DNMTs and TETs differs in h-PDLCs and l-PDLCs.

PDLCs isolated from third molars of healthy young donors were characterised for their ability to differentiate towards osteogenic lineage (Fig. 1a-c). The two isolates clearly had different capacity to form mineral nodules upon 21 days of osteogenic induction despite similar growth rates (Fig. 1d,e). Henceforth these two populations are referred to as h-PDLCs (high potential) and l-PDLCs (low potential).
To investigate the underlying basis for this variability, the basal/unstimulated transcript levels and associated promoter methylation were analysed for genes involved in pluripotency (*NANOG* and *OCT4*), early regulation of bone formation (*RUNX2* and *SP7*) and establishing DNA methylation pattern (*DNMT*s and *TET*s). The expression of *NANOG* and *OCT4* was much higher in l-PDLCs compared to h-PDLCs (Fig. 1f). In addition, l-PDLCs also had higher expression of *DNMT1*, *DNMT3B* and all *TET* genes, while *DNMT3A* transcript levels were found to be low in both PDLCs (Fig. 1g-h). However, *RUNX2* and *SP7* were expressed similarly in the two populations either at a moderate (*RUNX2*) or hardly detectable (*SP7*) levels (Fig. 1i), which agrees with the fact the cells were under unstimulated conditions All genes tested had DNA methylation levels permissive of transcription (21.87% - 37.79%) and low levels of DNA hydroxymethylation (~1%) at their promoters (Supplementary Fig. 1 and Table S1). For *DNMT1*, the anti-correlation between gene expression and promoter methylation was observed, suggesting methylation could play a role in modulating *DNMT1* expression in PDLCs (Fig. 1j).
These data indicate l-PDLCs could be locked in the multipotent state characterized not only by high gene expression of *NANOG* and *OCT4* but also by increased levels of both *DNMT*s and *TET*s.

Osteogenic induction triggers different gene expression changes in h-PDLCs and l-PDLCs

To study the events occurring in early osteogenesis, gene expression and DNA methylation changes were analysed in h- and l-PDLCs stimulated for 3 days *in vitro* in the osteogenic media (OM). In response to differentiation, the expression of *DNMT3A* and *DNMT3B* decreased in h-PDLCs (Fig. 2a), further increasing the difference between h- and l-PDLCs previously observed in unstimulated conditions (Fig. 1g). Additionally, *TET2* expression was also down-regulated in h-PDLCs while up-regulated in l-PDLCs (Fig. 2b).
In h-PDLCs, the expression of *NANOG* moderately decreased (Fig. 2c) while *RUNX2* and *SP7* increased (Fig. 2d), reflecting the changes expected at the start of osteo-differentiation. In l-PDLCs however, *OCT4* up-regulation and *RUNX2* and *SP7* down-regulation (Fig. 2c-d) were observed. Generally, the changes in gene expression were not accompanied by changes in DNA methylation and hydroxymethylation in the promoter regions analyzed

(Supplementary Fig. 2 and Table S1) in both epigenetic and multipotency markers, except in the promoter region of *DNMT3A* in h-PDLCs (Fig. 2e).
In summary, in h-PDLCs the osteogenic induction correlated with the decrease in multipotency marker *NANOG* and the expected increase in bone markers *RUNX2* and *SP7*. This was also accompanied by further down-regulation of *DNMTs*. In contrast, the opposite changes were observed in l-PDLCs suggesting the osteogenic stimulation at the early stage leads to the enhancement of multipotent state, therefore delaying the onset of differentiation.

RG108 enhances osteogenic potential in l-PDLCs which correlates with up-regulation of RUNX2 and down-regulation of DNMTs.
The data obtained so far suggest that the difference in the osteogenic potential of the two PDLCs populations correlates not only with the basal and stimulated levels of multipotency and bone markers but is also associated with the expression of *DNMT*s and *TET*s. Therefore, we next investigated whether inducing demethylation by using RG108 could promote changes in l-PDLCs favouring osteogenic differentiation. We have previously shown in BMSCs that RG108 treatment leads to 75% decrease in DNMTs activity and DNA methylation by 42%. 50 µM RG108 was used due to minimal effects on cell death and cell viability in l-PDLCs (Supplementary Fig. 3 a-d).
Firstly, we investigated the effect of RG108 on formation of mineral nodules *in vitro.* An enhanced mineralization was observed in l-PDLCs after 21 days of osteogenic differentiation when the cells were pre-treated with 50 µM RG108 for 72h (Fig. 3a, b). Interestingly, DMSO, the vehicle alone, also promoted the osteogenic phenotype. Both effects were independently confirmed in a second l-PDLC population where the effect of RG108 was significantly more pronounced than for DMSO alone (Supplementary Fig. 4).
The pro-osteogenic effect of RG108 in l-PDLCs was associated with changes in gene expression levels, most specifically down-regulation of *DNMT*s and *TET2* observed in both basal and stimulated conditions (Fig. 3c, d). DMSO treatment resulted in similar, although less pronounced changes (Fig. 3c, d). The expression of *TET1* and *TET3* was upregulated in the OM condition only and this effect was observed for RG108 but not for DMSO pre-treated cells (Fig. 3d). In addition, the levels of *NANOG* and *OCT4* also slightly increased upon RG108/OM treatment, at least at day 3 (Fig. 3e). Despite the observed gene expression changes, DNA methylation and hydroxymethylation levels were not affected (Supplementary Table 1). Most importantly, the level of *RUNX2* transcript significantly increased following RG108 as compared to both DMEM and OM treatments (Fig. 3f) with smaller effect observed for DMSO. This increase in *RUNX2* expression in l-PDLCs was associated with a decrease in DNA methylation at the promoter, especially when compared to the initial unstimulated state (Fig. 3g, h). This could be caused directly by RG108, or indirectly through low levels of *DNMTs* in combination with the observed up-regulation of *TET1* and *TET3* levels (as shown in Fig. 3d).
Interestingly, in h-PDLCs the pre-treatment with both RG108 and DMSO promoted a decrease in the formation of mineral nodules, associated with a decrease in *RUNX2* expression (Supplementary Fig. 5a-c). Whereas the expression of *DNMTs* (already low upon the OM stimulation in h-PDLCs), *TETs* and *NANOG*/*OCT4* (Supplementary Fig. 5d-f) did not change upon pre-treatments.
Therefore, in l-PDLCs, the enhanced mineral nodule formation observed following RG108 pre-treatment could be caused by an increase in *RUNX2* expression associated with the

promoter demethylation. The pre-treatment also led to down-regulation of *DNMTs* bringing their expression to the levels comparable with those observed in h-PDLCs (Supplementary Fig. 6).

RG108 promotes RUNX2 dominance in the nucleus.

Both RG108 and DMSO enhanced the osteogenic potential of l-PDLCs, with distinct increase in the *RUNX2* expression profile by RG108, suggesting different underlying mechanisms. Therefore, RUNX2 protein localization was next investigated.

RUNX2 is a transcription factor and as such nuclear localization is prerequisite to its function. As expected, RUNX2 was predominantly nuclear upon osteogenic stimulation. This was significantly enhanced in the presence of RG108 but not DMSO (Fig. 4a). However, the initial nuclear localization of NANOG and OCT4, the TFs maintaining pluripotency, was altered by DMSO alone with both proteins mostly retained within the cytoplasm (Fig. 4b, c). Therefore, both pre-treatments lead to a change in ratio between pro-osteogenic (RUNX2) and pluripotent (OCT4 and NANOG) factors in the nucleus resulting in RUNX2 dominance (Fig. 4d-e). Such change within the nuclear environment could subsequently enhance the early stages of osteogenic differentiation.

RG108 accelerates osteogenic program

The effects of RG108 and DMSO on osteogenic program were assessed through expression of several osteogenic markers over the course of osteogenesis (early - 3rd day, middle - 10th day, late -21st day): *RUNX2, miR32* (known to regulate osteoblastic gene markers - Liu et al., 2017), *SP7*, bone morphogenetic protein 2 (*BMP2*), alkaline phosphatase (*ALPL*), osteocalcin (*BGLAP*) and secreted phosphoprotein 1 (*SPP1*). An early expression peak was observed for the majority of the osteoblastic gene markers after RG108 pre-treatment compared to all other conditions and importantly, these included the main pro-osteogenic TFs, *RUNX2* and *SP7* (Fig. 5a). In detail, on 3rd day, RG108 increased *RUNX2*, *SP7*, *BMP2* and *BGLAP* transcripts, while on 10th day, it was observed for *SP7*, *BGLAP,* and *SPP1*. *miR32* was found to be expressed at a precocious manner after both pre-treatments with a peak as early as day 3, compared to day 10 in the OM-only media.

The expression changes of osteogenic markers were accompanied by time-dependent reduction in *NANOG* transcript over later time points in RG-108 pre-treated cells (Fig. 5a). However, mRNA for *OCT4* showed a peak on 10th day, following RG108 treatment. This was unexpected, although it is worth noting the expression of *OCT4* was generally very low in all conditions tested so far.

The data suggest that RG108-specific acceleration of *RUNX2* up-regulation and its nuclear dominance over the pluripotency TFs accompanied by a down-regulation of *DNMTs* and *NANOG* could result in the observed enhanced mineralization in l-PDLCs (Fig. 5b).

Discussion

PDLCs are good candidates for exploitation in tissue regeneration due to their availability, proliferation rates and differentiation potential (Y. Liu et al., 2014; Shi et al., 2005). However, mechanisms underlying their heterogenous commitment to osteogenesis are still poorly understood. Here, we minimized the possible bias by controlling for donors' age, methods for cell collection and cell culture, however, the heterogeneity was still apparent in the osteoblastic phenotype acquired. Subsequently, we identified at least some sources of

such heterogeneity by comparing h-PDLCs and l-PDLCs and investigating the role of epigenetic modulation in control of cell differentiation and mineral nodule production *in vitro*.

In h-PDLCs, low basal expression of pluripotency markers *NANOG* and *OCT4* as well as *DNMTs* and *TETs* was a prerequisite for high osteogenic potential. *NANOG* and *DNMT*s were further downregulated in h-PDLCs upon early osteogenesis, possibly allowing for phenotypic change and increase in *RUNX2* expression. Expression of genes related to stemness is known to decrease during osteogenic differentiation in MSCs (Dansranjavin et al., 2009; Mortada & Mortada, 2018). Similarly, *DNMT1* and *DNMT3B* have been reported to be downregulated upon differentiation *in vitro* (Sun et al., 2019). Interestingly, l-PDLCs not only had an opposite basal expression pattern of the studied genes but this pattern was enhanced upon osteogenic induction, followed by decrease in *RUNX2* expression and low level of mineralization.

Due to differential expression of genes implicated in DNA methylation we hypothesized that exposure to RG108 could enhance osteogenesis in l-PDLCs. The treatment resulted in significant downregulation of *DNMT*s, bringing them to the levels comparable to h-PDLCs. It was accompanied by a significant increase in *RUNX2* expression and, ultimately, mineralization. The timeline of differentiation in l-PDLCs after RG108 treatment is reminiscent of a known pattern of osteogenesis with *RUNX2* upregulation in early stages followed by a decline in later stages (Maruyama, 2007; Komori, 2018). Surprisingly, *TET1* and *TET3* genes were specifically upregulated by RG108 in l-PDLCs. Further investigation is needed regarding whether and how this increase contributes to enhanced osteogenesis in l-PDLCs, however, similar effect was previously observed in hBMSCs (Assis et al., 2018). In addition, *TETs'* up-regulation has been observed during *in vitro* differentiation (Gao et al., 2013) while loss of TET proteins caused differentiation block (Reimer et al., 2019). RG108 treatment also led to a sustained decrease of *NANOG* transcript over the 21 day of differentiation, the pattern not observed in the two controls. This could allow for the differentiation to proceed in l-PDLCs. Although, the peak of *OCT4* expression was as late as day 10 upon RG108/OM stimulation, this would occur within low expression levels and could have smaller impact compared to the highly expressed *NANOG*.

The promoter demethylation during osteogenesis is necessary to allow for expression of genes related to osteoblast lineage such as *BGLAP*, *RUNX2* and *SP7* (N. Fu et al., 2013; Villagra et al., 2002). Here, *RUNX2* promoter methylation is indeed slightly higher in l-PDLCs, compared to h-PDLCs and RG108 decreases it significantly in l-PDLCs. However, the magnitudes of methylation changes are rather small, therefore likely reflect on gene regulation rather than cause it. In addition, the expression changes in *DNMTs* and *TETs* following RG108 pretreatment were not accompanied by methylation and hydroxymethylation changes at their promoters. Therefore, the exact mechanism through which RG108 alters the expression profile remains to be established, although the results could also be explained by the fact all genomic regions analyzed were within proximal promoters and limited to one region per gene. It is possible that methylation changes at distant promoters or enhancers are primarily involved in the fine-tuning of transcriptional regulation during osteogenesis or that not relevant CpGs were targeted in the analysis. Regardless of the methylation levels, increase in *RUNX2* expression and nuclear localization appears to be the main effect underlying enhanced osteogenesis triggered by RG108 in l-PDLCs, likely facilitating the RUNX2-driven osteogenic program.

In addition to RG108, its vehicle DMSO itself also significantly enhanced osteogenic potential of l-PDLCs. DMSO is a hydrogen bond disrupter and cryoprotectant, used as a vehicle for drug therapy and its side-effects are rarely studied or reported (Pal et al., 2012). However, some studies have shown DMSO having impact on *DNMT*s (Assis et al., 2018; Iwatani et al., 2006; Thaler et al., 2012), *OCT4* and *NANOG* (Czysz et al., 2015), *RUNX2* and *SP7* (Cheung et al., 2006) transcripts. DMSO also enhanced the capacity of human embryonic stem cells to make definitive endoderm (Chetty et al., 2013) and induced osteoblastic differentiation in MC3T3-E1 cells (Cheung et al., 2006). However, the mechanism through which DMSO affects differentiation are not understood with one study in pre-osteoblastic MC3T3-E1 cells pointing towards induction of DNA hydroxymethylation (Thaler et al., 2012). Our results indicate DMSO can affect nuclear localization of OCT4 and NANOG leading to RUNX2 dominance in the nucleus without changes in *RUNX2* expression, however this observation requires further investigation. Altogether, the role of DMSO in cell differentiation and in altering epigenetic machinery enzymes, pluripotency and osteogenic markers should be considered when using DMSO as vehicle.

CONCLUSIONS

Here, we have highlighted the role of DNA methylation and *DNMTs* expression in supporting the osteogenic potential in PDLCs. This is shown by differential *DNMTs* and *TETs* basal levels in h- and l-PDLCs, their changes upon OM stimulation as well as in the response to RG108 treatment. These changes correlate with the expression and methylation of the main pro-osteogenic transcription factor, RUNX2. Altogether RG108 is shown to promote changes in enzymes in control of DNA methylation levels, multipotency and osteogenic markers, leading to an increase in the mineralization capacity of l-PDLCs, therefore opening a new avenue for therapeutic modulation of osteoblastic differentiation and promotion of osteogenesis.

ACKNOWLEDGEMENTS

This work was supported by São Paulo Research Foundation - FAPESP (DCA grant number 2013/09650-8), FAPESP and the University of Birmingham, UK Collaborative Research Program (DCA and MW grant number 2017/07944-5) and FAPESP (RIFA grant number 2015/02160-0).

AUTHOR CONTRIBUTIONS STATEMENT: Rahyza I F Assis: Investigation, Validation, Writing - Original Draft, Methodology. **Arthur G Schmidt:** Investigation, Validation, Writing - Original Draft, Methodology. **Francesca Racca:** Investigation, Validation, Methodology. **Rodrigo A da Silva:** Investigation, Validation, Writing - Review & Editing. **William Zambuzzi:** Resources, Writing - Review & Editing. **Karina G Silvério:** Resources, Writing - Review & Editing. **Francisco H Nociti Júnior:** Resources, Writing - Review & Editing. **Vanessa G Pecorari:** Formal analysis, Writing - Original Draft. **Malgorzata Wiench:** Conceptualization, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition. **Denise C Andia:** Conceptualization, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. All authors state there is no conflict to disclosure or no competing financial interests exist.

FUNDING: This work was supported by São Paulo Research Foundation - FAPESP (grant number 2013/09650-8, 2015/02160-0), FAPESP and the University of Birmingham, UK Collaborative Research Program (grant number 2017/07944-5).

ADDITIONAL INFORMATION

information accompanies this paper.

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