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Glycogen synthase kinase 3 inhibition enhances mineral nodule formation by cementoblasts *in vitro*

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Abstract: This study aimed to investigate whether GSK-3 inhibition (CHIR99021) effectively promoted mineralization by cementoblasts (OCCM-30). OCCM-30 cells were used and treated with different concentrations of CHIR99021 (2.5, 5, and 10 mM). Experiments included proliferation and viability, cellular metabolic activity, gene expression, and mineral nodule formation by Xylene Orange at the experimental time points. In general, CHIR99021 did not significantly affect OCCM-30 viability and cell metabolism (MTT assay) ($p > 0.05$), but increased OCCM-30 proliferation at 2.5 mM on days 2 and 4 ($p < 0.05$). Data analysis further showed that inhibition of GSK-3 resulted in increased transcript levels of *Axin2* in OCCM-30 cells starting as early as 4 h, and regulated the expression of key bone markers including alkaline phosphatase (*Alp*), runt-related transcription factor 2 (*Runx-2*), osteocalcin (*Ocn*), and osterix (*Osx*). In addition, CHIR99021 led to an enhanced mineral nodule formation *in vitro* under both osteogenic and non-osteogenic conditions as early as 5 days after treatment. Altogether, the results of the current study suggest that inhibition of GSK-3 has the potential to promote cementoblast differentiation leading to increased mineral deposition *in vitro*.

Keywords: *Wnt* Signaling Pathway; Glycogen Synthase Kinase 3; Dental Cementum.

Introduction

The periodontium is a topographically complex organ consisting of soft and mineralized tissues, including gingivae, periodontal ligament, cementum, and alveolar bone. The periodontium has been reported to be affected by a number of conditions, ultimately leading to the destruction of the connective tissue matrix and cells, loss of fibrous attachment and resorption of alveolar bone, and tooth loss. The ideal treatment outcome for periodontal defects resulting from periodontitis is the resolution of the inflammatory process (*e.g.*, absence of bleeding on probing) associated with complete periodontal regeneration (*e.g.*, new cementum and bone formation connected by functionally orientated, aligned periodontal ligament fibers) and absence of gingival recession. With this concept in mind, numerous approaches, often including various types of bone grafts and/or bone substitutes, root surface

demineralization, guided tissue regeneration, growth and differentiation factors, enamel matrix proteins, or the combinations of these procedures have been used to achieve periodontal regeneration.[1,2] Despite promising results reported for animal models and some successful clinical findings for many of the available regenerative techniques and materials compared to the conventional therapy alone (scaling and root planing), robust information on whether the reported clinical improvements reflect true periodontal regeneration is still lacking. Furthermore, the clinical use of potential regenerative approaches is limited to few situations, including intrabone and class II furcation defects, and the results have been unpredictable.[2,3]

Dental cementum is a mineralized tissue that covers the tooth root and has important functions in tooth attachment and position, which have been reported to be severely compromised by periodontitis progression. Current therapies to promote dental cementum repair are unpredictable, particularly in regard to the acellular cementum, which is the key tissue anchoring the periodontal ligament to the tooth root. Insights into the developmental biology of cementum have been useful showing the potential of novel approaches to promote its regeneration and restore periodontal function. The phosphate story is a good example of such a strategy of cementum regeneration. Evidence suggesting that cementum formation is highly sensitive to phosphate metabolism[4] led to subsequent studies demonstrating that cementum regeneration/neoformation was also modulated *in vivo* by local levels of pyrophosphate.[5]

The canonical Wnt/β-catenin signaling plays a broad role in the development and maintenance of many organs and tissues, including dental tissues. It is known that the Wnt/β-catenin system (canonical Wnt signaling pathway) is vital for bone formation,[6] and because bone and cementum share many similarities, several independent studies have investigated the relationship between the canonical Wnt signaling pathway and cementum development. In general, it has been suggested that activation of the Wnt signaling may impact cementum homeostasis.[7-12] Glycogen synthase kinase-3 (GSK-3) is a cytoplasmic serine/threonine protein kinase that phosphorylates and inhibits glycogen synthase, thereby inhibiting glycogen synthesis from glucose, and has been shown to regulate a variety of biological processes,[13] including biomineralization.[14,15] GSK-3 activation results in the inhibition of the canonical Wnt signaling pathway; therefore, targeting GSK-3 could represent a potential strategy to control biomineralization processes, including cementogenesis. In fact, such strategy has been reported as a promising approach to induce dentinogenesis.[16,17] In the current study, we evaluated the effect of a GSK-3 inhibitor, CHIR99021 (6-((2-((4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl)amino)ethyl)amino) nicotinonitrile), on the biological behavior of a cementoblastic cell line (OCCM-30).

Methodology

Cell line and cell culture

OCCM-30 cells were obtained by isolating tooth root surface cells from transgenic mice containing an SV40 large T-antigen under the control of an OCN promoter and were characterized as highly differentiated cementoblasts.[18,19] OCCM-30 were maintained in standard culture medium containing DMEM (Gibco, Life Technologies, City, USA) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (FBS) (Gibco), at 5% CO_2 atmosphere conditions and 37°C. All the assays described below were performed in triplicate in two independent experiments. The current study was granted a formal waiver of ethics approval (IACUC - Faculdade São Leopoldo Mandic – protocol # 2022-0158).

Cell proliferation and viability assays

A dose-response assay was performed to define the impact of CHIR99021 (Sigma Aldrich, St. Louis, USA) treatment on cell proliferation and viability. OCCM-30 were seeded at 1.5 x10^4 cells/well onto 48-well culture plates in standard culture medium. After 24 h, the culture medium was replaced by DMEM supplemented with 2% FBS (and penicillin, streptomycin, and L-glutamine) with or without

CHIR99021 at 2.5, 5, and 10 mM. The number of viable and non-viable cells were obtained at days 1, 2, and 4 by the trypan blue method.

MTT assay

The MTT assay was performed at days 1, 2, and 4 after treatment. OCCM-30 cells were seeded onto 48-well plates at $2x10^4$ cells/well and incubated (37°C, 5% CO_2, 95% humidity) for 24 h in standard culture medium. Thereafter, the growth medium was replaced by DMEM with 5% FBS (and penicillin, streptomycin, and L-glutamine) with or without CHIR99021 at 2.5, 5, and 10 mM. Cell metabolic activity was determined by the MTT assay according to the manufacturer's instructions. Briefly, culture medium was replaced by 900 µL of DMEM supplemented with 100 µL of MTT (5 mg/mL) (Life Technologies, Carisbad, USA) at 37°C, and 5% CO_2 and incubated for 4 h, under dark conditions. Subsequently, dimethyl sulfoxide (Sigma-Aldrich, St. Lousi, USA) was used to dissolve the formazan crystals, and the solution's optical density was read at 570 nm (VersaMax; Molecular Devices, USA).

Gene expression analysis

Transcripts levels for *Axin2* were assessed in OCCM-30 cells treated or not with CHIR99021 (2.5, 5, and 10 mM). OCCM-30 cells were seeded onto 12-well plates at $1x10^5$ cells/well and kept in standard culture medium until they reached 80% confluence, and culture medium was replaced by DMEM supplemented with 2% FBS (and penicillin, streptomycin, and L-glutamine) with or without CHIR99021. Total RNA was isolated after 30 minutes, 1, 4, 6, and 12 h using the Qiagen RNeasy Micro kit (Valencia, USA). In addition, complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction were performed on the Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA) using intron-spanning primers to assess the expression of Axin2 (*5′-AAGAAGGAGACCGGTCACAG-3′/5′-GGTCCTGGGTAAATGGGTGA-3′*). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as housekeeping gene (*5′-TGAGCAAGAGAGGCCCTATC-3′/5′-AGGCCCCTCCTGTTATTATG-3′*). In addition, the effect of CHIR99021 on the mRNA levels of bone markers, including alkaline phosphatase (*Alp*) (5′-ATCGGAACAACCTGACTGACCCTT-*3′/5′-ACCCTCATGATGTCCGTGGTCAAT-3′*), runt-related transcription factor 2 (*Runx-2*) (*5′-ATGATGACACTGCCACCTCTGAC-3′/5′-ACTGCCTGGGGTCTGAAAAAGG-3′*), osteocalcin (*Ocn*) (*5′-TGAACAGACTCCGGCG-3′/5′-GATACCATAGATGCGTTTG-3′*), and osterix (*Osx*) (*5′-GATGGCGTCCTCTCTGCTT-3′/5′-CGTATGGCTTCTTTGTGCCT-3′*) was also assessed by qPCR.

Mineral nodule formation

OCCM-30 were seeded at $2x10^4$ cells/well onto 12-well culture plates and incubated under differentiation or control conditions in an atmosphere of 5% CO_2 and 37 °C for 5, 8, and 12 days. Differentiation medium was composed by DMEM plus antibiotics (penicillin/streptomycin) (10 µg/mL) with 100 nM dexamethasone (Gibco, Life Technologies, Carisbad, USA), 10% FBS, 0.05 mM L-ascorbic acid and 10 mM β-glycerophosphate disodium salt hydrate whereas the control medium was DMEM with 10% FBS and antibiotics. CHIR99021 was added to both differentiation and control culture media at 2.5, 5, and 10 µM, and mineral nodules were assessed by Xylenol orange (XO) staining as previously described.[20]

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by the Tukey test in order to determine the effect of GSK-3 inhibition on mitochondrial activity, proliferation rate, gene expression, and mineral nodule formation. Significance levels were set at 5%.

Results

Cell metabolic activity and viability/ proliferation assays

In the current investigation, classical assays were performed in order to determine the impact of CHIR99021, a GSK-3 inhibitor, on cell behavior. First, we performed a dose-response assay to define the effect of different concentrations of CHIR99021, at 2.5, 5, and 10 mM, on cellular metabolism by the

MTT assay. In general, data analysis showed that CHIR99021 did not significantly impact the ability of OCCM-30 cells to metabolize MTT compared to the control group ($p > 0.05$) (Figure 1A). Next, we examined whether CHIR99021 (at 2.5, 5, and 10 mM) would impact cell proliferation using the Trypan blue approach and a hemocytometer. Data analysis showed that there was a trend for increased cell proliferation for CHIR99021-treated groups, with significant differences for CHIR99021 at 2.5 mM on days 2 and 4 ($p < 0.05$) (Figure 1B). It was additionally found that inhibition of GSK-3 with CHIR99021 treatment did not affect OCCM-30 cell viability ($p>0.05$) (data not shown).

Gene expression analysis

In order to test whether GSK-3 inhibition led to the activation of the canonical Wnt signaling pathway, we assessed *Axin2* mRNA levels in OCCM-30 cells treated with CHIR99021. *Axin2* is a downstream target of the canonical Wnt pathway, and increased expression of *Axin2* would serve as an evidence of the activation of this signaling pathway. Increased expression of *Axin2* was observed as early as 4 h after CHIR99021 treatment and reached a maximum increase after 12 h ($p < 0.05$). Fold-change increase for *Axin2* mRNA levels ranged from 5.8 to 28.1 across the CHIR9902-treated groups. Furthermore, to determine whether the rate of mineral nodule formation paralleled with the expression of mineralization markers, we assessed the expression of *Alp*, *Runx-2*, *Ocn*, and *Osx*. Overall, these findings showed that CHIR99021 regulated the expression of target genes as early as 6 h after treatment, featuring a trend towards increased transcript levels for *Alp*, *Runx-2*, and *Ocn*. Figure 2A-E summarize the qPCR findings.

Mineral nodule formation

To determine whether GSK-3 inhibition would impact OCCM-30 cell differentiation and mineral nodule formation *in vitro*, we cultured OCCM-30 cells with and without osteogenic condition associated with CHIR99021 at 2.5, 5, and 10 mM. Mineral nodule formation by OCCM-30 cells was examined using the XO assay. Fluorescent images from XO staining were obtained and are presented in Figure 3A. To quantitatively determine the impact of CHIR99021 treatment on the capacity of OCCM-30 to produce mineral nodules *in vitro*, fluorescence was assessed by Image J software (National Institutes of Health, version 1.53a). Data analysis showed that OCCM-30 cells cultured under osteogenic conditions featured mineral nodule formation starting at day 5, which was increased in a time-dependent manner ($p < 0.05$). Furthermore, data analysis demonstrated that

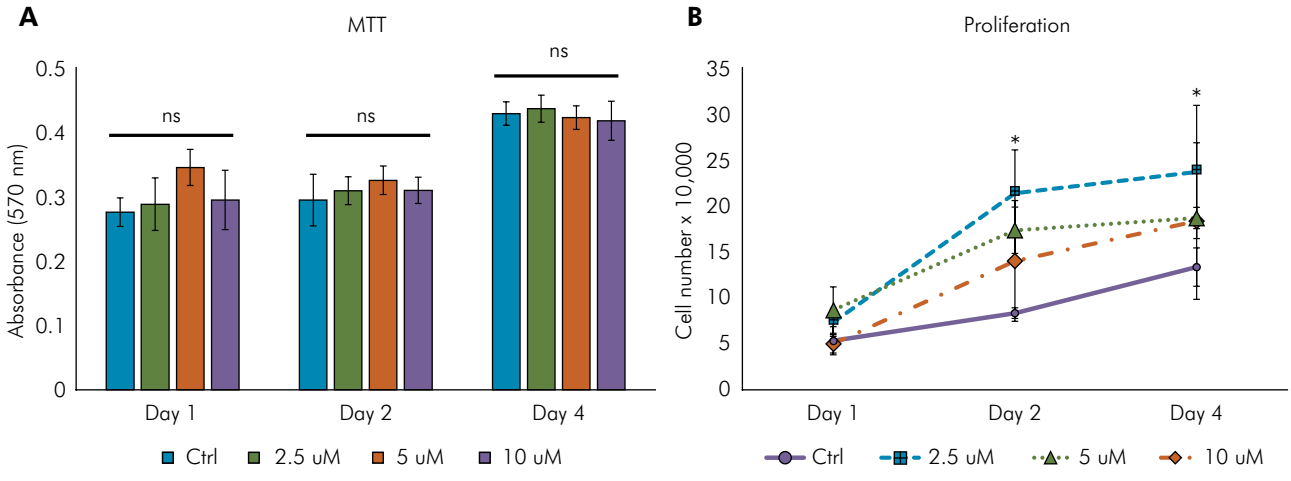
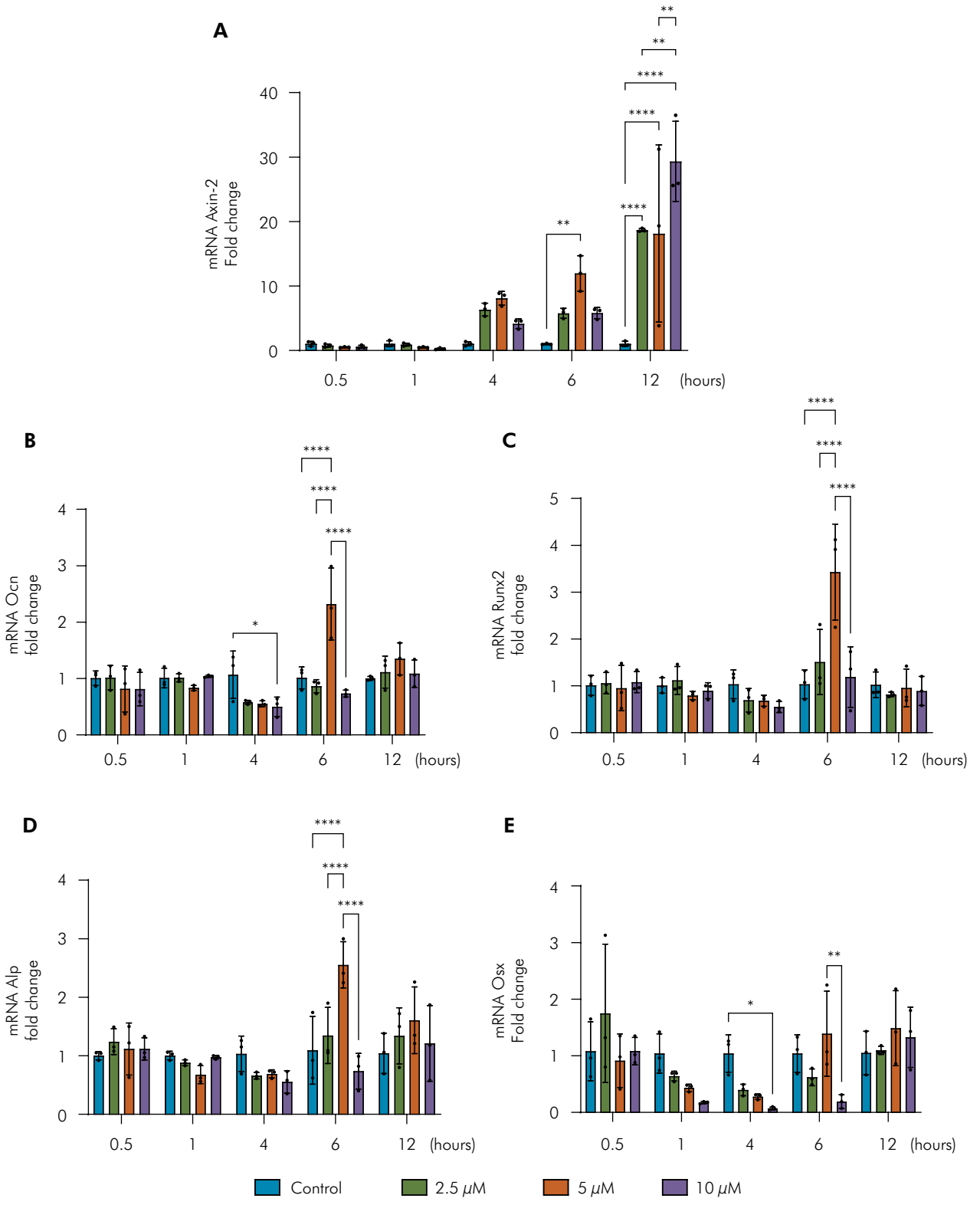


Figure 1. Representative figures of two independent experiments performed in triplicate illustrating the effect of CHI99021 treatment on the metabolic activity (MTT assay) (A) and proliferative rate (Trypan blue) (B) of OCCM-30 cells. Data are expressed as mean +/- SD and were collected at days 1, 2, and 4. The effect of treatment was statistically assessed by an intergroup analysis using one-way ANOVA followed by the post-hoc Tukey test (*$p < 0.05$, ns: non-significant differences).



Tukey test (*p< 0.05, **p < 0.01, ***p <0.001, ****p < 0.0001).

Figure 2. Mean fold-change and standard deviation values for target genes, including *Axin-2* (A), *Ocn* (B), *Runx2* (C), *Alp* (D), and *Osx* (E), at 0.5, 1, 4, 6, and 12 hours after treatment with CHIR99021. The effect of treatment was statistically assessed by an intergroup analysis using one-way ANOVA followed by the post-hoc

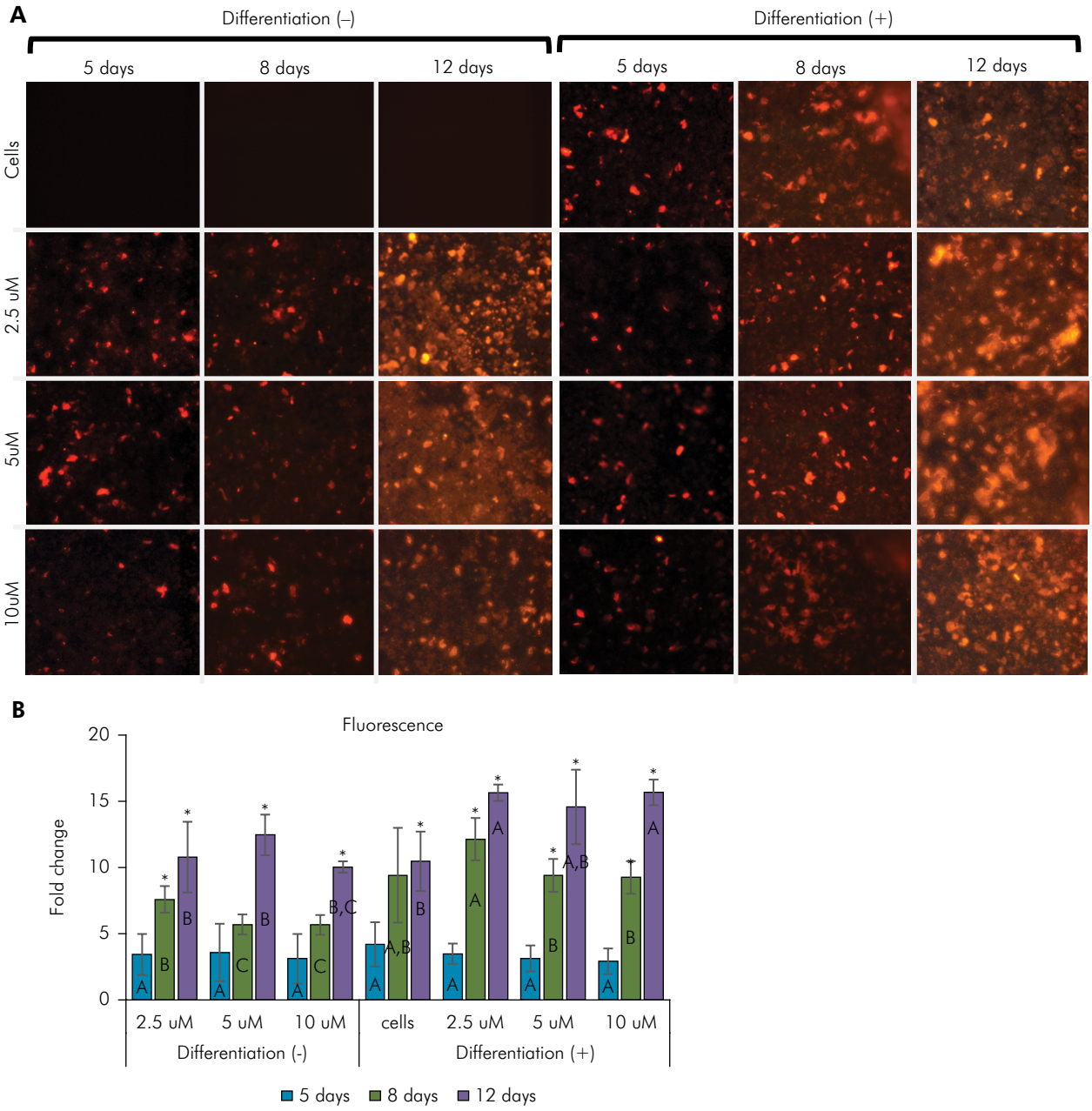


Figure 3. A) Representative illustration of the mineralization assay by Xylene Orange staining. OCCM-30 cells were cultured without or with osteogenic induction (Differentiation (-) and Differentiation (+), respectively), and treated with CHI99021 at 2.5, 5, and 10 uM. CHIR99021 clearly increased mineral nodule deposition overtime, regardless of its concentration or osteogenic induction, except for the control group (non-induced and non-treated). B) Bar graphs illustrating fluorescence intensity fold-change over the control group (non-induced and non-treated). GSK-3 inhibition led to time dependent increase in mineral nodule formation starting at day 5 with fold-change ranging from 3.1 to 12.4-fold in the non-induced group [Differentiation (-)]. Intra (*) (compared to 5 days - time effect) and intergroup (capital letters) (within the same time point across the experimental groups – treatment effect) analyses were performed by one-way ANOVA followed by the Tukey test, with alpha = 5%.

CHIR99021 treatment led to an increased mineral nodule formation on days 8 and 12, regardless of its concentration, in OCCM-30 cells cultured under osteogenic conditions ($p < 0.05$). Notably, the findings of the present study, clearly demonstrated that even in the absence of osteogenic conditions, CHIR99021-treated

groups featured increased mineral nodule formation by OCCM-30 cells in a time-dependent manner with fold change ranging from 3.1 to 12.4. Figure 3B illustrates the quantitative assessments of mineral nodule formation by OCCM-30 cells treated or not with CHIR99021.

Discussion

The Wnt family of secreted glycoproteins have been shown to play important roles in morphogenesis and cellular differentiation in many tissues through several distinct pathways.[21] Studies have found that Wnt/ß-catenin signaling plays a crucial role in mesenchymal tissue development, including skeletal maturation and tooth morphogenesis.[22,23] Inhibition of GSK-3 activity will allow β-catenin to enter the nucleus where it interacts with Lef/Tcf transcription factors to regulate expression of target genes, including Axin2.[24] Although, there is a large body of evidence demonstrating the potential impact of the Wnt signaling on periodontal ligament cell (PDL) differentiation and dental cementum development, very limited information is available on the potential significance of decreased GSK-3 activity on cementoblast biology. Therefore, in the current work, we aimed to perform a proof-of-principle study to determine the effect of a small molecule inhibitor of GSK-3 activity, CHIR99021, on the biological properties of a well-known cementoblastic cell line (OCCM-30). Together, our findings demonstrated that GSK-3 in cementoblast led to increased mineral nodule formation *in vitro* without affecting cellular viability and metabolic activity, and therefore, these results highlight the potential use of specific GSK-3 inhibitors to promote cementum regeneration.

Although CHIR99021 has been extensively used to experimentally elevate Wnt activity,[25] there was no report on OCCM-30 sensitivity to CHIR99021. Therefore, to establish a concentration of CHIR99021 that is non-cytotoxic to OCCM-30 cells, we performed a dose-response assay and assessed OCCM-30 proliferation and viability and metabolic activity (MTT) at days 1, 2, and 4. Three concentrations were chosen (2.5, 5, and 10 mM) based on a recent report where an immortalized cell line (17IA4 cell line) was cultured with increasing concentrations of CHIR99021,[17] and our results demonstrated that none of the concentrations used negatively affected OCCM-30 viability or mitochondrial metabolic activity. In contrast, OCCM-30 proliferative rates were significantly increased by CHIR99021 at 2.5 mM on days 2 and 4. Our findings confirmed previous studies that showed that activation of the Wnt/β-catenin signaling by Li^+ ions release was implicated with increased cellular proliferative rates of human-derived PDL cells.[8] Naujok et al.[26] reported that among four different GSK-3 inhibitors (BIO, SB-216763, CHIR99021, and CHIR98104), CHIR99021 presented the lowest toxicity in mouse embryonic stem cells, but in contrast to the current findings, it affected cell viability in a dose-dependent way. Similarly, inhibition of GSK-3 has been shown to induce an anti-proliferative effect on epithelioid sarcoma cell proliferation, with a dose-dependent effect on cell viability.[27] Therefore, the impact of GSK-3 inhibition on cell proliferation and viability seems to be highly dependent on CHIR99021 concentration and cell type.

Axin2 is a negative regulator and also a downstream target of this signaling pathway, and Axin2 mRNA and protein levels have been shown to be rapidly increased by activation of the Wnt pathway as a negative feedback response.[28,29] Therefore, in the current study, transcript levels of *Axin2* were assessed by qPCR to confirm the activation of the canonical Wnt signaling pathway. We anticipated that reduced GSK-3 activity resulting from CHIR99021 treatment would lead to increased mRNA levels of *Axin2* as a consequence of a strong activation of the Wnt signaling. We indeed found that *Axin2* mRNA levels were significantly increased by CHIR99021 as early as 4 h, reaching its peak at 12 h regardless of the CHIR99021 concentration used. These findings are in line with those reported by Neves et al.[17] who also found increased mRNA levels of Axin2 in 17IA4 cells cultured with GSK-3 inhibitors, including CHIR99021. More importantly, the findings of the present study clearly showed that OCCM-30 cells cultured with a GSK-3 inhibitor featured Wnt signaling activation.

It is currently established that activation of canonical Wnt signaling will promote bone formation. Studies applying distinct technologies to activate Wnt signaling have served as the basis for this understanding. Experiments include the use of neutralizing antibodies to inhibit sclerostin and the use of lithium chloride to inhibit GSK-3 activity to promote bone formation via activation of canonical Wnt.[30-32] Dental cementum phenotype has also been shown to be impacted by the activation of Wnt signaling with increased width of formed cementum.[33] It has also been shown that not only cementum formation is increased as a consequence of activation of Wnt signaling, but also cementum regeneration.[8,34] In general, it has been proposed that the enhanced Wnt signaling pathway will stimulate cementogenic differentiation of PDL-derived cells,[8] but there is evidence that constitutive stabilization of b-catenin in the dental mesenchyme leads to an altered expression of key mineralization markers in cementoblasts,[35] suggesting that differentiated cementoblasts may play a key role in regulating cementum formation/regeneration as a result of activation of the Wnt signaling pathway.

Here, we sought to perform a proof-of-principle study in order to define the effect of GSK-3 inhibition on mineral nodule formation *in vitro* and the expression of bone markers on a well-known cementoblastic cell line called OCCM-30. To the best of our knowledge, this is the first study to examine the effect of GSK-3 inhibition directly on cementoblasts focusing on its ability to form mineral nodules *in vitro* as well as on the expression of key cementoblastic markers. The findings of the present investigation showed that GSK-3 inhibition led to increased mineral nodule formation by cementoblasts and resulted in regulation of crucial differentiation markers of cementoblasts, including *Alp*, *Runx-2*, and *Ocn*. In the current study, gene expression analysis focused on the effect of GSK-3 inhibition alone on transcript levels of mineralization markers, as the effect of osteogenic induction on these markers has been extensively documented. Therefore, GSK-3 emerges as a potential target for therapies for dental cementum reconstruction. Currently, there are ongoing studies in our lab to combine the use of 3D-biopriting and GSK-3 inhibitors to create a reliable and reproducible strategy for reconstructing dental cementum lost due to periodontal diseases and external root resorptions.

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