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Metformin induces osteoblastic differentiation of human induced pluripotent stem cell-derived mesenchymal stem cells

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Abstract

Metformin, a first-line antidiabetic drug used by millions of patients, has been shown to have potential osteogenic properties. The present study was performed to test the hypothesis that clinically relevant doses of metformin promote the osteogenic differentiation and mineralization of induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs). iPSC-MSCs were treated with metformin (10 μ M) to assess cell viability, osteogenic differentiation, mineralization and activation of the LKB1/AMP-activated protein kinase (AMPK) signalling pathway, a surrogate marker of metformin action. To determine its potential application in MSC-based bone and periodontal tissue engineering, iPSC-MSCs were also treated with metformin when seeded on to calcium phosphate cement (CPC) scaffolds. Immunoblotting and cellular uptake assays showed that iPSC-MSCs express functional organic cation transporter-1 (OCT-1), a transmembrane protein that mediates the intracellular uptake of metformin. Although metformin treatment did not impair iPSC-MSC viability, it significantly stimulated alkaline phosphatase activity, enhanced mineralized nodule formation and increased expression of osteogenic markers, including Runt-related transcription factor 2 (RUNX2) and osterix. Inhibition of LKB1 activity, a common upstream AMPK kinase, markedly reversed metformin-induced AMPK activation, RUNX2 expression and nuclear localization. Moreover, metformin substantially increased mineralized nodule formation of iPSC-MSC seeded on CPC scaffolds. Collectively, functional OCT-expressing iPSC-MSCs responded to metformin by inducing an osteogenic effect in part mediated by the LKB1/AMPK pathway. Considering the widespread use of metformin in diabetics, this work may lead to novel tissue-engineering platforms where autogenous OCT-expressing iPSC-MSCs might be used to enhance bone and periodontal regeneration in diabetic patients prescribed with daily doses of metformin.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

Keywords

bone regeneration; metformin; osteoblasts; osteogenic markers; periodontal regeneration; tissue engineering

1 INTRODUCTION

The prevalence of diabetes mellitus (DM), in particular adult-onset or type 2 (T2DM), continues to rise worldwide. Around 415 million people (9% of adults) have diabetes, and it is predicted that over the next decade this number will increase to 642 million people (Jaacks, Siegel, Gujral, & Narayan, 2016). Long-established evidence indicates that DM-associated hyperglycaemia is linked to more complex, accelerated periodontal tissue destruction, which remains a critical clinical challenge (Chapple, Genco, & Working group 2 of Joint EFPAAPw, 2013). Poorly controlled hyperglycaemia and its detrimental effects on bone marrow mesenchymal stem cell (MSC) viability and differentiation under diabetic conditions are major factors potentially compromising treatment outcomes of bone and periodontal tissue regeneration (Fadini, Ferraro, Quaini, Asahara, & Madeddu, 2014). Novel therapeutic strategies addressing both factors simultaneously and effectively may offer a more predictable approach to control bone and periodontal regeneration.

Metformin, a relatively inexpensive antihyperglycaemic biguanide, is widely used by millions of diabetic patients as first-line treatment for T2DM. As an insulin-sensitizing drug, it can lower high glucose blood levels without increasing insulin secretion (Viollet et al., 2012). Moreover, it is safe, non-toxic and well tolerated. Several studies have recently reported that metformin has a potential osteogenic effect by promoting the differentiation of pre-osteoblasts and MSCs (Cortizo, Sedlinsky, McCarthy, Blanco, & Schurman, 2006; Gao, Xue, Li, Jia, & Hu, 2008; Jang et al., 2011; Kanazawa, Yamaguchi, Yano, Yamauchi, & Sugimoto, 2008). Likewise, metformin can also revert the deleterious effects of high glucose on osteoblastic cells (Schurman et al., 2008; Zhen, Chen, & Tang, 2010). Clinical studies have also indicated that locally delivered metformin seems to improve clinical and radiological parameters of chronic periodontitis (Pradeep et al., 2015). Although therapeutically attractive, there are still pending questions with significant clinical implications that must be addressed. As a highly hydrophilic cationic compound, metformin relies on polyspecific cell membrane organic cation transporters (OCTs) of the solute carrier 22A (SLC22A) gene family to facilitate its intracellular uptake and action (Nies, Koepsell, Damme, & Schwab, 2011). Therefore, for metformin to act as a translationally relevant osteoanabolic agent, OCT expression and proper function must be a biological prerequisite to positively affect osteodifferentiating cells.

Cell-based therapy has the potential to greatly improve current methods of tissue regeneration through increased bioactivity of scaffolds and localized growth factor delivery (Pilipchuk et al., 2015; Rosa, Della Bona, Cavalcanti, & Nor, 2012). Induced pluripotent stem cells (iPSCs) are regarded as a promising cell source for tissue engineering (Takahashi & Yamanaka, 2006). For the purpose of periodontal and bone tissue regeneration, efficient *in vitro* differentiation of iPSCs into downstream cells, such as MSCs, prior to their clinical

application is necessary to limit undesired tumorigenesis associated with iPSC pluripotency (Hynes, Menicanin, Gronthos, & Bartold, 2012; Lian et al., 2010; Pagni et al., 2012). Indeed, iPSC-derived MSCs (iPSC-MSCs) exhibit a higher proliferative capability than bone marrow MSCs and are less tumorigenic than undifferentiated iPSCs and bone marrow MSCs (Villa-Diaz et al., 2012; Zhao et al., 2015). Therefore, iPSC-MSCs hold promise, especially for patients with compromised health conditions, such as T2DM, whose autologous bone marrow MSCs might be rather compromised for tissue regeneration (Schwartz, 2003).

Considering the broad use of metformin in T2DM patients, we propose that metformin-controlled T2DM patients in need of periodontal and bone regeneration might take advantage of their circulating levels of metformin to pharmacologically enhance iPSC-MSC-driven regeneration. Here, we present evidence that iPSC-MSCs express functional OCT-1 and respond to clinically relevant doses of metformin by enhancing osteogenic differentiation and mineralization in an LKB1/AMP-activated protein kinase (AMPK) signalling pathway-dependent manner. Moreover, we show that metformin promotes mineral deposition of iPSC-MSCs seeded on calcium phosphate cement (CPC) constructs. This study provides a significant step forward in the emerging area of iPSC-MSC-based tissue regeneration in the oral and maxillofacial field. Most significantly, this work may lead to future applications of tissue-engineering platforms that incorporate autologous iPSC-MSCs in combination with metformin as a novel therapeutic option to enhance periodontal and bone tissue regeneration in diabetic and non-diabetic patients.

2 MATERIALS AND METHODS

2.1 Derivation of iPSC-MSCs from iPSCs

The human iPSC-BC1 cell line was maintained on mitotically inactivated murine embryonic fibroblasts feeder as previously reported (Chou et al., 2011). After proliferation, iPSCs were detached from murine embryonic fibroblasts and dissociated into clumps by incubation with type IV collagenase for 10 min. Dissociated iPSC clumps were collected, resuspended and transferred to ultra-low attachment cell culture flasks (Corning, Corning, NY, USA) to form embryonic bodies. After 10 days of suspension culture in ultra-low attachment cell culture flasks, embryonic bodies were transferred on to 0.1% gelatin-coated culture dishes for cell re-attachment. Cells growing out from embryonic bodies were collected and cultured in MSC growth media, which consisted of low glucose Dulbecco's modified Eagle's media (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). The differentiated cells were passaged until a homogeneous fibroblastic morphology appeared and were referred to as iPSC-MSCs. The iPSC-MSCs generated from this method fulfilled the International Society of Cellular Therapy's minimal criteria for defining multipotent MSC: (1) they had plastic adherent properties; (2) they expressed key MSC-associated markers (CD29, CD44, CD166, CD73) and were negative for typical haematopoietic (CD34), endothelial (CD31) and pluripotent markers (TRA-1-81 and OCT-3/4) and (3) they had the capacity to undergo tri-lineage differentiation (Tang et al., 2014). The sixth to seventh passage iPSC-MSCs were used in the following experiments.

2.2 OCT expression in iPSC-MSCs

Protein expression levels of OCT-1, OCT-2 and OCT-3, encoded by the SLC22A gene family, facilitate metformin transport across cellular membranes (Nies et al., 2011). We tested OCT expression in iPSC-MSCs via Western blot analysis, as previously demonstrated (Patel, Younis, Ord, Basile, & Schneider, 2013). Briefly, after isolation of whole cell lysates, equal amounts of protein were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis, electrophoretically transferred on to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with rabbit antihuman OCT-1, OCT-2 and OCT-3 primary antibodies (Sigma, St. Louis, MO, USA), respectively. Blots were stripped and reprobed with an antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Sigma).

2.3 [¹⁴C]-metformin uptake assay

To test metformin cellular uptake, iPSC-MSCs were washed once with prewarmed KRH buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM HEPES, 5.6 mM glucose, pH 7.4). After 10 min incubation with 10 μM [¹⁴C]-metformin plus 40 μM unlabelled metformin with or without 10 μM cimetidine, a known OCT inhibitor, the uptake was halted by removing the buffer and washing the cells with ice-cold KRH buffer three times. Metformin was obtained from Calbiochem (Gibbstown, NJ, USA). Cells were lysed in 300 μl 1% Triton X-100 and 250 μl cell lysates were transferred to scintillation tubes containing 3 ml Econo-Safe™ economical biodegradable cocktail (Research Products International, Mount Prospect, IL, USA). Radioactivity was counted in a Tri-Carb 2910TR liquid scintillation analyser (Perkin Elmer, Boston, MA, USA) (Chen et al., 2014).

2.4 iPSC-MSC viability assay

The effect of metformin on cell viability was evaluated through a colorimetric assay by using the CellTiter 96® Aqueous One Solution (MTS) reagent following the manufacturer's protocol (Promega, Madison, WI, USA) as previously reported (Patel et al., 2013). iPSC-MSCs were plated in 96-well plates at a density of 5000 cells/cm². The following day, cells were either exposed to MSC growth media alone (control) or MSC growth media containing 10 μM metformin (*n* = 5 wells/condition). Media were changed every 2 days. At 1, 3, 5 and 7 days, MTS reagent was added directly to each culture well, incubated at 37°C for 1 h and absorbance at 490 nm was measured in a microplate reader.

2.5 Alkaline phosphatase (ALP) activity and staining

iPSC-MSCs were plated in six-well plates at a density of 1.5×10^5 cells/well. At 7 days, ALP activity in iPSC-MSCs treated with MSC growth media alone (control) or MSC growth media containing 10 μM metformin was measured according to an ALP fluorometric assay kit (Abcam, Cambridge, MA, USA) following the manufacturer's protocol. Cells were washed with cold phosphate-buffered saline and resuspended in the assay buffer provided by the kit. After centrifugation, the supernatant was collected for the ALP assay using 4-methylumbelliferyl phosphate disodium salt (MUP) as a phosphatase substrate. ALP cleaved the phosphate group of MUP, resulting in an intense fluorescent signal. The absorbance was

measured at Ex/Em = 360/440 nm ($n = 3$ wells/condition). ALP activity was normalized to the total protein level. The protein level was quantified using a Bio-Rad protein assay kit (Hercules, CA, USA) following manufacturer's instructions. For ALP staining, cells were fixed for 30 s using citrate-acetone-formaldehyde fixative. A leukocyte ALP kit (Sigma-Aldrich) was used to stain control and metformin-treated iPSC-MSCs following the manufacturer's instructions. The ALP-positive cells displayed a blue-like staining.

2.6 Mineralization assays

To test the formation of calcium-containing mineral nodules from iPSC-MSCs treated with metformin, cells were plated in six-well plates at a density of 1.5×10^5 cells/well. At 14 days, iPSC-MSCs were stained at room temperature for 45 min with 2% Alizarin Red S (ARS, Lifeline Cell Technology, Walkersville, MD, USA) following the manufacturer's protocol. In another set of experiments, we used xylenol orange (XO), a calcium-chelating fluorochrome that stains minerals into a red fluorescent colour. At 14 and 21 days, iPSC-MSCs were incubated overnight in 2 ml osteogenic medium (OS) containing 100 μ M XO. Mineral nodules were observed by using an epifluorescence microscope (Eclipse TE-2000S, Nikon). The percentage of mineral area was calculated as $A_{\text{Mineral}}/A_{\text{Total}}$, where A_{Mineral} is the area of mineralization (red fluorescence) in the image and A_{Total} is the total area of the image ($n = 3$). OS-treated cells were used as the positive control, whereas cells cultured in MSC growth media were used as the negative control. OS consisted of MSC growth medium supplemented with 100 nM dexamethasone, 10 mM β -glycerophosphate, 0.05 mM ascorbic acid and 10 nM 1 α ,25-dihydroxyvitamin D3 (Sigma), as previously reported (Wang et al., 2016).

2.7 Osteogenic gene expression markers in metformin-treated iPSC-MSCs

Quantitative real-time reverse transcription polymerase chain reaction (qPCR) was used to measure the expression of osteogenic markers in iPSC-MSCs following treatment with MSC growth media alone (control) or MSC growth media containing 10 μ M metformin. Total cellular RNA was extracted with the PureLink RNA Mini Kit (Invitrogen, Waltham, MA, USA) and then reverse-transcribed into cDNA by a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Expression levels of the following genes were quantified by qPCR using SYBR Green PCR Master Mix (Applied Biosystems): ALP, collagen type I, Runt-related transcription factor 2 (RUNX2), osterix (OSX) and GAPDH. The sequences of human-specific primers were synthesized commercially (Sigma) and are listed in Table 1. Relative expression was evaluated using the 2^{-C_t} method and normalized by the C_t of the housekeeping gene GAPDH. C_t of iPSC-MSCs in MSC growth media cultured for 1 day served as the calibrator ($n = 3$). qPCR analyses were performed using an Applied Biosystems Prism 7000 Sequence Detection System. Western blotting was also used to detect protein expression levels of RUNX2 (rabbit anti-RUNX2 antibody, Cell Signaling Technology, Danvers, MA, USA) and OSX (rabbit anti-OSX antibody, Abcam) in control-and metformin-treated cells following the same immunoblotting procedures as described above.

2.8 Generation of LKB1 wild-type (LKB1-WT) and LKB1 kinase-dead (LKB1-KD) iPSC-MSCs

The serine threonine kinase LKB1 is the main upstream AMPK activating kinase (Shaw et al., 2004). To assess the effects of the LKB1/AMPK signalling pathway in metformin-induced iPSC-MSC osteogenic differentiation, we transiently transfected iPSC-MSCs with either WT, catalytically active LKB1, or KD, catalytically inactive LKB1, plasmid constructs. Plasmids encoding LKB1-WT (pcDNA3-FLAG-LKB1, Addgene plasmid #8590) or LKB1-KD (pcDNA3-FLAG-KD LKB1, Addgene plasmid #8591) were gifts from Lewis Cantley (Addgene, Cambridge, MA, USA) (Shaw et al., 2004). Transfections were performed by nucleofection according to the protocol provided by the manufacturer (Amaxa Biosystems, Köln, Germany). Briefly, cells were resuspended in 100 µl Human Mesenchymal Nucleofector Solution (Amaxa Biosystems), mixed with plasmids and pulsed with the program C-17 (high cell survival). Immediately after, cells were transferred into prewarmed fresh medium in six-well plates at a density of 5×10^5 /well. The following day, cells were serum-free treated overnight. Then, cells were treated with DMEM media with or without 10 µM metformin for 24 h. Protein expression levels of activated, phospho-AMPK α (pAMPK) (rabbit anti-pAMPK; Thr-172) and total AMPK α (rabbit anti-AMPK) (Cell Signaling Technologies) were detected by Western blotting in whole cell lysates from metformin-treated or control iPSC-MSCs cells, as previously reported (Schneider, Younis, & Gutkind, 2008). Nucleofection of hMSCs was performed according to the optimized protocols provided by the manufacturer (Amaxa Biosystem).

2.9 RUNX2 immunofluorescence staining

LKB1-WT- and LKB1-KD-transfected iPSC-MSCs were plated on glass coverslips in six-well plates at 5×10^5 /well. The following day, cells were serum starved overnight. Then, cells were either treated with serum-free MSC growth media in the absence (control) or presence of 10 µM metformin for 24 h. iPSC-MSCs were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 0.5% bovine serum albumin (BSA) prior to antibody addition. Indirect immunofluorescence experiments were performed using the primary rabbit anti-RUNX2 antibody (Cell Signaling Technologies) at 1:200 overnight at 4 °C. Coverslips were then incubated for 60 min at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:250 in 0.5% BSA, followed by incubation for 60 min in streptavidin Texas Red (Vector Laboratories) diluted 1:200 in 0.5% BSA. Nuclei were counterstained with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Carlsbad, CA, USA). Imaging was carried out using an epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY, USA).

2.10 CPC scaffold fabrication

To investigate whether metformin also induces an osteogenic effect on iPSC-MSCs seeded on bioscaffolds for potential use in periodontal and bone tissue engineering, we used a well-investigated CPC scaffold with good biocompatibility and osteoconductivity. CPC scaffolds were prepared following similar procedures as previously described (Wang et al., 2015). Briefly, TTCP ($\text{Ca}_4(\text{PO}_4)_2\text{O}$) and DCPA (CaHPO_4) powders at 1:3 molar ratio were thoroughly mixed in a micromill (Bel-Alert Products, Pequannock, NJ, USA) to form a CPC powder. Water-soluble mannitol particles (Sigma) were used as a porogen to create

macropores in the CPC. The mannitol particles were mixed with CPC powder at a mannitol/ (mannitol + CPC powder) mass fraction of 40%. The CPC liquid was 0.2 M Na₂HPO₄ in distilled water. A powder: liquid mass ratio of 2:1 was used to form a flowable CPC paste. The paste was placed in 8 mm diameter and 1 mm thickness moulds. After incubation in a humidifier for 1 day at 37 °C, disks were demoulded and immersed in distilled water at 37 °C for 3 days to dissolve the mannitol. CPC disks were autoclaved and then placed into 24-well plates with one disk per well prior to iPSC-MSC seeding.

2.11 Metformin treatment of iPSC-MSCs seeded on CPC scaffolds

iPSC-MSCs were seeded on to CPC disks in 24-well plates at a density of 1.5×10^5 cells/well ($n = 5$). After 21 days of being treated with MSC growth media in the absence (control) or presence of 10 μ M metformin, cell-free or cell-containing disks were stained with ARS to visualize the presence of mineralized nodules. ARS staining quantification was determined after eluting ARS with 10% cetylpyridinium chloride (Sigma). The extracted solution was transferred to 96-well plates. ARS was quantified colorimetrically by measuring absorption at 550 nm using a microplate reader.

2.12 Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 19.0, Chicago, IL, USA). All data were expressed as the mean value $\pm$ standard deviation. Statistical significance was analysed by using the one-way analyses of variance (ANOVA) and Student-Newman-Keuls test. A confidence level of 95% ($p < 0.05$) was considered significant.

3 RESULTS

3.1 iPSC-MSCs express functional OCT-1

Western blotting was performed to determine OCT expression levels in iPSC-MSCs. Among the members of the SLC22A family of transporters frequently associated with metformin uptake, only OCT-1 was expressed in these cells (Figure 1a). An uptake assay with radiolabelled metformin was conducted to confirm its intracellular transport, which was significantly inhibited by the OCT inhibitor cimetidine (Figure 1b).

3.2 Metformin does not impair iPSC-MSC viability

A colorimetric assay was carried out to assess the impact of increasing doses of metformin on iPSC-MSC viability. Our data showed no significant differences in cell viability between metformin-treated groups and the control group, indicating that metformin did not affect iPSC viability (Figure 1c). Moreover, sustained cell growth was observed overtime regardless of treatment conditions.

3.3 Metformin markedly increases mineral formation in iPSC-MSCs

Mineral synthesis by iPSC-MSCs in response to metformin, as evidenced by calcium-containing nodule formation, was analysed through two different staining methods, ARS and XO. At 14 days, both the ARS and XO staining showed that metformin treatment markedly

increased mineral deposition in iPSC-MSCs when compared with the negative control group (MSC growth media only). From 14 to 21 days, the fluorescent red staining of the synthesized mineral matrix increased and became denser in metformin- and OS-treated groups (Figure 2a, b). We found no statistically significant differences between metformin- and OS-treated groups at 14 and 21 days (Figure 2c).

3.4 Metformin induces osteogenic gene expression in iPSC-MSCs

Osteogenic gene expression in iPSC-MSCs was evaluated following treatment with metformin for 7 and 14 days. First, we found that ALP staining was more pronounced in metformin-treated iPSC-MSCs than in cells cultured only in MSC growth medium (Figure 3a). Likewise, ALP activity and gene expression levels were significantly elevated when compared with control (Figure 3b, c). Gene and protein expression levels of the osteogenic transcription factors RUNX2 and OSX were also significantly upregulated in response to metformin (Figure 3d–f).

3.5 LKB1/AMPK pathway mediates metformin-induced RUNX2 expression and nuclear localization

To determine whether metformin triggered AMPK signalling pathway activation, iPSC-MSCs were treated with metformin for 24 h. To be activated, AMPK needs to be phosphorylated by its upstream kinase LKB1 at threonine 172 within its catalytic α subunit, as previously reported (Schneider et al., 2008). Metformin treatment significantly induced AMPK phosphorylation (pAMPK). pAMPK upregulation also correlated with an increase in RUNX2 expression (Figure 4a). To further explore the role of the LKB1/AMPK pathway in metformin-induced RUNX2 expression, we transiently overexpressed either a LKB1-WT or a LKB1-KD plasmid construct in iPSC-MSCs and exposed them to metformin or control. Following metformin treatment, pAMPK and RUNX2 expression were markedly increased in LKB1-WT iPSC-MSCs, but were downregulated in the LKB1-KD-transfected cells (Figure 4b). These results were supported by RUNX2 immunofluorescence staining, which showed a strong RUNX2 nuclear expression after metformin treatment in LKB1-WT iPSC-MSCs. However, no RUNX2 nuclear expression was detected in metformin-treated LKB1-KD iPSC-MSCs (Figure 4c).

3.6 Metformin enhances mineral formation in iPSC-MSCs seeded on CPC scaffolds

CPC, first developed in 1986 then approved in 1996 by the US Food and Drug Administration for craniofacial defect repair, is one of the promising bone substitutes, with excellent bioactivity, biocompatibility and osteoconductivity. iPSC-MSCs were seeded on CPC scaffolds to investigate the potential adjunctive application of metformin in bone tissue engineering. When iPSC-MSCs were seeded on CPC scaffolds, ARS staining showed that mineralized nodule formation by iPSC-MSCs was significantly enhanced following metformin treatment for 3 weeks when compared with control CPC-seeded iPSC-MSCs (Figure 5).

4 DISCUSSION

Considerable evidence has emerged in recent years on the potential use of metformin, a first-line antidiabetic biguanide, in promoting osteoblastic differentiation (McCarthy, Cortizo, & Sedlinsky, 2016). Cortizo et al. (2006) were the first to report a pro-osteogenic effect of metformin in MC3T3E1 mouse calvaria-derived osteoblast-like cells, which dose-dependently stimulated collagen type I production and calcium deposition. Metformin has also been shown to significantly activate AMPK in MC3T3E1 cells in a dose- and time-dependent manner, and induce the expression of endothelial nitric oxide synthase and bone morphogenetic protein-2 (Kanazawa et al., 2008). Interestingly, this study showed that the addition of the antiviral agent adenine 9- β -D-arabinofuranoside, a specific AMPK inhibitor, blunted the reported metformin-induced responses (Kanazawa et al., 2008). These results suggested that metformin triggered the differentiation and mineralization of osteoblast-like cells via activation of AMPK signalling. Similarly, metformin-induced osteogenic gene expression in MC3T3E1 cells was either genetically inhibited by a dominant negative form of AMPK or chemically downregulated by compound C, a widely used inhibitor of AMPK activation (Jang et al., 2011). This study concluded that the osteogenic activity of metformin is probably mediated by RUNX2 transactivation through an AMPK-driven regulatory cascade (Jang et al., 2011).

Apart from the effect of metformin on osteoblasts, recent studies also showed that metformin regulates MSCs osteogenic differentiation. In rat-derived bone marrow MSCs, metformin induced osteoblastic differentiation while inhibiting an adipogenic phenotype (Gao et al., 2008). Another group also demonstrated in rat bone marrow MSCs that metformin could revert the pro-adipogenic effect of rosiglitazone in favour of an osteoblastic phenotype partly mediated by AMPK and RUNX2 activation (Molinuevo et al., 2010). Likewise, metformin treatment markedly influenced the osteogenic differentiation of adipose tissue-derived stem cells, while preventing their differentiation into adipocytes (McCarthy et al., 2016).

To the best of our knowledge, the present study represents the first to report an osteogenic action of metformin in iPSC-MSCs. iPSC-MSCs are regarded as an alternative source for bone marrow MSCs in regenerative medicine (Jung, Bauer, & Nolta, 2012). However, a key translationally relevant issue that still remains elusive is how to enhance the osteogenic potential of iPSC-MSCs, as tumorigenic activity is greatly reduced following differentiation when compared with iPSCs (Jung et al., 2012; Wu et al., 2017). Obtaining pure and functional osteogenic cells from iPSC-MSCs is of paramount importance before clinical application. Therefore, it seemed reasonable to investigate whether metformin administration might also induce iPSC-MSC osteoblastic commitment, especially as metformin is often prescribed to T2DM patients who may require bone and periodontal regenerative procedures.

As a highly hydrophilic cationic drug, metformin intracellular effects rely on uptake mechanisms mediated by OCTs belonging to the solute carrier 22A (SLC22A) gene family (Nies et al., 2011). Here, we found that iPSC-MSCs expressed functional OCT-1. Moreover, OCT inhibition by cimetidine significantly reduced metformin uptake in iPSC-MSCs. These

results strongly indicate that to act as a promising osteoanabolic drug, metformin must access functional OCT-expressing cells with potential osteogenic capacity. Furthermore, OCTs could eventually serve as cellular biomarkers to predict positive osteogenic responses to metformin treatment. Our data also demonstrate that metformin increased ALP activity and mineral deposition and markedly stimulated gene and protein expression of critical osteoblastic transcription factors, like RUNX2 and OSX. We also validate and extend previous observations by demonstrating, in the particular case of iPSC-MSCs, that LKB1/AMPK pathway activation in response to metformin is associated with an increase in RUNX2 gene and protein expression as well as nuclear localization. These responses were markedly inhibited in cells transfected with a catalytically inactive, kinase dead form of LKB1, which pointed to a positive regulatory role of the LKB1/AMPK pathway in metformin-induced iPSC-MSC osteogenic differentiation. This is in agreement with previous findings showing AMPK involvement in metformin-mediated osteoblastic differentiation in bone marrow MSCs and osteoblasts (Gao et al., 2008; Jang et al., 2011; Kanazawa et al., 2008; McCarthy et al., 2016). Of note, following transfections, we found a marked reduction in LKB1-KD surviving cells when compared with LKB1-WT cells. This undesirable effect could have been related to the fact that LKB1/AMPK signalling activation is essential for cell survival under bioenergetic stress. Thus, by inhibiting AMPK activation, cell responses to conditions that compromise intracellular energy (i.e. serum starvation) might have led to increased cell death (Lee et al., 2015).

Compelling studies reporting on the potential application of iPSC-MSCs in oral and craniofacial tissue regeneration have recently emerged. In one study, human-derived foreskin iPSC-MSCs were implanted into a periodontal fenestration defect model in nude rats with clotted fibrinogen and thrombin without any other scaffolds. After 2 weeks, the percentage of defect area regenerated with newly mineralized tissue in animals receiving iPSC-MSCs was as high as 51.31% when compared with the 'defect only' group (23.8%) and the 'clotting factors only' group (29.77%) (Hynes et al., 2013). Also, in rats, another research group created a periodontal defect model by using wire ligatures and *P. gingivalis* inoculation. They derived iPSC-MSCs from rat iPSCs and transfected them with tumour necrosis factor α -stimulated gene-6 (TSG-6), which has strong anti-inflammatory effects. iPSC-MSCs and iPSC-MSCs/TSG-6 were administered to the animals through intravenous and topical injection once weekly for 3 weeks. Compared with controls, both iPSC-MSCs and iPSC-MSCs/TSG-6 groups significantly decreased serum levels of pro-inflammatory cytokines interleukin-1 β and tumour necrosis factor- α . In addition, the iPSC-MSCs/TSG-6-treated group showed a significant reduction in alveolar bone resorption (Yang et al., 2014). These results demonstrated that iPSC-MSCs have an anti-inflammatory effect, and the combination with TSG-6 greatly enhanced the therapeutic effects of iPSC-MSCs in periodontal bone loss. Overall, these studies demonstrate that iPSCs and iPSC-MSCs have the potential to differentiate into cells essential to enhance bone and periodontal regeneration.

5 CONCLUSIONS

Our study demonstrates that the antidiabetic drug metformin exerts an osteogenic action on OCT-1-expressing iPSC-MSCs. This effect appears to be dependent on the LKB1/AMPK

signalling pathway. The concept of utilizing the osteoanabolic effects of metformin to promote the osteogenic commitment of autogenous iPSC-MSCs as a therapeutic option to enhance periodontal and bone tissue regeneration is promising. In particular, this is translationally attractive as it could be applied to a subset of T2DM patients in need of bone or periodontal tissue regenerative procedures who are already being prescribed with daily, highly tolerable doses of systemic metformin. Our work also provides preliminary evidence to support metformin/iPSC-MSC-based localized therapy with CPC scaffolds. CPC has excellent biocompatibility, osteoconductivity, *in situ* hardening and moulding capabilities and injectability, and can be resorbed and replaced by new bone *in vivo* (Wang et al., 2014). Thus, the proposed metformin/iPSC-MSCs/CPC-based therapy has great potential for bone and periodontal tissue regeneration to be used in the general population. We plan to conduct future preclinical studies to evaluate the feasibility of using CPC-seeded iPSC-MSCs for bone and periodontal tissue regeneration in response to clinically relevant doses of systemic as well as locally delivered metformin.

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References

- Chapple ILC, Genco R. Working group 2 of Joint EFPAAPw. Diabetes and periodontal diseases: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *Journal of Clinical Periodontology*. 2013; 40(Suppl 14):S106–S112. [PubMed: 23627322]
- Chen L, Shu Y, Liang X, Chen EC, Yee SW, Zur AA, ... Giacomini KM. OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111(27):9983–9988. [PubMed: 24961373]
- Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LM, ... Cheng L. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Research*. 2011; 21(3):518–529. [PubMed: 21243013]
- Cortizo AM, Sedlinsky C, McCarthy AD, Blanco A, Schurman L. Osteogenic actions of the anti-diabetic drug metformin on osteoblasts in culture. *European Journal of Pharmacology*. 2006; 536(1–2):38–46. [PubMed: 16564524]
- Fadini GP, Ferraro F, Quaini F, Asahara T, Madeddu P. Concise review: diabetes, the bone marrow niche, and impaired vascular regeneration. *Stem Cells Translational Medicine*. 2014; 3(8):949–957. [PubMed: 24944206]
- Gao Y, Xue J, Li X, Jia Y, Hu J. Metformin regulates osteoblast and adipocyte differentiation of rat mesenchymal stem cells. *Journal of Pharmacy and Pharmacology*. 2008; 60(12):1695–1700. [PubMed: 19000376]
- Hynes K, Menicanin D, Gronthos S, Bartold PM. Clinical utility of stem cells for periodontal regeneration. *Periodontology 2000*. 2012; 59(1):203–227. [PubMed: 22507067]
- Hynes K, Menicanin D, Han J, Marino V, Mrozik K, Gronthos S, Bartold PM. Mesenchymal stem cells from iPS cells facilitate periodontal regeneration. *Journal of Dental Research*. 2013; 92(9):833–839. [PubMed: 23884555]

- Jaacks LM, Siegel KR, Gujral UP, Narayan KM. Type 2 diabetes: A 21st century epidemic. *Best Practice & Research Clinical Endocrinology & Metabolism*. 2016; 30(3):331–343. [PubMed: 27432069]
- Jang WG, Kim EJ, Bae IH, Lee KN, Kim YD, Kim DK, ... Koh J-T. Metformin induces osteoblast differentiation via orphan nuclear receptor SHP-mediated transactivation of Runx2. *Bone*. 2011; 48(4):885–893. [PubMed: 21147283]
- Jung Y, Bauer G, Nolta JA. Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: Progress toward safe clinical products. *Stem Cells*. 2012; 30(1):42–47. [PubMed: 21898694]
- Kanazawa I, Yamaguchi T, Yano S, Yamauchi M, Sugimoto T. Metformin enhances the differentiation and mineralization of osteoblastic MC3T3-E1 cells via AMP kinase activation as well as eNOS and BMP-2 expression. *Biochemical and Biophysical Research Communication*. 2008; 375(3):414–419.
- Lee SW, Li CF, Jin G, Cai Z, Han F, Chan CH, ... Lin H-K. Skp2-dependent ubiquitination and activation of LKB1 is essential for cancer cell survival under energy stress. *Molecular Cell*. 2015; 57(6):1022–1033. [PubMed: 25728766]
- Lian Q, Zhang Y, Zhang J, Zhang HK, Wu X, Zhang Y, ... Tse HF. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation*. 2010; 121(9):1113–1123. [PubMed: 20176987]
- McCarthy AD, Cortizo AM, Sedlinsky C. Metformin revisited: Does this regulator of AMP-activated protein kinase secondarily affect bone metabolism and prevent diabetic osteopathy. *World Journal of Diabetes*. 2016; 7(6):122–133. [PubMed: 27022443]
- Molinuevo MS, Schurman L, McCarthy AD, Cortizo AM, Tolosa MJ, Gangoiti MV, ... Sedlinsky C. Effect of metformin on bone marrow progenitor cell differentiation: in vivo and in vitro studies. *Journal of Bone and Mineral Research*. 2010; 25(2):211–221. [PubMed: 19594306]
- Nies AT, Koepsell H, Damme K, Schwab M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. *Handbook of Experimental Pharmacology*. 2011; 201:105–167.
- Pagni G, Kaigler D, Rasperini G, Avila-Ortiz G, Bartel R, Giannobile WV. Bone repair cells for craniofacial regeneration. *Advanced Drug Delivery Reviews*. 2012; 64(12):1310–1319. [PubMed: 22433781]
- Patel H, Younis RH, Ord RA, Basile JR, Schneider A. Differential expression of organic cation transporter OCT-3 in oral premalignant and malignant lesions: Potential implications in the antineoplastic effects of metformin. *Journal of Oral Pathology & Medicine*. 2013; 42(3):250–256. [PubMed: 22861817]
- Pilipchuk SP, Plonka AB, Monje A, Taut AD, Lanis A, Kang B, Giannobile WV. Tissue engineering for bone regeneration and osseointegration in the oral cavity. *Dental Materials*. 2015; 31(4):317–338. [PubMed: 25701146]
- Pradeep AR, Patnaik K, Nagpal K, Karvekar S, Ramamurthy BL, Naik SB, ... Raju A. Efficacy of locally-delivered 1% metformin gel in the treatment of intrabony defects in patients with chronic periodontitis: A randomized, controlled clinical trial. *Journal of Investigative and Clinical Dentistry*. 2015; 7(3):239–245. [PubMed: 25787121]
- Rosa V, Della Bona A, Cavalcanti BN, Nor JE. Tissue engineering: From research to dental clinics. *Dental Materials*. 2012; 28(4):341–348. [PubMed: 22240278]
- Schneider A, Younis RH, Gutkind JS. Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma. *Neoplasia*. 2008; 10(11):1295–1302. [PubMed: 18953439]
- Schurman L, McCarthy AD, Sedlinsky C, Gangoiti MV, Arnol V, Bruzzone L, Cortizo AM. Metformin reverts deleterious effects of advanced glycation end-products (AGEs) on osteoblastic cells. *Experimental and Clinical Endocrinology & Diabetes*. 2008; 116(6):333–340. [PubMed: 18273753]
- Schwartz AV. Diabetes mellitus: Does it affect bone? *Calcified Tissue International*. 2003; 73(6):515–519. [PubMed: 14517715]
- Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in

- response to energy stress. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(10):3329–3335. [PubMed: 14985505]
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006; 126(4):663–676. [PubMed: 16904174]
- Tang M, Chen W, Liu J, Weir MD, Cheng L, Xu HH. Human induced pluripotent stem cell-derived mesenchymal stem cell seeding on calcium phosphate scaffold for bone regeneration. *Tissue Engineering Part A*. 2014; 20(7–8):1295–1305. [PubMed: 24279868]
- Villa-Diaz LG, Brown SE, Liu Y, Ross AM, Lahann J, Parent JM, Krebsbach PH. Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. *Stem Cells*. 2012; 30(6):1174–1181. [PubMed: 22415987]
- Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: An overview. *Clinical Science*. 2012; 122(6):253–270. [PubMed: 22117616]
- Wang P, Liu X, Zhao L, Weir MD, Sun J, Chen W, ... Xu HHK. Bone tissue engineering via human induced pluripotent, umbilical cord and bone marrow mesenchymal stem cells in rat cranium. *Acta Biomaterialia*. 2015; 18:236–248. [PubMed: 25712391]
- Wang P, Song Y, Weir MD, Sun J, Zhao L, Simon CG, Xu HHK. A self-setting iPSMSC-alginate-calcium phosphate paste for bone tissue engineering. *Dental Materials*. 2016; 32(2):252–263. [PubMed: 26743965]
- Wang P, Zhao L, Chen W, Liu X, Weir MD, Xu HHK. Stem cells and calcium phosphate cement scaffolds for bone regeneration. *Journal of Dental Research*. 2014; 93(7):618–625. [PubMed: 24799422]
- Wu Q, Yang B, Hu K, Cao C, Man Y, Wang P. Deriving osteogenic cells from induced pluripotent stem cells for bone tissue engineering. *Tissue Engineering Part B, Reviews*. 2017; 23:1–8. [PubMed: 27392674]
- Yang H, Aprecio RM, Zhou X, Wang Q, Zhang W, Ding Y, Li Y. Therapeutic effect of TSG-6 engineered iPSC-derived MSCs on experimental periodontitis in rats: a pilot study. *PLoS One*. 2014; 9(6):e100285. [PubMed: 24979372]
- Zhao Q, Gregory CA, Lee RH, Reger RL, Qin L, Hai B, ... Liu F. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112(2):530–535. [PubMed: 25548183]
- Zhen D, Chen Y, Tang X. Metformin reverses the deleterious effects of high glucose on osteoblast function. *Journal of Diabetes Complications*. 2010; 24(5):334–344.

FIGURE 1.

Expression of organic cation transporters (OCTs) in induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs). (a) Whole cell extracts were analysed by Western blotting to determine expression levels of OCTs in iPSC-MSCs. As shown, only OCT-1 expression was detected in iPSC-MSCs. (b) A metformin uptake assay was conducted in iPSC-MSCs in the presence or absence of cimetidine (10 μM). Cimetidine significantly inhibited metformin intracellular uptake (* $p < 0.05$). (c) iPSC-MSC cell viability in response to 10 μM metformin or vehicle control. Metformin treatment for 1 week was not detrimental to iPSC-MSC viability when compared with controls

FIGURE 2.

Metformin promotes mineralized nodule formation in induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs). (a) Following treatment with control media, metformin (10 μM) or osteogenic media (OS) for 14 days, iPSC-MSCs were stained with Alizarin Red S to detect calcium-containing mineralized nodules. (b) In another set of experiments, iPSC-MSCs treated with control media, metformin (10 μM) or OS media for 14 and 21 days were stained with xylenol orange (XO), a calcium-chelating fluorochrome that stains minerals. Mineral nodules were observed by using an epifluorescence microscope. Scale bar: 100 μM. (c) Quantification of the red fluorescent, XO-stained mineral area was performed by calculating $A_{\text{Mineral}}/A_{\text{Total}}$, where A_{Mineral} is the area of mineralization in the image and A_{Total} is the total area of the image ($n = 3$) (* $p < 0.05$, ** $p < 0.01$)

FIGURE 3.

Induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) osteogenic markers in response to metformin. (a) Alkaline phosphatase (ALP) staining in iPSC-MSC cells treated with 10 μ M metformin for 7 days display a more pronounced blue-like staining than cells cultured in MSC growth media (control). (b) An ALP activity assay was also performed in iPSC-MSCs treated for 7 days with MSC growth media (control) or containing 10 μ M metformin. ALP activity was significantly elevated in the metformin-treated group ($*p < 0.05$). Fold change gene expression levels for ALP (c), Runt-related transcription factor 2 (RUNX2) (d) and osterix (OSX) (e) were determined following similar treatments for 7 and 14 days as described in (a). The Ct value of iPSC-MSCs in MSC growth media for 1 day served as the calibrator. All osteogenic markers were upregulated in response to metformin ($*p < 0.05$). (f) Whole cell extracts were analysed by Western blotting to determine expression levels of RUNX2 and OSX following treatment with metformin for 7 days. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoblotting served as the loading control

FIGURE 4.

Metformin induces Runt-related transcription factor 2 (RUNX2) via LKB1/AMP-activated protein kinase (AMPK) signalling activation in induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs). (a) Whole cell extracts were analysed by Western blotting to determine expression levels of phosphorylated and total AMPK and RUNX2 following treatment with control media or metformin (10 μ M). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoblotting served as the loading control. (b) Whole cell extracts were obtained following a 24 h treatment with metformin or control media of iPSC-MSCs transiently transfected with empty vector (ev), LKB1-WT (LKB1 wild-type) plasmid or LKB1 kinase dead (LKB1-KD) plasmid. Western blotting was performed to determine expression levels of phosphorylated AMPK, total AMPK and RUNX2. GAPDH served as the loading control. (c) RUNX2 immunofluorescence staining in LKB1-WT and LKB-KD transfected iPSC-MSCs following a 24 h treatment with 10 μ M metformin (Met) or control media (Ctl). Scale bar: 100 μ M

FIGURE 5.

Metformin induces the formation of calcium-containing mineralization nodules in induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) seeded on calcium phosphate cement (CPC) scaffolds. (a) Alizarin Red S (ARS) staining of iPSC-MSC-seeded CPC scaffolds treated with or without 10 μ M metformin. (b) ARS staining was quantified colorimetrically following elution of ARS dye with 10% cetylpyridinium chloride (** $p < 0.01$)

TABLE 1

List of primer sequences used to analyse induced pluripotent stem cell-derived mesenchymal stem cell osteogenic gene expression

Gene	Primers
Alkaline phosphatase (ALP)	Forward: cggatcctgacaaaaacc Reverse: tcatgatgtccgtgggtcaat
Runt-related transcription factor 2 (RUNX2)	Forward: gactgtggttaccgtcatggc Reverse: acttggttttcataacagcgga
Osterix (OSX)	Forward: tctccatctgcctgactcct Reverse: agcgtatggcttcttctgc
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Forward: tcaacgacccttcattgac Reverse: atgcaggatgatgtctgg