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Effect of Metformin on Cell Growth and Differentiation in Cultured Odontoblasts

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Metformin (1,1-dimethylbiguanide hydrochloride), derived from French lilac (*Galega officinalis*), is a first-line anti-diabetic drug prescribed for patients with type 2 diabetes. However, the role of metformin in odontoblastic cell differentiation is still unclear. This study therefore undertook to examine the effect of metformin on regulating odontoblast differentiation in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells. As compared to controls, metformin significantly accelerated the mineralization, significantly increased and accelerated the expressions of ALP and Col I mRNAs, and significantly increased the accelerated expressions of DSPP and DMP-1 mRNAs, during differentiation of MDPC-23 cells. There was no alteration in cell proliferation of MDPC-23 cells, on exposure to metformin. These results suggest that the effect of metformin on MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells, facilitates the odontoblast differentiation and mineralization, without altering the cell proliferation.

Key words: metformin, odontoblasts, differentiation, mineralization

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Introduction

Tooth formation is regulated by sequential and reciprocal epithelial- mesenchymal interactions [1]. Dental epithelial cells from the dental organ differentiate into ameloblasts, while ectomesenchymal cells from the dental papilla differentiate into odontoblasts [1,2]. Dentin, which forms the bulk of the tooth, is a mineralized tissue composed of odontoblasts [3,4]. Odontoblasts are differentiated from ectomesenchymal cells and are involved in the secretion of the organic matrix during odontoblast differentiation [3-5]. This matrix contains a mixture of collagenous and non-collagenous proteins, which subsequently mineralize to form dentin, the main hard tissue of a tooth [3-5]. Dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1), which are synthesized and secreted by odontoblasts, are regarded as odontoblast differentiation markers [1,4,5].

Many researchers suggest that signaling molecules in the bone morphogenetic protein, fibroblast growth factor and wingless families as well as transcription factors such as Runx2 are involved in the odontoblast differentiation [5-7]. Indeed, the exquisite balance between conserved signaling pathways and transcription factors is important for all aspects of odontoblast differentiation [5-7]. However, the exact molecular mechanisms underlying odontoblast differentiation

are not well understood.

Metformin (1,1-dimethylbiguanide hydrochloride), derived from French lilac (*Galega officinalis*), is an anti-diabetic drug that belongs to the biguanide class, and a first-line drug prescribed for patients with type 2 diabetes [8-10]. It exerts its anti-diabetic effect by reducing hepatic glucose production [11,12]. Moreover, it has been known to used in the treatment of polycystic ovarian syndrome [13] and non-alcoholic fatty liver disease [14]. In addition, metformin has been reported to have anti-cancer effects in variety of cancers including colon cancer, ovarian cancer, lung cancer, breast cancer and prostate cancer and so on [15-20]. Nevertheless, at present, the role of metformin in regulating odontoblast differentiation remains unclear.

In this study, therefore, the effect of metformin on regulating odontoblast differentiation was investigated in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells.

Materials and Methods

Materials

Metformin, Alizarine red S and 3-[4,5-dimethylthiazol-2-yl] -2,5- diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other reagents were of analytical grade. In preliminary studies to investigate the effect of metformin on the differentiation of MDPC-23 odontoblastic cells, the 100 μM metformin started to show the difference from the control group (metformin non-treated control group). In the subsequent experiments of this study, therefore, MDPC-23 odontoblastic cell differentiation was examined using the concentration of 100 μM metformin.

Cell cultures

MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells were kindly provided by Dr. J. E. Nör (University of Michigan, Ann Arbor, MI, USA). The MDPC-23 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37°C [21,22]. To induce cell differentiation and mineralization, confluent MDPC-23 cells were treated with 50 μg/ml ascorbic acid and 10 mM β -glycerophosphate for up to 7 days [4,23].

Alizarine red S staining

The cells were fixed with 70% ethanol for 20 min and stained with 1% Alizarine red S (Sigma–Aldrich Corp., St. Louis, MO, USA) in 0.1% NH_4OH at pH 4.2–4.4. The mineralization assays were performed by staining MDPC-23 cells with Alizarin red S solution. The cells were evaluated at 0, 4 and 7 days. To quantify the intensity of mineralization, we measured density of stained nodules by colorimetric spectrophotometry. The stained cells were collected by centrifugation at 13,000 rpm for 10 min at 4°C. Cell lysate was solubilized with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature. Solubilized stain (0.1 ml) was transferred to wells of a 96-well plate, and absorbance was measured at 405 nm.

Quantitative PCR analysis

To perform qPCR, the total RNA was isolated using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was carried out with 1 μg of total RNA using the TermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA). The levels of alkaline phosphatase (ALP) (ALP-F, 5'-CTCTCCGAGA TGGTGGAGGT-3'; ALP-R, 5'-GTC TTCTCCACCGTGGGT CT-3'), type I collagen (Col I) (Col I-F, 5'-TAAGTTGCC AAGAACGTGCC-3'; Col I-R, 5'-AATTGAAAGCCAGGA GGCAT-3'), dentin sialophosphoprotein (DSPP) (DSPP-F, 5'-ATAGCACCAACCATGAGGCT-3'; DSPP-R, 5'-CTTTTGTT GCCTTTGTTGGG-3'), dentin matrix protein-1 (DMP-1) (DMP-1-F, 5'-CGGCTGGTGGTCTCTCTAAG-3'; DMP-1-R, 5'-ATCTTCCTGGG ACTGGGTCT-3'), bone sialoprotein (BSP) (BSP-F, 5'-AAGAAAATGGAGACGGC GAT-3'; BSP-R, 5'-CACCTGCTTCAGTGACGCTT-3') and GAPDH (GAPDH-F, 5'-TGCATCCTGCACCACCAACT-3'; GAPDH-R, 5'-CGCCT GCTTCACCACCTTC -3') inductions were measured by qPCR, and visualized by DNA agarose gel electrophoresis. The differences in expression were presented as a histogram after densitometry using a VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay

The MDPC-23 cells were seeded at a density of 4 X 10^4 cells/well in 24-well plates and allowed to attach to the well overnight. After incubation, the cultured cells were treated with metformin. The cells were incubated with metformin at various defined concentrations (1, 3, 10, 30, 100 and 300 μ

M) for 1 and 2 days at 37°C. After incubation under the defined conditions, the cells were incubated for another 4 h in 20 ㎕ of 5 mg/ml MTT solution. To dissolve the formazan transformed from MTT, the cells were resuspended in 150 ㎕ dimethyl sulfoxide and the optical density of the solution was determined using a spectrometer at an incident wavelength of 495 nm [24]. The experiments were repeated four times, independently.

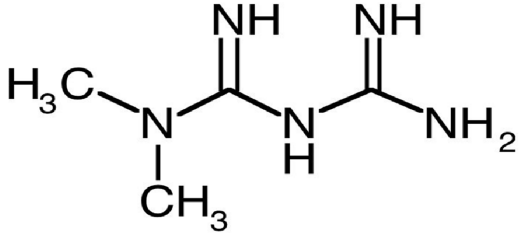
Statistical analysis

All experiments were performed at least in triplicate. The results were presented as the mean ± SEM. Statistical significance was analyzed using a Student's *t*-test for two groups and one-way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

Metformin accelerates mineralization in MDPC−23 odontoblastic cells

To examine the effect of metformin on odontoblastic cell mineralization, the mineralization assays were performed by staining MDPC-23 cells with Alizarine red S solution. MDPC-23 cells were cultured for 7 days in differentiation media treated with 100 μM metformin and the mineralized nodules were evaluated by Alizarin red S staining. In control MDPC-23 cells and metformin treated MDPC-23 cells, mineralized nodules appeared after 4 days of culture (Fig. 2). The metformin-treated MDPC-23 cells showed mineralized nodules after 4 and 7 days, and the mineralized nodules significantly increased compared to control MDPC-23 cells (Fig. 2).



Metformin
(1,1-Dimethylbiguanide)
(*N*,*N*-Dimethylimidodicarbonimidic diamide)

Fig. 1. Chemical structure of metformin.

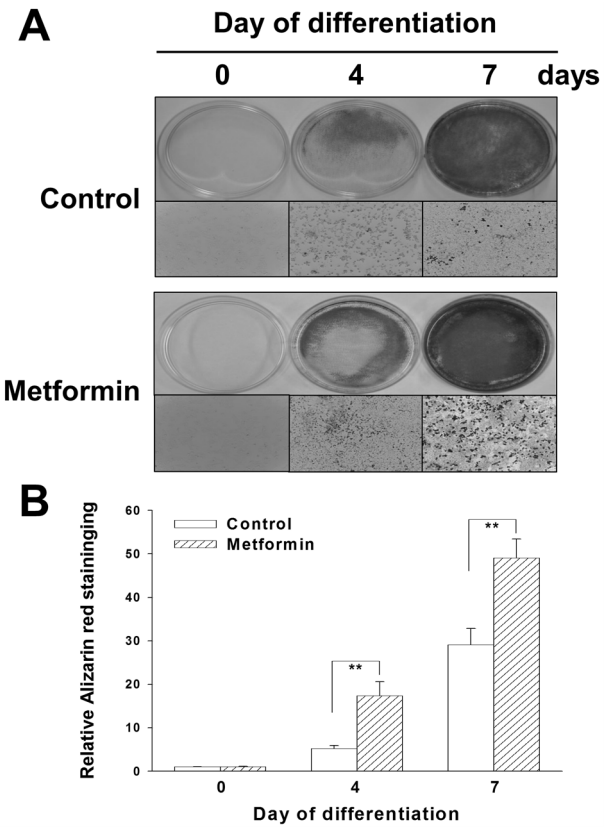


Fig. 2. Effect of metformin on the mineralization in MDPC-23 odontoblastic cells. Confluent cultures of MDPC-23 cells were maintained in complete medium with the addition of differentiation cocktail (50 ㎍/ml ascorbic acid and 10 mM β -glycerophosphate). MDPC-23 odontoblastic cell mineralization was altered by metformin. (A) Mineralized nodule formation in MDPC-23 cells. MDPC-23 cells were treated with 100 μM metformin for 7 days, and the mineralization was evaluated by Alizarine red S staining (0; 0 day after treatment with differentiation media, 4; 4 days after treatment with differentiation media, 7; 7 days after treatment with differentiation media, Control; treatment with differentiation media only, Metformin; treatment with differentiation media and 100 μM metformin). (B) Quantification of mineralization was accessed by colorimetric spectrophotometry. Each data point represents the mean ± SEM of four experiments. $^{**}p<0.01$ vs. control (the control cells were measured in the absence of metformin treatment).

Metformin changes the expressions of differentiation marker genes in MDPC−23 odontoblastic cells

To study the potential role of metformin in MDPC-23 cell differentiation, the qPCR analyses were performed using ALP, Col I, DSPP, DMP-1, BSP and GAPDH primers in MDPC-23 cells treated with metformin. In control MDPC-23 cells, the ALP expression appeared after 7 days of culture (Fig. 3). In contrast, to control MDPC-23 cells, the ALP expression appeared after 4 days of culture in metformin-treated MDPC-23 cells, and significantly increased compared

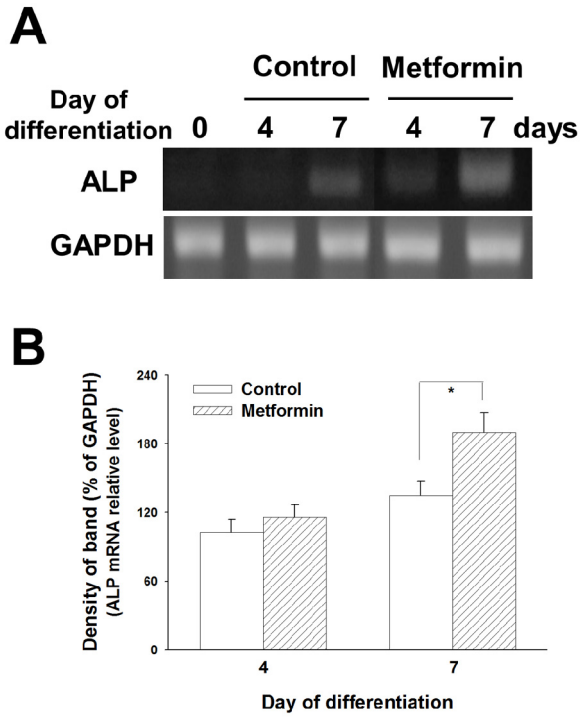


Fig. 3. Effect of metformin on alkaline phosphatase (ALP) mRNA expression. Total RNA was isolated using TRIzol reagent and reverse transcription was carried out with 1 μg of total RNA using the TermoScript RT-PCR system. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The quantitative data for (A) were analyzed by using Imagegauge 3.12 software after GAPDH normalization. The percentage of ALP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

to control MDPC-23 cells (Fig. 3). Furthermore, in control MDPC-23 cells, the Col I expression appeared after 7 days of culture (Fig. 4). In contrast to control MDPC-23 cells, the Col I expression appeared after 4 days of culture in metformin treated MDPC-23 cells, and significantly increased compared to control MDPC-23 cells (Fig. 4). In addition, the qPCR results showed that metformin enhanced the higher expression of DSPP, along with an increase in their activities compared to control cells (Fig. 5). And also, the qPCR results showed that the expression of DMP-1 was gradually up-regulated in MDPC-23 cells treated with metformin (Fig. 6). On the other hand, the expression of BSP, one of osteogenic differentiation marker gene, was not observed in control MDPC-23 cells and metformin-treated MDPC-23 cells (Fig. 7)

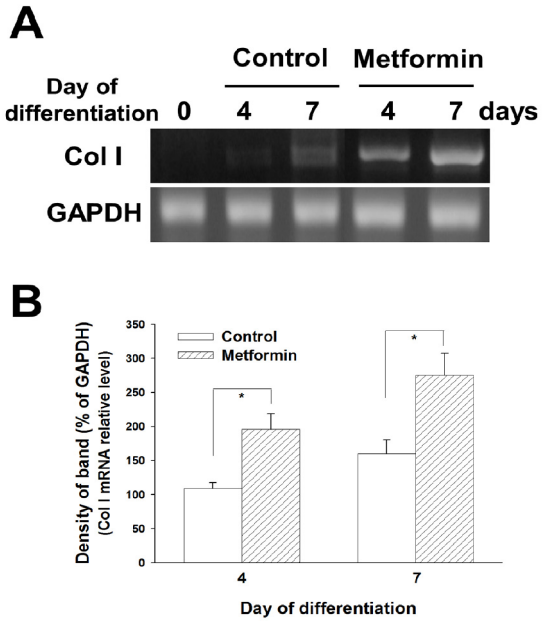


Fig. 4. Effect of metformin on type I collagen (Col I) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of Col I mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

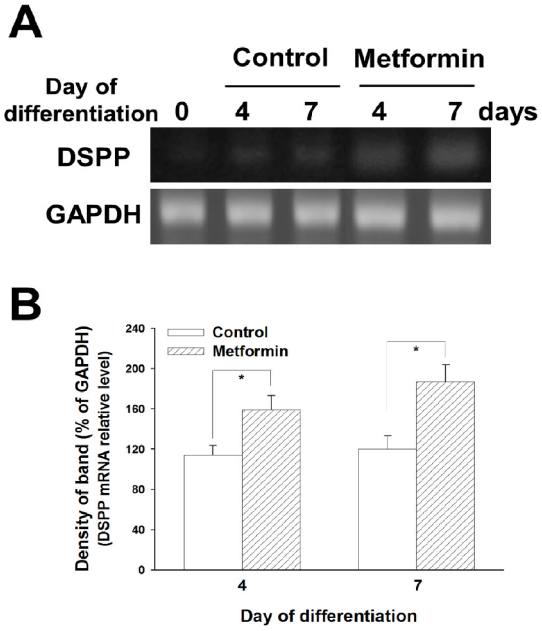


Fig. 5. Effect of metformin on dentin sialophosphoprotein (DSPP) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of DSPP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

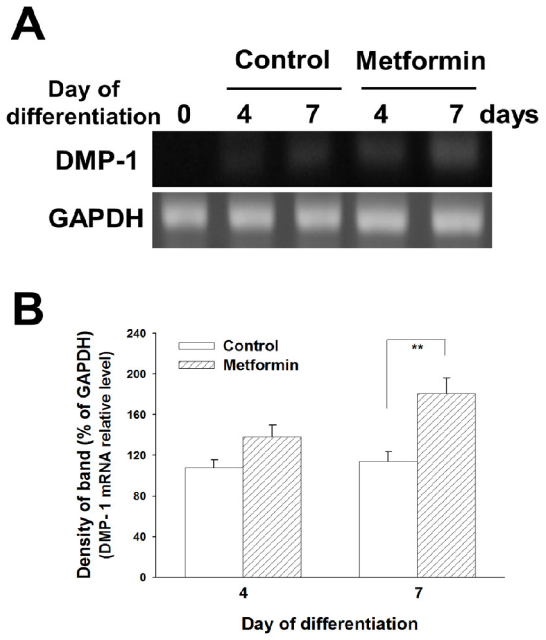


Fig. 6. Effect of metformin on dentin matrix protein-1 (DMP-1) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of DMP-1 mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{**}p<0.01$ vs. control (the control cells were measured in the absence of metformin treatment).

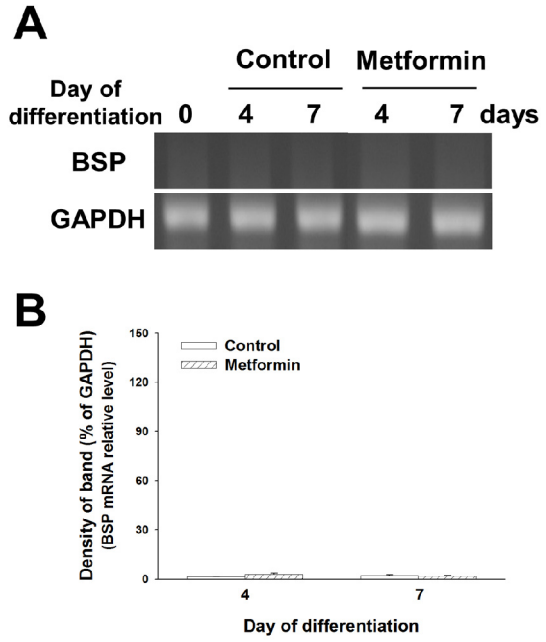


Fig. 7. Effect of metformin on bone sialoprotein (BSP) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of BSP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments.

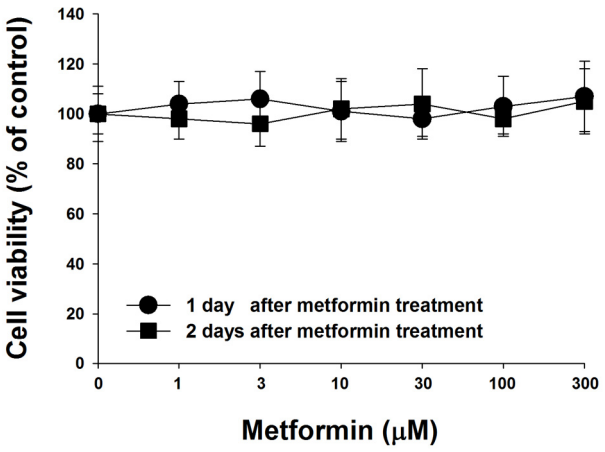


Fig. 8. Measurement of MDPC cell proliferation by metformin. The cells were treated with metformin at various defined concentrations (1, 3, 10, 30, 100 and 300 μM) for 1 and 2 days at 37°C. The cell proliferation was determined by MTT assays after metformin treatment. The experiments were repeated four times, independently.

Metformin does not alter the cell proliferation in MDPC−23 odontoblastic cells

To investigate the effect of metformin in MDPC-23 cell proliferation, the MTT assays were performed in MDPC-23 cells treated with metformin. As shown in Fig. 8, metformin did not alter the cell proliferation in the MDPC-23 cells. Taken together, these results were suggesting that metformin was promoting the odontogenic specific differentiation without alteration the cell proliferation in MDPC-23 odontoblastic cells.

Discussion

Metformin, derived from French lilac, is an oral hypoglycemic drug that is widely used in the world [8-10]. Moreover, it has been studied in biological events, including anti-cancer activities [15-20], polycystic ovarian syndrome [13] and non-alcoholic fatty liver disease [14]. However, the physiological role of metformin in the regulation of odontoblast differentiation is not entirely clear. In this study, therefore, the effect of metformin on regulating odontoblast differentiation was examined in MDPC-23 odontoblastic cells. This study reports here that metformin promoted the MDPC-23 odontoblastic cell differentiation.

The MDPC-23 cells are immortalized undifferentiated dental papilla cells that are capable of differentiating into odontoblasts that express DSPP (a dentin-specific gene) and forming mineralized nodules [1]. To determine the effect of

metformin on the stage of odontoblast differentiation *in vitro*, MDPC-23 cells were cultured in differentiation medium for up to 7 days with metformin, and the formation of mineralized nodules was evaluated by Alizarin red S staining. Our results revealed the presence of mineralized nodules from 4 days in control MDPC-23 cells and metformin treated MDPC-23 cells, and the mineralized nodules significantly increased compared to control MDPC-23 cells in the metformin-treated MDPC-23 cells (Fig. 2).

In addition, the expression levels of ALP and Col I mRNAs, well known markers of odontoblastic differentiation [1,4], gradually increased from days 4 to 7, and up-regulated compared to control MDPC-23 cells in the metformin-treated MDPC-23 cells (Fig. 3 and 4). Furthermore, to determine whether metformin induces the odontogenic specific differentiation in MDPC-23 cells, it was measured the expressional levels of DSPP and DMP-1, which are well known representative markers to identify the odontogenic differentiation [1,4,6,25]. Our qPCR results showed that the expressions of DSPP and DMP-1 were gradually up-regulated in MDPC-23 cells treated with metformin (Fig. 5 and 6). By contrast, the expression of BSP, one of osteogenic differentiation marker genes [1,4,26], was not observed in control MDPC-23 cells and metformin-treated MDPC-23 cells (Fig. 7). Taken together, these data suggest that metformin may positively accelerate the differentiation of MDPC-23 odontoblastic cells.

Next, to examine the effect of metformin on MDPC-23 cell proliferation, the MTT assays were performed in MDPC-23 cells treated with metformin. In the present study, while metformin accelerated mineralization in MDPC-23 odontoblastic cells, metformin did not alter the cell proliferation in the MDPC-23 cells (Fig. 8).

In conclusion, these results suggested that the metformin in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells facilitate the odontoblast differentiation and mineralization without alteration the cell proliferation. However, the mechanisms of odontoblast differentiation induced by metformin are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of odontoblast differentiation induced by metformin. Overall, metformin might be considered a critical candidate as an odontoblastic cell differentiation molecular target for the development of therapeutic agents in the dental medicine.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflicting interest.

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