O-YS-03

Effect of Metformin on Reducing Self-renewal and Proliferative Ability in Neuroblastoma SH-SY5Y Cells by Induction of Cell Differentiation

Thunwa Binlateh[1], Supita Tanasawet[2] and Pilaiwanwadee Hutamekalin[1]

[1]Department of Physiology, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand

[2]Department of Anatomy, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand

**Corresponding author. E-mail: pilaiwanwadee.h@psu.ac.th*

DOI:

ABSTRACT

Neuroblastoma (NB), the most common extracranial solid tumor, is challenged by high rate of relapse and drug-resistance. These may due to a presence of cancer stem cell (CSC) subpopulation in NB which relates to high capacity of self-renewal and differentiation. Recently, it has been believed that the promotion of cancer cells differentiation can irreversible change in the cancer phenotypes, particularly in loss of self-renewal. Consequently, drugs that trigger differentiation in cancers have currently being explored. In the present study, we showed the role of metformin in promoting differentiation and its effect on self-renewal capacity. The results revealed that metformin can promote cell differentiation with increasing neurite length and neuronal markers, β-tubulin III and MAP-2. Furthermore, the ability of cell proliferation at day 2 and 3 after metformin-mediated cell differentiation was significantly diminished. Likewise, self-renewal capacity was decreased in metformin-promoted cell differentiation as determined by significant reduction of spheroid number and size. The percentage of cell in G0/G1 phase was markedly increased in metformin-treated cells compared to control untreated cells. Together, Metformin has a potential role to promote differentiation and reduces self-renewal capacity and ability of cell proliferation after promoting differentiation.

Keywords: Neuroblastoma, Metformin, Self-renewal, Differentiation, Proliferation

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor of infants arising from pluripotent sympathetic cells or neuroblasts (Desai et al., 2009). It accounts for approximately 8-10% of all childhood tumors and around 15% of all cancer-related death in pediatric population. The available treatments of NB are challenged by high rate of relapse and drug-resistance (Colon and Chung, 2011). These may due to a presence of CSC subpopulation in NB (Bahmad et al., 2019) as a presence of efficient DNA repair, over-activation of survival pathways, enhanced cellular plasticity and immune evasion in CSC population (Turdo et al., 2019). Thus, the finding of targeting molecular drivers to cope with distinct stemness cell population within NB tumor may improve treatment outcome with long-lasting responses.

Currently, cancer cells can be induced to become normal cells by differentiation therapy. The therapy aims to resume the differentiation process and reduce cancer phenotypes by restimulating endogenous differentiation process (Yan and Liu, 2016). The prototype of differentiation therapy was success in acute promyelocytic leukemia (APL) by which terminal differentiation with loss of self-renewal capacity (Lo-Coco et al., 2013). Consequently, the chemical substances that can trigger cell differentiation in various types of tumor have recently been identified.

Biguanide drug, metformin, has been noted to promote cell differentiation in a variety of progenitor cells such as bone marrow progenitor cells (Molinuevo et al., 2010) and mesenchymal stem cells (Ma et al., 2018). Furthermore, our previous study revealed that metformin can promote neuronal differentiation via Cdk5/Sox6 crosstalk in NB (Binlateh et al., 2019). Moreover, it has been documented that metformin has anti-tumor effect by which inhibition of cell proliferation and cell cycle activity (Mogavero et al., 2017; Xie et al., 2017). However, the relation between differentiation potential and anti-tumor effect of metformin has not yet been thoroughly investigated. Therefore, this study aimed to investigate the role of metformin in promoting cells differentiation and its effect on self-renewal capacity after metformin promoted differentiation.

MATERIALS AND METHODS

Chemical and cell culture

Metformin was purchased from Apex Biotech Company, Taiwan. SH-SY5Y cells were purchased from ATCC (Bethesda, USA). Cells were inoculated at 37 ^{0}C in the humidified atmosphere of 95% under 5% of CO_2. Dulbecco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1 U/ml penicillin/streptomycin was conducted in this study. The medium was changed every second consecutive day.

Cell morphological observation

SH-SY5Y cells were treated with or without 5 mM metformin for 24 h. The morphological changes were observed and captured using bright-field microscope (Olympus IX73). The alteration of neurite outgrowth was analyzed using Image J software (NIH free download). Three-independent random area of each replication was calculated (see more in Binlateh et al., 2019).

Proliferative detection by MTT assay

Cell proliferation was examined after metformin withdrawal. Briefly, SH-SY5Y cells were seeded on 96-well plate (1.5×10^4 cells/well). The cells were treated with or without 5 mM metformin for 24 h. Following the treatment, metformin was removed and the cells were subsequently incubated with completed DMEM for 1, 2 and 3 day. The ability of proliferation was measured in each time point by incubating the cells with 5 mg/ml of MTT for 3 h. The formazan pellet was dissolved in DMSO. An optical density was detected at 540 nm from triplicate experiments.

Flow cytometry

In brief, SH-SY5Y cells-treated with or without 5 mM metformin for 24 h were trypsinized and fixed with 70% cold ethanol for 2-3 h. The cells then were incubated with Guava cell cycle reagent (Millipore, USA) for 30 min in the dark at room temperature. The proportion of cells in each cell cycle stage was determined using Guava EasyCyte Plus Flow Cytometry System (Millipore, USA).

Sphere formation assay

The treated and untreated cells with 5 mM metformin were incubated in serum-free media for inducing spheroid formation. The cells were then trypsinized and gently dissociated to be a single cells. Cells at the density 1.5×10^4 cells/well were cultured in the low attachment 24-well plate for 7 days. At the end of incubation, the number and diameter of spheroid were analyzed and imaged using bright-field microscope (Olympus IX73).

Western blot analysis

The cell lysate was prepared SH-SY5Y cells-treated with or without 5 mM metformin for 24 h by incubation with RIPA lysis buffer for 30 min. The concentration of lysate protein was examined by Bradford assay (Hercules, USA). The protein were equally subjected in SDS-PAGE, transferred to PVDF membrane (Millipore, USA) and blocked by 5% skimmed milk. The immunoblots were determined using specific primary antibodies against rabbit polyclonal anti-MAP-2 (Cell Signaling, USA), mouse-monoclonal anti-β-tubulin III, rabbit polyclonal anti-Cdk5 and rabbit polyclonal anti-Sox6 (all from Santa Cruz, USA). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Abcam, USA). The density of each protein band was detected using SuperSignal West Pico chemiluminescence substrate (ThermoFisher, USA) and determined by Image J software with β-actin normalization.

Statistical analysis

All experiment data were represented as mean ± standard error of mean (S.E.M.). The difference of statistical comparisons was analyzed using pair-independent *t-test*. The statistical significance was considered as $p\text{-}value < 0.05$.

RESULTS

Effect of metformin on cell differentiation

Our previous study revealed the potential role of metformin in neuronal differentiation (Binlateh et al., 2019). In order to verify this, the morphological changes of metformin-treated cells and untreated cells were visually determined. Metformin-treated cells displayed the longer neurites outgrowth than control untreated cells. Furthermore, the cell density of metformin-treated cells was lower than control untreated cells (Figure 1A).

Subsequently, the quantitative data of neurite length was remarkably increased in metformin-treated cells (Figure 1B). Meanwhile, the expression levels of neuronal markers, β-tubulin II and MAP-2, exhibited the obvious increase in metformin-treated cells (Figure 1C), suggesting that metformin has an effect on promoting neuronal differentiation.

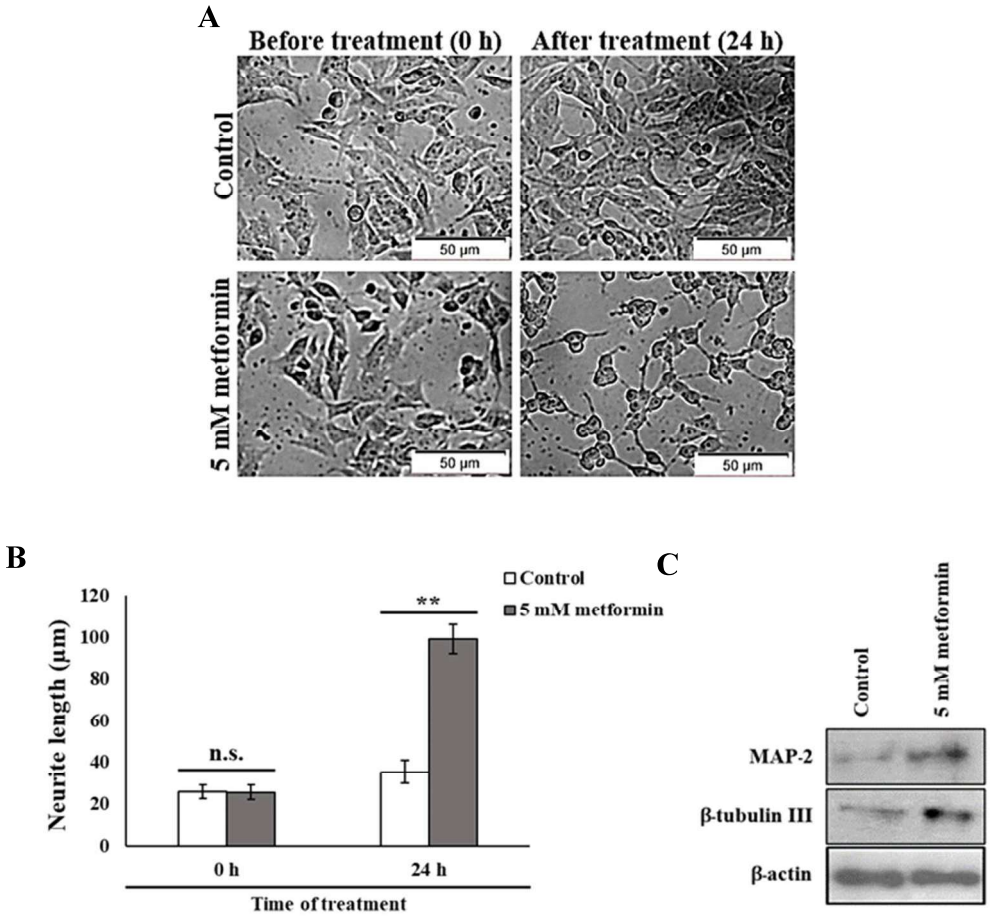

Figure 1. Effect of metformin on cell differentiation. (A) Morphological alterations under bright field microscope. (B) Quantitative data of neurite length (µm). Data represents the mean ± S.E.M. (n=3). $^{**}p<0.01$ versus control untreated group. (C) Expression levels of MAP-2 and β-tubulin III of cells-treated with or without 5 mM metformin for 24 h.

The expression of Cdk5 and Sox6 is changed in metformin-induced cell differentiation

Cdk5 and Sox6 are essential for regulating neuronal differentiation (Batista-Brito et al., 2009: Tsai et al., 1993). Our previous study reported that their function related to metformin-induced neuronal differentiation (Binlateh et al., 2019). To ascertain whether both of Cdk5 and Sox6 could relate to metformin-induced cell differentiation, the expression levels of both proteins were performed. The results showed that the differentiated cells induced by metformin significantly decreased Cdk5 expression while Sox6 expression was markedly increased compared to untreated control cells (Figure 2).

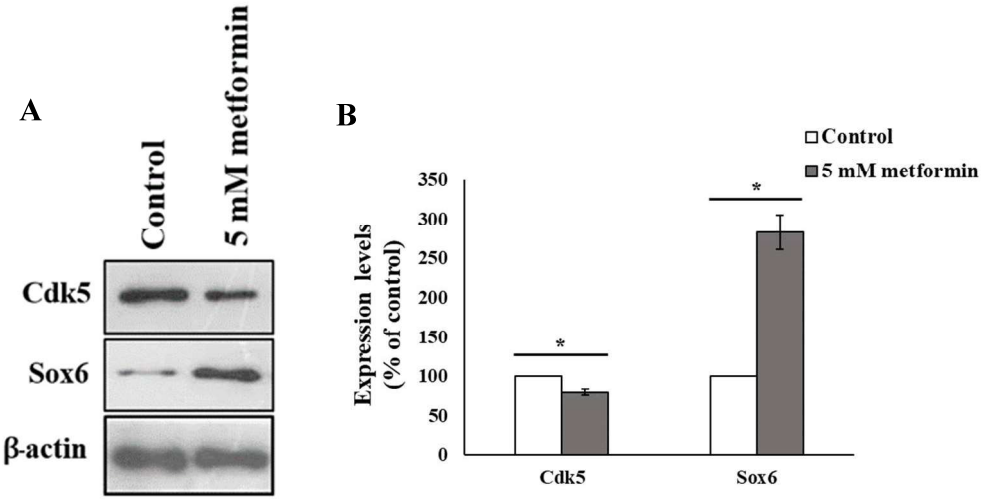

Figure 2. Expression levels of Cdk5 and Sox6 in differentiated cells induced by metformin. (A) Protein expression of Cdk5 and Sox6. (B) Quantitative result of Cdk5 and Sox6 expression levels. Data represents the mean ± S.E.M. (n=3). $^{*}p<0.05$ versus control untreated group.

The ability of cell proliferation is reduced in metformin-mediated cell differentiation with increasing cell proportion in G0/G1 phase of cell cycle

It has been reported that cell differentiation plays a remarkable inverse association with cell proliferation (Ruijtenberg and van den Heuvel, 2016). According to previous results that metformin can promote cell differentiation (Figure 1), therefore, we next hypothesized that whether the metformin-

mediated cell differentiation could reduce the ability of cell proliferation. The proliferative ability was determined at day 1, 2 and 3 after metformin-mediated cell differentiation. The results demonstrated that cell proliferative ability of metformin withdrawal trended to decrease in day1 while displayed a significant decrease in day2 and day3 compared to control untreated cells (Figure 3A). Consequently, the flow cytometric result noted that the percentage of cell in G0/G1 phase of metformin-induced differentiated cells was significantly increased (Figure 3B). Together, the findings strengthen our hypothesis that cell proliferative ability of cell differentiation induced by metformin is reduced after metformin withdrawal. This may be due to the increase of cell percentage in G0/G1 phase of cell cycle.

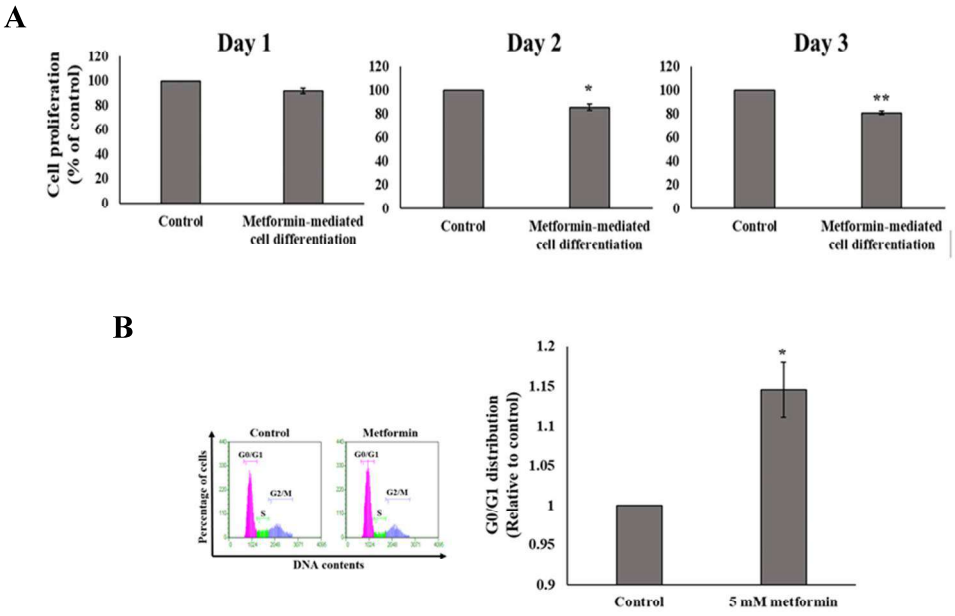

Figure 3. Cell proliferative ability is reduced after metformin withdrawal with increasing cell proportion in G0/G1 phase of cell cycle. (A) Representative results of cell proliferation as detected by MTT assay at day 1, 2 and 3 after metformin- mediated cell differentiation. (B) Flow cytometry results and quantitative result of G0/G1 cell distribution in metformin treatment relative to control untreated cells. Data represents the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ versus control untreated group.

Effect of metformin-induced cell differentiation on the ability of self-renewal

Since metformin reduced cell proliferative ability even though metformin was withdrawn (Figure 3A). Currently, it has been known that the mechanism that control the decision of self-renewal, cell cycle activity or division and differentiation are intermingled (Viatour, 2012). Thus, we next examined the ability of self-renewal using spheroid formation assay. The results revealed that the number and size of spheroid formation were significantly decreased in metformin withdrawal compared to control untreated cells (Figure 4).

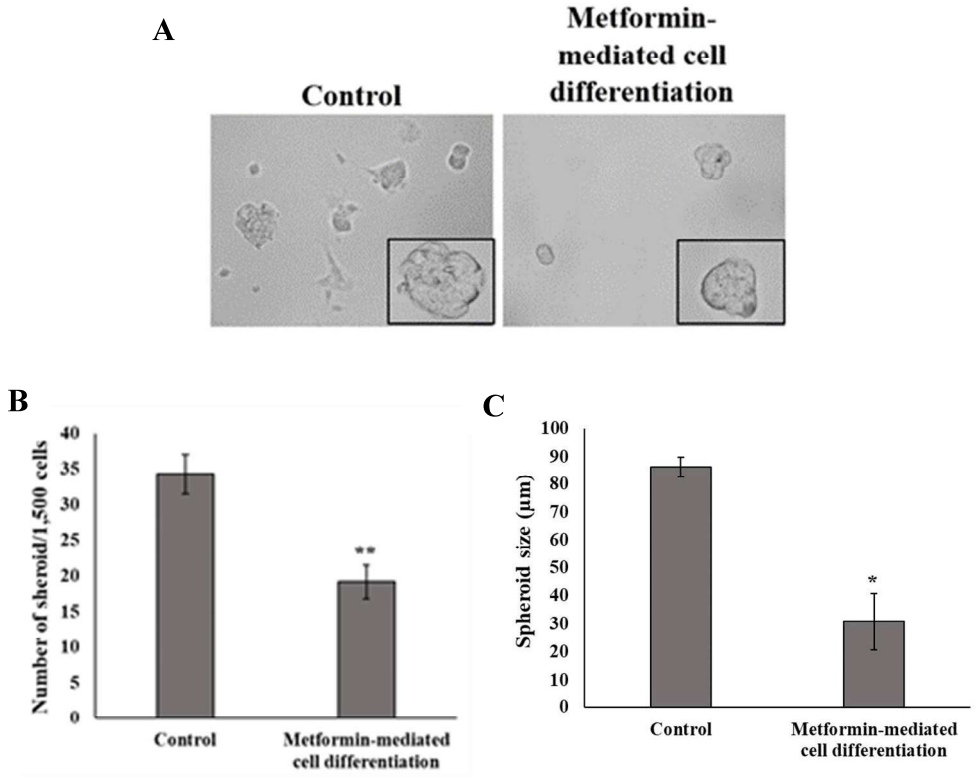

Figure 4. Effect of metformin-induced cell differentiation on the ability of self-renewal. (A) Morphology of spheroid formation of control and metformin withdrawal. (B) Quantitative result of spheroid number. (C) Quantitative result of spheroid size. Data represents the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ versus control untreated group.

DISCUSSION

Our results agree with previous study that metformin promoted neuronal differentiation by reducing Cdk5 while elevating Sox6 expression levels. This might be possible that metformin plays a role in regulating Cdk5/Sox6 crosstalk through ROS production during differentiation induction (Binlateh et al., 2019). Another interesting results was that differentiated cells induced by metformin reduced the capacity of self-renewal even though metformin was withdrawn. This is consistent with previous study, activation of terminal differentiation/maturation of APL by treating with retinoic acid and arsenic combination results in significant loss of self-renewal (Lo-Coco et al., 2013). It is likely that terminal differentiation constitutes the different transcriptional regulators with self-renewal (Hugues, 2018). However, the different transcriptional regulators of differentiation and self-renewal in tumor cells are elusive, since a presence of heterogeneity and functional hierarchy within tumor type cell is obvious difference (Hugues, 2018). Furthermore, the ability of cell proliferation was found to decrease after metformin-mediated cell differentiation. Consistent with result, the proportion of cells-treated with metformin in G0/G1 phase was increased, suggested that metformin reduce cell proliferative ability via induction of cell cycle arrest in G0/G1 phase. This result leads to fail G1 to S phase transition, resulted in inhibition of cell proliferation (Futatsugi et al., 2012). Previous study revealed that metformin downregulated cyclin D1 while upregulated $p27^{kip1}$ Cdk inhibitor to promote cell cycle arrest and growth inhibition (Xie et al., 2017). Thus, metformin-mediated cell cycle arrest and cell proliferative inhibition may relate to suppress cyclin expression whereas induced expression of Cdk inhibitors. These findings may open a new door to treat NB and other cancers-related to stemness of CSC.

CONCLUSION

Metformin has a potential role in promoting cell differentiation and reduces self-renewal capacity and ability of proliferation after promoting differentiation even though metformin is withdrawn. The findings could apply for the idea of using metformin as an alternative treatment strategy for NB and other cancers-related to stemness of CSC.

ACKNOWLEDGEMENTS

This research was supported by grants funded by Graduate School Dissertation Funding for Thesis and Revenue Budget Fund from Prince of Songkla University, Thailand and science achievement scholarship of Thailand (SAST). The authors thank the Department of Physiology, Faculty of Science, Prince of Songkla University for providing instruments.

REFERENCES

Bahmad, H. F., Chamaa, F. O., Assi, S., Chalhoub, R. M., Abou-Antoun, T. and Abou-Kheir, W. 2019. Cancer stem cells in neuroblastoma: Expanding the therapeutic frontier. Frontiers in Molecular Neuroscience. 12(131): 1-17.

Batista-Brito, R., Rossignol, E., Hjerling-Leffler, J., Denaxa, M., Wegner, M., Lefebvre, V. and Fishell, G. 2009. The cell-intrinsic requirement of Sox6 for cortical interneuron development. Neuron. 63(4): 466-481.

Binlateh, T., Tanasawet, S., Rattanaporn, O., Sukketsiri, W. and Hutamekalin, P. 2019. Metformin promotes neuronal differentiation via crosstalk between Cdk5 and Sox6 in neuroblastoma cells. Evidence-Based Complementary and Alternative Medicine. 2019: 1-14.

Colon, N. C. and Chung, D. H. 2011. Neuroblastoma. Advances in Pediatrics. 58(1): 297-311.

Desai, G., Filly, R. A. and Rand, L. 2009. Prenatal detection of an extra-adrenal neuroblastoma with hepatic metastases. Journal of Ultrasound in Medicine. 28(8): 1085-1090.

Futatsugi, A., Utreras, E., Rudrabhatla, P., Jaffe, H., Pant, H. C. and Kulkarni, A. B. 2012. Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons. Cell Cycle. 11(8): 1603-1610.

Hugues, D. T. 2018. Differentiation therapy revisited. Nature Reviews Cancer. 18(2): 117-128.

Lo-Coco, F., Avvisati, G., Vignetti, M., Thiede, C., Orlando, S. M., Iacobelli, S. and Specchia, G. 2013. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. New England Journal of Medicine. 369(2): 111-121.

Ma, J., Zhang, Z. L., Hu, X. T., Wang, X. T. and Chen, A. M. 2018. Metformin promotes differentiation of human bone marrow derived mesenchymal

stem cells into osteoblast via GSK3β inhibition. European Review for Medical and Pharmacological Sciences. 22(22): 7962-7968.

Mogavero, A., Maiorana, M. V., Zanutto, S., Varinelli, L., Bozzi, F., Belfiore, A. and Gariboldi, M. 2017. Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. Scientific Reports. 7(1): 1-12.

Molinuevo, M. S., Schurman, L., McCarthy, A. D., Cortizo, A. M., Tolosa, M. J., Gangoiti, M. V. and Sedlinsky, C. 2010. Effect of metformin on bone marrow progenitor cell differentiation: in vivo and in vitro studies. Journal of Bone and Mineral Research. 25(2): 211-221.

Ruijtenberg, S. and van den Heuvel, S. 2016. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle. 15(2): 196-212.

Tsai, L. H., Takahashi, T., Caviness, V. S. and Harlow, E. 1993. Activity and expression pattern of cyclin- dependent kinase 5 in the embryonic mouse nervous system. Development. 119(4): 1029-1040.

Turdo, A., Veschi, V., Gaggianesi, M., Chinnici, A., Bianca, P., Todaro, M. and Stassi, G. 2019. Meeting the challenge of targeting cancer stem cells. Frontiers in cell and developmental biology. 7: 1-16.

Viatour, P. 2012. Bridges between cell cycle regulation and self- renewal maintenance. Genes and Cancer. 3(11-12): 670-677.

Xie, W., Wang, L., Sheng, H., Qiu, J., Zhang, D., Zhang, L. and Zhang, K. 2017. Metformin induces growth inhibition and cell cycle arrest by upregulating microRNA34a in renal cancer cells. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 23: 29.

Yan, M., and Liu, Q. 2016. Differentiation therapy: a promising strategy for cancer treatment. Chinese Journal of Cancer. 35(1): 1-3