The Stemness and Quiescence Maintenance of Human Iliac Bone Marrow Mesenchymal Stem Cells by Resveratrol: An In Vitro Study

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Abstract

Resveratrol is one of the active substances in grape seeds that may maintain the stemness and quiescence of MSCs. The aim of this study was to investigate the effect of Resveratrol administration in maintaining the human iliac bone marrow stem cells (hIBM-MSCs) stemness and quiescence.

The examination processes in passage 3 of hIBM-MSCs for Cluster of Differentiation (CD)14, CD34, CD44, CD45, CD73, and CD105 expressions were done by using a method called flow cytometry. This study divided the samples into four experimental groups; they were a control group (hIBM-MSCs only), a group of hIBM-MSCs with 0.1µg Resveratrol and a group of hIBM-MSCs with 1µg Resveratrol. Moreover, SOX2 expression in passage 3 and 5 of hIBM-MSCs was examined by applying RT-qPCR. Meanwhile, cdk, p21 and pRb expression in passage 3 and 6 of hIBM-MSCs were investigated by employing of Immunocytochemistry. The proliferation rate of hIBM-MSCs in passage 3 and 6 between control and treatment groups were examined. Data were then analyzed by applying the analysis of variance continued with post hoc test ($p<0.05$).

This investigation revealed that hIBM-MSCs were positively expressed by CD44, CD73, CD105 and CD105 but not with CD13, CD34, and CD45 expression. The proliferation rates in hIBM-MSCs were significantly different between passage 3 and 6 in resveratrol groups compared to control group ($p<0.05$). Meanwhile, Sox expression was fundamentally enhanced in treatment groups after resveratrol administration in contrast to the control group, especially in passage 3 and 5 ($p<0.05$). Additionally, the expressions of cdk, p21 and pRb were significantly improved in resveratrol groups compared to the control groups in passage 3 and 6 ($p<0.05$)

During the post administration, Resveratrol proved that it was able to maintain hIBM-MSCs' stemness through the enhancement of Sox2 expression and their quiescence through the increased expressions of cdk, p21 and pRb in late passage.

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Introduction

Resveratrol is a natural compound found in red wine which was often suggested to exert its potential health benefit through the activation of situin1 (sirt1), an endogenous enzyme found in mammalian NAD^+-dependent deacetylases. Resveratrol may be a strong candidate to maintain Sox2 by increasing sirt1 activity in the bone marrow mesenchymal stem cells (BM-MSCs).[1,2] It should be noted that Sirt1 has emerged as an attractive therapeutic target for many aging related diseases, however, the way on how its activity can only be activated by resveratrol has been poorly studied. Furthermore,

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the substrate-dependent sirt1 activation by resveratrol needs the specific N-terminal Domain (NTD) which is essential as the "loose-binding" substrate for resveratrol. The binding of resveratrol leads to a conformational change in NTD of sirt1 that promotes a tighter binding between sirt1 and the substrates. Since the NTD is unique to yeast Sir2 and mammalian sirt1, this may explain the reason why resveratrol is an isoform-selective sirtuin activator.[3,4,5]

Besides Oct4 and Nanog, Sox2 is one of transcription factor keys with a diverse role in stem cell potency and maintenance, embryonic development and cancer. Recently, Sox2 has been implicated in the maintenance and differentiation of adult stem cells. While, Sox2 expression also has been reported in bone marrow, neuronal tissues and sensory epithelial.[6] Moreover, Sox2 gene encodes Sox2 protein consisting of 317 amino acids. It has the High Mobility Group (HMG) domain built from approximately 80 amino acids. Through the HMG domain, Sox2 proteins bind to the ATTGTT motif in DNA. The level of Sox2 protein expression depends on the cell type and degree of differentiation. The function of this protein in the cell is strictly determined by its concentration, which is regulated on many levels, including the transcription, post transcription, and post translational levels.[7]

Furthermore, as sirt1 of Sox2 and resveratrol interactions maintain the stemness of BM-MSC, there are several limitations which reduce self-renewal capacity and multipotency ability over long term cultivation to obtain the required number of MSCs. According one of the nuclear exporting sequence (NES) theories, acetylation in Sox2 can export the nuclear then undergo proteasomal degradation, which is able to decrease the cell's renewal capacity and multipotency ability. This contributes to the prolonged growth by slowing down the aging over long-term cultivation resulting in the senescence or aging MSCs. Apart from this, Sox2 instability can be prevented by activating Sirt1 which is an endogenous enzyme protein that serves as an oxidizer in various processes in cells including cellular aging, bone homeostasis, and metabolic pathways.[8]

The contribution of Sox2 expression in cell cycle prevents G1 to S1 phase transition, which maintains the adult stem cell stemness. In the differentiated mammalian cells, the progressions from G1 to S are regulated by hypophosphorylated Rb gene or its related proteins, which inhibit the expression of genes required for an entry into S phase by sequestering E2F family of transcription factors. During G1 phase, Rb/HDAC repressor complex binds to E2F-DP1 transcription factors inhibiting the downstream transcription. Even though the real mechanism is unclear, thus, it needs further study.[9,10] The aim of this study was to investigate the effect of Resveratrol administration in maintaining the stemness and quiescence of human iliac bone marrow stem cells (hIBM-MSCs).

Materials and methods

Study Design and Ethical Clearance

This study was a quasi-experimental non-equivalent type control group design. An ethical clearance certificate of approval for experimental study was issued by the Faculty of Medicine, Brawijaya University, Malang, Indonesia with a reference number of 152/EC/KEPK-S3/06/2018.

Isolation, culture and resveratrol administration on Human Illiac Bone Marrow Mesenchymal Stem Cells

Bone marrow aspirates were obtained from the iliac bone of three adult donors under the patient's approval and written informed consent. The isolation, the culture, and the chemical treatment were done in the Stem Cell Laboratory, Universitas Airlangga, Surabaya. BM-MSCs were then selected based on their ability to adhere into the plastic cell culture flasks. The cells were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; Invitrogen, US) supplemented with 10% fetal bovine serum (FBS, Gibco, US) and 1% antibiotic-antimycotic solution (Invitrogen, US) at 37^0C in a 5% CO_2 atmosphere. Later, BM-MSCs were grown to 80-90% confluence and then harvested by incubation with 0.25% trypsin/EDTA (Invitrogen, US) followed by centrifugation at 1.300 rpm for 3 min. The harvested cells were replanted at a density of 5.000 cells per square centimeter and then sub-cultured when cells were 80-90% confluent. To evaluate the effect of resveratrol on Sirt1 activation in BM-MSCs, the studied groups were organized into one control group and two experimental groups of resveratrol treatment with doses as many as 1 μM and 0.1 μM.

Human iliac bone marrow mesenchymal stem cells characterization

The characteristics of the harvested cells were confirmed with a flow cytometry by applying cluster of differentiation (CD) 14, CD 34, CD 44, CD 45, CD 73, and CD 105 as markers. The flow cytometry was performed following the experimental protocol as follows: BM-MSCs were washed three times with PBS then with 1:100 dilution of primary antibodies from CD 14, CD 34, CD 44, CD 45, CD 73, CD 105 (Santa Cruz Biotechnology, US) were added with fluorescein isothiocyanate (FITC)-conjugated antibodies. Meanwhile, antibodies against the human antigens of CD34 (FITC mouse monoclonal anti-human CD34), CD 44 (FITC mouse monoclonal anti-human CD 44), CD45 (FITC mouse monoclonal anti-human CD45), CD73 [phycoerythrin (PE) mouse monoclonal anti-human CD73) and CD105 (PE mouse monoclonal anti-human CD105) were purchased from BD Biosciences. In addition, a total of $5x10^5$ cells were resuspended in 0.2 ml DPBS and incubated with 10 µl FITC- or PE-conjugated antibodies for 30 min at room temperature. The fluorescence intensity of cells was evaluated by applying flow cytometri (FACScan; BD Biosciences) and the data were then analyzed using CellQuest software version 6.0 (BD Biosciences).[11]

Immunocytochemistry examination for quiescence marker.

The quiescence stage in the cycle of MSCs was examined by performing immunocytochemistry rabbit polyclonal markers p21 (bs-10129R-FITC, Bioss, US), cdk2 /Thr14 (bs-5277R-FITC, Bioss, US), and Rb/P105 RB (Ser807+ser811) (bs-3380R-FITC, Bioss, US). Then, MSCs were seeded in 35 mm Petri dishes. Medium was removed and the cell layers were rinsed in ice cold PBS, fixed in 4% paraformaldehyde for 15 min and washed in PBS again. The next step was the cells got permeabilized with 0.25% Triton-X-100 (Packard) and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. This study used the labelled primary antibody diluted for 1:100 in 1% BSA all antibodies were applied for 1 hour at room temperature. Additionally, the cells were then further counterstained for 1 mm with Hoechst solution (1µg/ml) and imaged with CCD camera with 100x magnification (Olympus $CKX_{53}SF$, lighting Olympus U-RFLT, PC DELL $D_1$8M, Japan).

Examination Sox2 expression with qRT-PCR.

Sox2 expression was examined by using the automatic machine PCR MyGo Mini, viral RNA mini kit Qiagen, Goscript promegaUSA, GoTaq qPCR master mix, Promega, USA, with the quick system protocol by employing Mygo Mini connection modes. Primer Sox2 was utilized according to the manufacturer's instructions from idtdna-USA, with a sequence Sox2 Forward 5'-GGG AAA TGG GAG GGG TGC AAA AGA GG -3' and Reverse 5'- TTG CGT GAG TGT GGA TGG GAT TGG TG -3'. And GADPH F 5'- TGT CAT CAA CGG AAA GGC-3' and Reverse 5'-GCA TCA GCA GAA GGA GCA-3'. Then, the total RNA was collected from the cultures as well as from undifferentiated hI-MSCs with difference passages. TRIzol reagent (Invitrogen Life Technologies, US) was performed in accordance with the manufacturer's instructions. Briefly, cell layers were washed with PBS, scrapped and homogenized in 1 ml TRIzol. Later, RNA was treated with DNase I (Invitrogen, US) to remove genomic DNA and 3 µg of total RNA was reverse transcribed to cDNA using Superscript III First-Strand Synthesis System for RT-PCR (Mygo mini machine, US) in conformity with the manufacturer's instructions. Furthermore, the reverse transcription was performed in thermomixer (Eppendorf, US) at these following conditions: 10 minutes at 20°C, 1 hour at 42°C, 5 minutes at 99°C and 5 minutes at 5°C. Gene expression levels were then determined by qRT-PCR using Gotaq qPCR Master mix (Promega, USA). Meanwhile, the used primers to determine gene expression levels were presented and the PCR reaction conditions were as follows: 10 minutes at 95°C for 1 cycle, 15 seconds at 95°C and 1 minute at 60°C for 40 cycles. Quantitative RT-PCR was performed on 7500 Fast PCR system. Expression levels were normalized to β-actin. Relative expression of targeted genes was calculated using the ΔΔCt method. In addition, the expressions of stem cell markers in differentiated cultures (day 14) were normalized to 1 as well as BSP and AP expression in 14 days old hMSCs cultures (day 14).

Statistical Analysis: The data were then described as mean and standard deviation (SD). All data in each group were analyzed by employing Duncan's test to investigate the significant difference between groups ($p<0.05$).

The statistical package for social science (SPSS) version 20.0 for windows (IBM corporation, Illinois, Chicago, US) was applied to analyze the data statistically.

Results

Human iliac hIBM-MSCs was successfully isolated from bone marrow aspirate of orthopedic patient that underwent the surgery. The spindle shape or fibroblast like morphology was shown at 3rd, 4th, 5th, and 6th sub-cultured hIBM-MSCs showed and attached in the culture plate. Meanwhile, a more confluent, less apoptosis and senescence cells than control group was presented at 3rd, 4th, 5th and 6th sub-cultured hIBM-MSCs with resveratrol administration at dose of 1 μM. The proliferation rates in hIBM-MSCs were significantly different between passage 3 and 6 in resveratrol groups compared to the control group ($p<0.05$) (Figure 1).

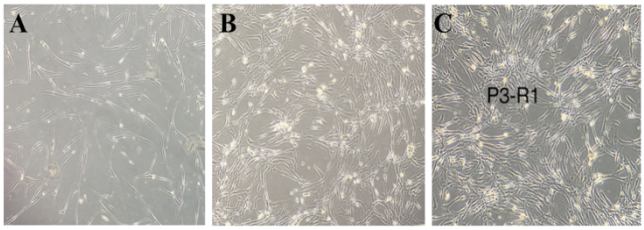

Figure 1. HIBM-MSCs population type. Class I colonies are comprised of a heterogenous and weak density cell population; Class II colonies are comprised of a homogenous and weak density cells population; Class III colonies are comprised of a homogenous and overcrowded-density. A. Class I, heterogenous, weak density; B. Class II, homogenous, weak density; Class I heterogenous, weak density; Class III, homogenous overcrowded density.

The characterization of hIBM-MSCs at early and 3rd sub-culture exhibited the positive expression of CD73 and CD105 as MSCs surface markers, but lack of CD14, CD 34, CD 44 and CD 45 as HSCs surface marker (Figure 2).

As the result, Sox expression was significantly enhanced after resveratrol administration than the control group in passage 3 and 5 ($p<0.05$) especially at the reservation dose of 1 indicating that the presenting resveratrol can maintain the stability of Sox2 expression (Figure 3). In the same manner, the expressions of cdk, p21 and pRb were significantly enhanced in resveratrol group compared to the control group in passage 3 and 6 ($p<0.05$) (Table 1), demonstrating that co-cultivation hIBM-MSCs with resveratrol can maintain stemness and quiescence in hIBM-MSCs (Figure 4).

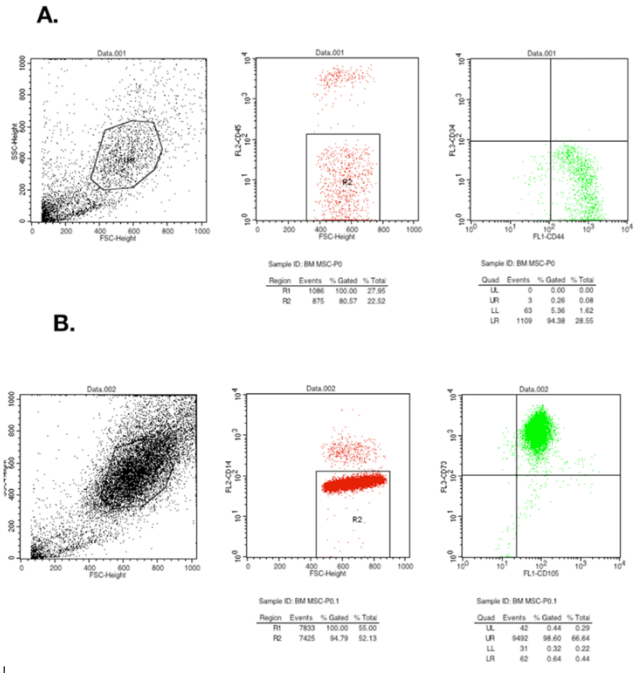

Figure 2. Flowcytometry result of hIBM-MSCs' surface markers. A. The characterization of hIBM-MSCs at P0 results on panel CD45, CD34, and CD44 (the expression of the cell is seen in the column % gated of upper right cell population that expressed CD45-/CD34-/CD44+). B. The characterization of hIBM-MSCs at P0 results on panel CD14, CD73, and CD105 (the expression of the cell is presented in the column % gated of upper right cell population that expresses CD14-/CD73+/CD105+).

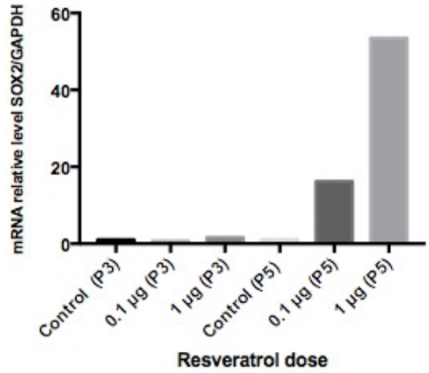

Figure 3. RT-qPCR result of Sox2 in hIBM-MSCs in passage 3 and 5 between control group and resveratrol 0.1μg and 1μg groups.

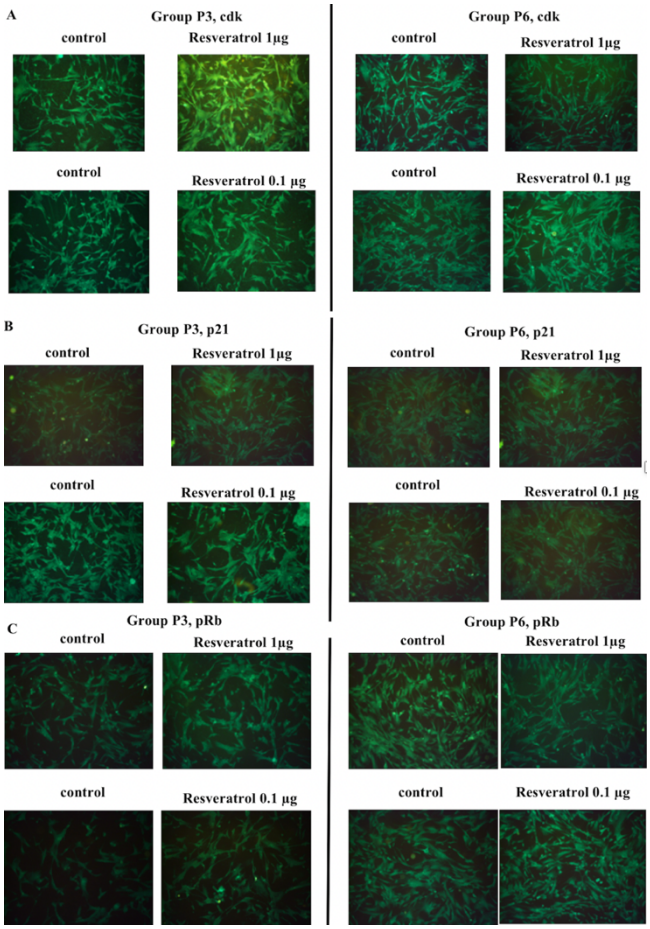

Figure 4. Result of ICC examination from the quiescence markers in hIBM-MSCs between groups. A. cdk expression on 3rd and 6th passage. B. p21 expression on 3rd and 6th passage. C. pRb expression on 3rd and 6th passage.

Expression marker	3rd passage SD ± Mean	6th passage SD ± Mean	*p-value*
cdk (0.1 mg)	14.53±0.98	41.88±2.70	*0.001
p21 (0.1 mg)	15.08±2.63	40.73±0.91	*0.001
pRb (0.1 mg)	7.92±0.65	33.19±0.85	*0.001
cdk (1 mg)	15.08±2.54	43.77±2.23	*0.001
p21 (1 mg)	15.08±2.63	40.75±0.62	*0.001
pRb (1 mg)	8.34±0.84	33.77±1.11	*0.001

Table 1. The ICC examination result of CDK, p21, and pRB expression between 3rd passage and 6th passage of hIBM-MSCs.
p-value<0.05 it was a significant difference.

Discussion

This investigation result indicates that the isolated hIBM-MSCs from human iliac bone marrow aspirates was the MSCs that according to the minimal criteria as MSCs from The International Society for Cellular Therapy.[11] Furthermore, the expanded cells maintain the potential to proliferate the populations of MSCs.[12] This study observed the growing and profiling of BM-MSCs.

The impaired proliferation factors of MSCs consist in many factors of source of MSCs such as the stage of cells or the culture condition.[13,14] However, no optimal culture conditions could alter MSC proliferation. Meanwhile, the cell seeding density may have the impact on MSC growth.[15,16] The initial cell number of mononuclear cells from the whole fraction from bone marrow is a key factor to obtain an efficient population of pure MSCs.[17,18]

In this study, the hIBM-MSCs at its 3rd, 4th, 5th, and 6th sub-cultured spheres administrated with resveratrol at dose 1 µM exhibited more confluent, less apoptosis and senescence cells than the control group (without resveratrol administration). This investigation also revealed that some groups of MSC culture suffer from the senescent cell, smaller and weak-density cell population, especially in the control cell group within late passage. In the other hand, on treatment culture cells, there are homogeneous and overcrowded-density cell population resulting in both of the early passage (P3) and late passage (P6). In that case, the resveratrol-mediated Sirt1 activation increases the proliferation potentials of hIBM-MSCs. Furthermore, it suggests that Sirt1 is a major epigenetic regulator that suppresses acetylation and nuclear translocation of Sox2 resulting in the prevention of proteasomal degradation of Sox2 via ubiquitination in human hIBM-MSCs. The regulation of Sox2 by Sirt1 is due to the direct interaction between the two proteins. As the results, it was indicated that the regulation of Sox2 by Sirt1 occurs at the post-translational level due to the knockdown of Sirt1 does not affect Sox2 mRNA expression.[1,19]

In addition, hIBM-MSCs provide an excellent source of multipotent progenitor cells that are presented in the bone marrow as well as in the most connective tissues. Due to their proliferation and differentiation capacities, they are able to differentiate toward multiple mesodermal lineages. This research has examined the Sox2 expression of hIBM-MSCs in the early passage as P3 and late passage as P5.

Thus, there was a significant difference of Sox2 expression. Significantly, Sox2 is a transcription factor co-expressed with OCT-4 that has been identified a crucial player in the maintenance and differentiation of adult stem cells such as in neural stem cells. Sox2 also maintains its self-renewal and proliferation of the osteoblast precursors. In contrast, Sox2 inactivation in cultured human MSCs has been shown to cause the exhaustion of their proliferative ability and senescence.[5] The downregulation of Sox2 after the differentiation is consistent with the suggested role of Sox2. Despite the fact that probably there is no universal stem-cell marker which could be applied to all types of stem cells., it is important to define stem cell markers specifically. Furthermore, Sox2 and Oct4 have evaluated two potential markers specifically for the clinically relevant cell types such as MSCs.[6,20-26] Accordingly, it is important to be able to evaluate the quality of stem cells because the stem cell potential is certainly associated with the potential results of cell therapy. However, one of the biggest problems in cell therapy is a potential malignant transformation of stem cells. This is another reason why it is necessary to investigate the role of pluripotency genes in somatic stem cells. This investigation concludes that the culture conditions do have an effect on the expression of stem cell marker and it is important to standardize the cultivation conditions in order to better predict the cell behavior. It is necessary to clarify better the role and mechanism of action of core pluripotency genes in mesenchymal stem cells following their cultivation in vitro.[6,27,28]

Meanwhile, SRY-Related HMG-Box Gene 2 encodes the Sox2 protein composed with 317 amino acids and has HMG domain in which SOX proteins bind to ATTGTT motif in DNA. In the nucleus cell, the expression of sox2 is high in passage 3 and decreases in the later passage. One of Sox2 stability theories is influenced by the nucleus exporting sequence (NES) where the Sox2 protein undergoes acetylation which pushes the Sox2 protein out of the nucleus and in turn also experiences a degraded ubiquitination process.[19,29,30] It proved that the deacetylation of Sox2 protein does occur by administering resveratrol which stimulates Sirt1. Thus, the stability of Sox2 can be maintained.

In this study, Sox2 gene expression was measured by qRT-PCR at passage 3 and in the late passage. The results were compared in the control cell group and the treatment cell group with resveratrol administration and it was found that hIBM-MSCs proliferation was higher in the treatment cell group. In the group of treated cells with the late passage, it turned out that the proliferation of MSCs was still high compared to the early passage. The resveratrol, a small molecule of 3, 5, 4'- trihydroxy stilbene with chemical formula $C_{14}H_{12}O_3$, plays an important role in stabilizing sirt1, via the N-terminal domain. Whereas, sirt1 is an endogenous cell enzyme in which NAD^+-dependent deacetylase serves as Sox2 deacetylation. Mechanistically, Sirt1 regulates the transcription of Sox2 gene through a way of chromatin-based epigenetic changes which are dependent on DNA methylation. This effect is achieved by an alternation of histone modification and interaction with DNA methyltransferase 3A, resulting in of hypermethylation the Sox2 promoter. Besides that, Sirt1 regulates Sox2 expression at the transcriptional level by epigenetic modification. Furthermore, Sirt1 is identified as a histone Deacetylase to regulate gene expression through histone modification by deacetylating the lysine residue of Sox2.[31-34] Accordingly, Sirt1 also regulates Sox2 expression through Sox2 protein acetylation modification in mouse's embryonic stem cells. It is also indicated that Sirt1's knockdown expression or overexpression significantly regulates Sox2 messenger RNA (mRNA).[35] Furthermore, Sirt1 is required for the long-term cultivation of MSCs and its overexpression improves the cellular senescence by altering aged MSCs resulting in an increase in cell proliferation. Sirt1 positively regulates the self-renewal and multipotency of human MSCs by deacetylating the lysine residue of Sox2.[1,36] Additionally, Sirt1-resveratrol interactions have led to the intense search for compounds that can enhance life-span and delay the process of senescence in tissues and organisms.[4,37-39]

Meanwhile, Sox2 is a transcription factor that belongs to a family of proteins having DNA binding domains that specifically recognize a short DNA sequence and an activation or repression domain that influences gene transcription. The different roles for Sox2 in MSCs maintenance including variable activities of Sox2 protein are controlled at the translational and post-translational modifications. In translational regulation, post-translational impacts the activities of Sox2 protein, while Sox2

also possesses 317 amino acids and many sites that can be modified through phosphorylation, acetylation, ubiquitination, methylation, and SUMOylation.Based on these modifications, Sox2 displays different activities. A lysine 47 residue of Sox2 located in the DNA binding domain is acetylated by p300/CBP and the status of acetylated lysine 47 is the nuclear export of Sox2. Furthermore, the increased level of acetylated Sox2 leads to the ubiquitination and proteasomal degradation of Sox2. Additionally, acetylation blocking at this site sustains the expression of its target genes under hyperacetylation or differentiation conditions. As a matter of fact, it has been known that the activities of Sox2 are variable through translational and post-translational modifications, and thereby, regulate pluripotency and differentiation. In most cases, Sox2 contributes greatly through many modifications including transcriptional level mediated by signal transduction, translational level mediated by microRNA, post-translational levels such as phosphorylation, acetylation, methylation, SUMOylation and ubiquitination.[40-44]

Lastly, Sox2 stabilization in the nucleus will affect the cell cycle through the signal path. On the other hand, Sox2 instability can be prevented by activating the protein sirtuin1 (Sirt1) which is an endogenous enzyme protein acting as an oxidizer in various processes in the cells including cellular aging, bone homeostasis, and metabolic pathways.[11-13] The contribution of Sox2 expression suggested in cell cycle prevents G1 to S1 phase transition which maintains the adult stem cell's stemness. In differentiated mammalian cells, G1 to S progression is regulated by the hypophosphorylated Rb genes or its related proteins which inhibit the expression of genes required for entry into S phase by sequestering E2F family of transcription factors. During G1 phase, Rb/HDAC repressor complex binds to E2F-DP1 transcription factors inhibiting the downstream transcription in which SOX2 is a recurrent molecular hallmark and Sox2 expression control converges in PI3K/AKT signaling with the versatile implications for stemness and cancer.[46-48]

Conclusions

As Sox2 Expression is still intact at the late passage, therefore, the cell cycle is still in the G0 quiescence phase. Resveratrol has an effect to regulate Sox2 expression that can maintain human iliac BM-MSCs proliferation, self-renewal and stemness as documented *in vitro*. Furthermore, the regulation of Sox2 by Sirt1 is suggested to occur at the after translational modification level, although the real mechanism is still unclear and needs further study.

Declaration of Interest

The authors report no conflict of interest.

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