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Basic Research

Rhynchophylline promotes stem cell autonomous metabolic homeostasis

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ABSTRACT

Rhynchophylline (Rhy) effectively obstructs the expansive signaling pathways of degenerative diseases, including Alzheimer disease, Parkinson disease, epilepsy and amyotrophic lateral sclerosis, and stimulates neurogenesis. Maintenance of stemness and cell proliferation requires sophisticated intracellular environments to achieve pluripotency via specific expression of genes and proteins. We examined whether Rhy promotes this regulation in bone marrow human mesenchymal stromal cells (BM-hMSCs). Results revealed (i) Rhy modulated biological activity by regulating the mitochondria, N-methyl-D-aspartate receptor subunit, and levels of FGFβ (basic fibroblast growth factor), BDNF (brain-derived neurotrophic factor), OXTR (oxytocin receptor) and ATP (Adenosine triphosphate); (ii) Rhy altered expression level of BM-MSC proliferation/differentiation-related transcription genes; and (iii) interestingly, Rhy amplified the glycolytic flow ratio and lactate dehydrogenase activity while reducing pyruvate dehydrogenase activity, indicating a BM-hMSC metabolic shift of mitochondrial oxidative phosphorylation into aerobic glycolysis. Altogether, we demonstrated a novel mechanism of action for Rhy-induced BM-hMSC modification, which can enhance the cell transplantation approach by amplifying the metabolic activity of stem cells.

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Introduction

Mesenchymal stem cells (MSCs) are capable of differentiating into cells of the mesoderm (bone, cartilage or fatty tissue), endoderm (muscle, gut epithelial cell or lung cell) and ectoderm (epithelial and neuronal cells) [1]. Based on their multi-lineage differentiation ability, they have been used in therapeutic interventions for myocardial infarction, neurodegenerative diseases, skeletal tissue injuries and organ failure [2]. Moreover, because MSCs do not express major histocompatibility complex II, they are considered highly viable candidates for allogeneic transplantation [3]. Lineage determination of human MSCs is important for human health, thus, dysfunction of this process is the direct cause of age-related disease [1]. Owing to their pleiotropic function, MSCs are relevant therapeutic targets to enhance tissue regeneration, although there is scarcity of insight into both opposing transcriptional and epigenetic networks that control divergent lineage determination of multipotent MSCs [4]. MSCs have heterogeneous metabolism that use both glycolysis and oxidative phosphorylation (OXPHOS) for Adenosine

triphosphate (ATP) production under normoxic conditions. Undifferentiated MSC metabolism during proliferation is associated primarily with glycolysis (glucose consumption and lactate production) [5] under both anaerobic/aerobic conditions [5,6], whereas OXPHOS within the mitochondria is associated with differentiated MSCs, which is correlated with impaired stem cell multipotency [5]. This suggests that the metabolism of proliferating MSCs is predominantly dependent on glycolytic metabolism with the Warburg Effect, termed “aerobic glycolysis,” rather than OXPHOS [7].

While in a strict sense, glycolysis occurs under purely anaerobic condition, immediately upon completion of its final step, the cell must continue respiration in either an aerobic or anaerobic direction. Hence, the terms aerobic or anaerobic respiration have been used. However, that glycolysis is purely anaerobic has been challenged, and the occurrence of aerobic glycolysis has been advanced. Aerobic glycolysis refers to a condition in which glucose is converted to lactate in the presence of oxygen [8]. This condition is also referred to as the Warburg Effect. This term has been used in the literature dating back almost 100 years. The final stages of aerobic glycolysis yield the production of extracellular lactic acid, which is counterintuitively produced even though sufficient oxygen is present to allow OXPHOS to proceed [8].

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The Warburg Effect in mammalian cells has been widely studied in cancer cells [9]. Cancer cells rewire their metabolism to promote growth, survival, proliferation and long-term maintenance. The common feature of this altered metabolism is increased glucose uptake and fermentation of glucose to lactate. This phenomenon is observed even in the presence of completely functioning mitochondria and together is known as the Warburg Effect. As noted above, the Warburg Effect has been documented for almost 100 years with thousands of articles reporting to have established either its causes or its functions. In the last 5 years, this Warburg Effect has been demonstrated to be robustly displayed not just by cancer cells, but also by pluripotent stem cells [10]. This hallmark of cancer cells involving a metabolic switch from OXPHOS to glycolysis, a phenomenon referred to as the ‘Warburg Effect,’ has now been observed in stem cells. Of interest, recent studies show that the metabolism of different types of stem cells, in particular primed pluripotent stem cells (such as human embryonic stem cells and human induced pluripotent stem cells), is also biased toward glycolysis rather than OXPHOS, exhibiting a Warburg-like effect [11–14]. Indeed, more recent studies demonstrate that this metabolic switch from OXPHOS to glycolysis is critical for bioenergetics, biosynthetic capacity and epigenetic regulation in human pluripotent stem cells (hPSCs) [15–17], which is further supported by metabolomics analyses [18–20]. Moreover, some stem cells, such as mouse embryonic stem cells, exhibit a more naive bioenergetics state that is energetically bivalent, and can dynamically switch from glycolysis to OXPHOS and vice versa on demand [16]. Altogether, these studies suggest that metabolic bioenergetics in stem cells, including MSCs, just like cancer cells, do not strictly follow established glycolytic or cell respiration pathways, and that these cells may be more primed toward the Warburg Effect.

The *N*-methyl-*D*-aspartate receptor (NMDAR) is a glutamate-gated ion channel that is critically involved with both physiological and pathological function within the central nervous system [21]. NMDAR is activated by allosteric modulation and is composed of seven subunits: the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) and a pair of GluN3 subunits (GluN3A and GluN3B). The NMDAR functions in a series of heterotetrameric assemblies that typically associate two GluN1 subunits with the same two GluN2 subunits, or a mixture of GluN2 (for example, 2GluN1/2GluN2A or 2GluN1/GluN2A/GluN2B). NMDARs are principally tetrameric assemblies of two obligatory, glycine-binding GluN1 subunits, and two glutamate-binding GluN2A/B subunits [22,23]. Equilibrium shift of NMDAR subunit expression pattern is a key control point for determination of receptor diversity of the plasma membrane [23].

Erythropoietin-producing hepatocellular A4 (EphA4) is a member of the A subclass of Eph receptor tyrosine kinases, in which their transductions induce receptor-expressing cells (forward signaling) and ligand-expressing cells (retrograde signaling) [24]. These signaling pathways are tightly regulated, and are largely attributed to neurodegenerative disorders, such as Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and epilepsy [24]. EphA4 acts as a negative regulator of NMDAR neurotransmission [25], which has been implicated in the pathophysiology of depression [26]. Activation of the EphA4 signaling pathway results in dendritic spine loss and reduction of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor expression [27], which together serve as potential mechanisms that underlie synaptic dysfunction in AD [28].

The alkaloid Rhy, derived from the extraction of *Uncaria rhynchophylla*, is a component of Kampo medicine, *Choto-san* [29], and has potential to serve as a suitable therapeutic tool for rescuing patients with chronic and regenerative disease. Rhy is able to penetrate into the blood-brain barrier and stimulate the formation of complex neuronal networks by upregulating neurogenesis [30] and by acting as noncompetitive antagonist of NMDAR [31]. Most interestingly, Rhy has demonstrated its ability to directly bind EphA4 and block the EphA4-dependent signaling pathway [32]. Rhy has also emerged as

an attractive candidate for eliciting ameliorative effects on AD [32], PD [33], ALS [34] and epilepsy [35]. However, it remains unknown whether Rhy enhances human mesenchymal stromal cells (BM-hMSC) efficacy for allogeneic transplantation, and further investigation regarding Rhy-induced up/downregulation of molecular mechanisms must be explored. The purpose of this study was to evaluate Rhy’s mechanistic link between proliferation control and cellular metabolism for BM-hMSCs. That Rhy’s biological and metabolic activities enhance stemness properties, namely, cell proliferation and differentiation, highlights the translational relevance of Rhy treatment in improving stem cell transplantation outcomes.

Methods

Cell culture

BM-hMSCs were purchased from the American Type Culture Collection, according to previous cell culture methods [36]. Rhynchophylline (Methyl (7 β , 16 E , 20 α)-16-(methoxymethylene)-2-oxocorynoxan-17-oate) was obtained from Toronto Research Chemical (R414180, Toronto, Ontario, Canada). BM-hMSCs were suspended in low-glucose DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) in the absence of antibiotics. The cells were plated at a density of 1.2×10^4 cells/cm² in poly-L-lysine-coated 96-well or ϕ 10-cm culture plate at 37°C in a humidified atmosphere containing 95% O₂ and 5% CO₂ (Figure 1).

Measurement of cell-proliferation, BrdU (bromodeoxyuridine) activity, and protein synthesis

Following treatment (Figure 1), cell proliferation assay was performed using WST-1 (05015944011; Roche, Basel, Switzerland) and Calcein-AM/EthD-1 (L3224; Invitrogen, Waltham, MA, USA) [23]. BrdU activity was measured using Cell Proliferation Enzyme-Linked Immunosorbent Assay (ELISA) BrdU (11647229001; Roche). In addition, trypan blue (15250; Gibco, Waltham, MS, USA) exclusion method was conducted, and mean viable cell counts were calculated in 16 randomly selected areas (1 mm²; *n* = 10) to reveal the cell viability. Active protein synthesis of BM-hMSCs was measured by incorporation of *O*-propargyl-puromycin [37] (OPP, ab239725; Abcam, Cambridge, MA, USA) according to the manufacturer’s protocols.

Measurement of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-, cytochrome c oxidase-activity, mitochondrial membrane potential ($\Delta\psi_m$) and intracellular ATP levels

MTT (catalog number 11465007001; Roche) by mitochondrial dehydrogenase was used as a measure of mitochondrial activity as previously described [23]. The activity of cytochrome c oxidase was measured using cytochrome c oxidase detection ELISA kit (ab239111; Abcam). For the measurement of $\Delta\psi_m$, BM-hMSCs were incubated with 25 nM tetramethyl rhodamine methyl ester (TMRM,

Fig. 1. Experimental design. After 3 days in culture, BM-hMSCs were exposed to 50 μ mol/L Rhy 3 times. At Day 7, we performed the following experiments. BrdU (bromodeoxyuridine) chemical was added into the cell culture medium at Day 6, and its activity was measured at Day 7. (Color version of figure is available online).

88065; Sigma-Aldrich, St. Louis, MI, USA) for 45 min at 37°C, and the fluorescence intensity was measured. The measurement of ATP content was analyzed using ATP bioluminescence assay kit (11699709001; Roche) according to the manufacturer's instructions.

Measurement of extracellular BDNF (brain-derived neurotrophic factor) and FGFβ (basic fibroblast growth factor) concentration

Following BM-hMSC treatments as detailed above, the culture medium was centrifuged at 3000g, 4°C for 15 min, and the supernatant was processed for BDNF ELISA kit (ab9978; Abcam) and FGFβ ELISA kit (ab99979; Abcam).

Real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from BM-hMSCs using RNase mini Kit (74104; QIAGEN, Vento, Netherlands) according to the manufacturer's instructions. The A260/280 ratio of RNA extraction corresponded to 2.0, which is considered high quality. The messenger RNAs (mRNAs) were quantified using a One-step RT-ddPCR Advance kit with EvanGreen system (186-4034/4022, BIO-RAD, Hercules, CA, USA). The primers (GAPDH [DHSAG5006642], KLF4 [DHSAG5009344], OCT4 [DHSAG518822], SOX2 [DHSAG5005966], cMYC [DHSAG5000932], NANOG [DHSAG518181], RUNX2 [DHSAG5008761], RUNX3 [DHSAG5018614], PPARG (peroxisome proliferator-activated receptor γ) [DHSAG5018088] and COL1A [DHSAG5000944]) were obtained from BIO-RAD. The reaction mixture (20 ng total RNA/20 μ L reaction mixture) was heated at 50°C for 1 h, and 95°C for 10 min followed by 40 cycles, each consisting of incubation for 30 s at 95°C, 1 min at 58°C. The amplified transcripts were quantified using the comparative C_t method.

Western blot analysis

BM-hMSCs were treated with Cellytic MT mammalian lysis reagent (C3228; Sigma-Aldrich) with protease inhibitor cocktail (I3786; Sigma-Aldrich). Protein samples (5–35 μ g/lane) were processed on 4–15% Tris/Glycine SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel (456-1084; BIO-RAD) and were then transferred onto a nitrocellulose membrane (162-0112; BIO-RAD) at 30 V, 4°C for

14 h. The nitrocellulose membranes were treated with phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 3% non-fat milk (170-6404; BIO-RAD) for 45 min at room temperature [23]. Membranes were then incubated with the primary antibodies, anti-oxytocin receptor rabbit monoclonal antibody (1/10,000, ab181077; Abcam), anti-BDNF rabbit monoclonal antibody (1/3,000, ab108319; Abcam), anti-GluN1 rabbit polyclonal antibody (1/1,000, ab52177; Abcam), anti-GluN2A rabbit polyclonal antibody (1/1,000, ab169873; Abcam), anti-GluN2B rabbit polyclonal antibody (1/1,000, ab565783; Abcam), anti-GluN3A rabbit polyclonal antibody (1/1,000, ABIN571959; RRID Antibodies-Online), anti-GluN3B rabbit polyclonal antibody (1/1,000, ABX135999; Antibodies), anti-EphA4 rabbit polyclonal antibody (1/1,000, ab5396; Abcam) and anti- β -actin mouse monoclonal antibody (1/3,000, ab8226; Abcam) at 4°C for 14 h [38].

Measurement of HK (hexokinase), G6PD (glucose 6-phosphate,) PFK1 (phosphofructokinase-1), PK (pyruvate kinase), LDH (lactate dehydrogenase), and PDH (pyruvate dehydrogenase) activity

The enzyme kinetics of HK-, G6PD-, PFK1-, PK-, LDH- and PDH activity were performed according to the manufacturer's protocols for HK assay kit (ab136957; Abcam), G6PD assay kit (ab102529; Abcam), PFK1 assay kit (MAK093; Sigma-Aldrich), PK assay kit (ab83432; Abcam), LDH assay kit (ab10526; Abcam) and PDH (ab10992; Abcam), respectively.

Measurement of intracellular glucose and lactate concentration

After BM-hMSC culture as detailed above, the cells were treated with cell lysis reagent (C3228; Sigma-Aldrich) and protease inhibitor cocktail (P8340; Sigma-Aldrich) at 4°C for 5 min. The lysate was processed for a Glucose Detection Kit (TR15421; Thermo Fisher Scientific) and a Lactate Assay Kit (MET-5013; Cell Biolabs Inc. San Diego, CA, USA), respectively.

Data analysis

Data were evaluated using one-way analysis of variance followed by post hoc compromised *t* tests (GraphPad Prism 6 software).

Fig. 2. Rhy promotes proliferation of BM-hMSCs accompanied by varying bioactivities. Following incubation of BM-hMSCs in the absence of reagents (control; CONT), 50 μ Mol/L Rhy for 3 days at 37°C (Rhy). (A) Cell proliferation, (B) BrdU activity with representative images of BM-hMSCs stained with the nuclear marker DAPI (4',6-diamidino-2-phenylindole) (left panel), BrdU (middle panel) and merged (right panel) under 10X, (C) protein synthesis, (D) intracellular ATP content, (E) mitochondrial membrane potential ($\Delta\psi_m$) % indicates the ratio of fluorescence intensity, (F) MTT activity, (G) cytochrome c oxidase activity, (H, I) extracellular concentration of FGFβ and BDNF, respectively, (J) Western blot analysis of BDNF and OXTR (oxytocin receptor) and (K) relative quantification of expression levels of BDNF and OXTR were measured. Experiments of A–I were independently conducted 4–6 times, and Western blot experiments were independently conducted 3 times. Data are presented as means $\pm$ SD. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. AU, arbitrary unit. (Color version of figure is available online).

Fig. 3. Rhy modulates mRNA expression level of transcription factors. Following incubation of BM-hMSCs in the absence of reagents (control; CONT), 50 $\mu\text{mol/L}$ Rhy was administered for 3 days at 37°C (Rhy). qRT-PCR was used to analyze the mRNA level of Rhy-induced expression of transcription factors (*Oct4*, *Sox2*, *cMyc*, *Klf4*, *Nanog*, *Runx2*, *Runx3*, *Pparγ* and *Col1A*) in BM-hMSCs. Data are presented as means $\pm$ SD. *** $P < 0.001$. Experiments were independently conducted in triplicate.

Statistical significance was preset at $P < 0.05$. Data are represented as means $\pm$ SD from 3–6 times of each treatment condition.

Results

Rhy exerts BM-hMSC biological activity

To determine Rhy's effect on BM-hMSCs biological activity, BM-hMSCs were treated with 50 $\mu\text{mol/L}$ Rhy, a dose found to be optimal in enhancing BrdU-positive proliferative BM-hMSCs (Figure 1 and Supplemental Figure 1). As shown in Figure 2A and 2B, Rhy significantly increased BM-hMSC proliferation and BrdU activity. BrdU is a marker of S-phase cell cycle [39], which firmly regulates mRNA and protein expression [40]. Active protein synthesis per cell was quantified by incorporation of OPP into nascent protein [37]. We confirmed that global protein synthesis was significantly lower in Rhy-treated BM-hMSCs (Figure 2C), although ATP content in BM-hMSCs was significantly higher than that of the control (Figure 2D).

The energy requirements of cell proliferation is, at least in part, reliant on complete catabolism of glucose using mitochondrial OXPHOS to maximize ATP production. Despite each $\Delta\psi_m$ value (Figure 2E), there was significant decrease in MTT and cytochrome c oxidase activity within Rhy-treated BM-hMSCs, as compared with control cells (Figure 2F and 2G). An essential parameter of mitochondrial function is its crucial role in maintaining the electron transport chain reaction for ATP production. There was no change in $\Delta\psi_m$, which indicated that mitochondrial function was maintained. These results imply that Rhy is capable of asymmetrically allocating mitochondrial OXPHOS influx materials to the TCA cycle (tricarboxylic acid cycle).

Next, we measured the extracellular concentrations of FGFβ and BDNF, and determined protein expression level of BDNF and OXTR (oxytocin receptor). Rhy administration was found to not only significantly attenuate the extracellular concentration of FGFβ (Figure 2H) and BDNF (Figure 2I), but also intracellular levels of BDNF (both

mature and immature types; Figure 2J and 2K) and OXTR (Figure 2J and 2K).

Rhy regulates gene expression of differentiation markers, but not stemness markers

BM-MSCs are capable of enhancing tissue regeneration, thus emphasizing their potential to serve as a therapeutic target [1]. We next investigated whether Rhy regulates gene expression of BM-hMSC transcription factors, as compared with non-Rhy-treated BM-hMSCs. Quantitative real-time PCR (qRT-PCR) analyses revealed that the mRNA expression levels of stemness genes (*Oct4* and *Sox2*) [41], genetic stability gene (*cMyc*) [41] and *Runx2* (osteogenic marker) [42] were stable; *Klf4* (differentiation/chondrogenic marker) [43], *Nanog* (pluripotency marker) [41,44] and *Col1A* (chondrogenic marker) [45] were significantly suppressed, and that of *Runx3* (neurogenic marker) [46] and *Pparγ* (adipogenic marker) [47] were significantly increased (Figure 3).

Rhy shifts NMDAR subunit expression patterns

The interactional signaling of distinct receptor-receptor may reveal disease pathophysiology [23,38]; indeed, EphA4 is shown to act as a negative regulator of NMDAR signal transduction [12]. Rhy strongly inhibits the EphA4 signal transduction pathway [32]. We next measured Rhy-induced alteration of protein expression levels for NMDAR subunits and/or EphA4 in BM-hMSCs (Figure 4). Rhy administration significantly augmented expression levels of GluN1, GluN2B and GluN3A, but did not change the levels of GluN2A, GluN3B and EphA4. Expression levels of GluN2C and GluN2D were not detected (Supplemental Figure 2A).

Glycolytic flux: efficient ATP production

It is essential to maintain glucose influx and metabolism to sustain cell survival and growth [48]. To investigate if Rhy modulated glycolytic

Fig. 4. Rhy regulates the protein expression levels of NMDAR subunits on BM-hMSCs. Following incubation of BM-hMSCs in the absence of reagents (control; CONT), 50 μmol/L Rhy was administered for 3 days at 37°C (Rhy). Western blot was performed. (A) Western blot analysis. (B) Relative quantification of protein expression levels. Data are presented as means ± SD. * $P < 0.05$ and ** $P < 0.01$. Experiments were independently conducted in triplicate.

metabolism, we compared the kinetic parameters of crucial glycolysis-associated enzymes and levels of glucose and lactate in BM-hMSCs. Rhy significantly enhanced the kinetic reaction of HK, G6PD, PFK1 and LDH, and decreased PDH activity, although the parameter of PK, as well as glucose and lactate, demonstrated no significant difference between Rhy-treated BM-hMSCs and control cells (Table 1). The expression levels of glucose transport 1 (GLUT1) and GLUT3 were not changed, and that of GLUT4 in Rhy-treated BM-hMSCs was significantly increased (Supplemental Figure 2B and 2C).

Discussion

This study demonstrated that Rhy promoted BM-hMSCs proliferation, as long as $\Delta\psi_m$ was maintained, while mitochondrial activity was suppressed, in addition to protein synthesis expression of FGFβ, BDNF and OXTR (Figure 2). Rhy downregulated mRNA expression of *Klf4*, *Nanog* and *Col1A*, upregulated *Runx3* and *Pparγ* (Figure 3) and increased protein expression of GluN1, GluN2B and GluN3A (Figure 4). Rhy elicited an amplification effect on glycolytic metabolism, which was drawn from our kinetic analyses (Table 1). These results suggest that the upregulation of Rhy-induced BM-hMSC proliferation may be attributed to its connection between signaling pathways that drive cell growth and cell metabolism regulation.

Accordingly, our present study demonstrating enhanced stemness of MSCs with Rhy treatment appears to support the Warburg Effect. Although the initial cell respiration readouts may seem oxymoronic and paradoxical (e.g., aerobic glycolysis and increased ATP content coupled with maintained $\Delta\psi_m$ indicating functional mitochondrial

but with reduced Cytochrome c oxidase activity), upon careful examination, our results resemble Warburg-like effects, where there is a shift from OXPHOS to aerobic glycolysis. We observed a metabolic switch in our stem cells from OXPHOS to glycolysis, even in the presence of high levels of oxygen, reminiscent of the Warburg Effect.

The ability to specifically up/downregulate genes provides an advantageous therapeutic contract. FGFβ increases RUNX2 expression, which induces osteoblast differentiation and chondrocyte maturation [42]. OXTR-mediated signaling facilitates differentiation of MSCs toward osteogenesis [49] and cardiomyogenesis [50]. BDNF expression synergistically modulates osteogenic differentiation and RUNX2 expression of BM-MSCs [51]. Rhy promotes neuronal stem cell expression of BDNF, which propagates axon extension and synapse formation [30]. Following demonstration of FGFβ-, BDNF- and OXTR- expression downregulation (Figure 2), we next investigated Rhy-induced regulation of BM-hMSC transcription factors' gene expression.

KLF4, a zinc-finger transcription factor, regulates a variety of cellular processes, including proliferation, differentiation, apoptosis and somatic cell reprogramming, as well as maintenance of tissue homeostasis by controlling the expression of a large number of genes with GC/GT-rich promoters, while suppressing RUNX2 expression and function. RUNX2 has a core-binding factor subunit α-1, which is essential for both osteoblast and hypertrophic chondrocyte differentiation, and can upregulate expression of bone matrix protein genes *Col1A*. The resulting abnormal balance between osteoclast and osteoblast can lead to bone disease, such as osteoporosis, periodontal disease, Paget's disease of bone and rheumatoid arthritis [52]. We measured the expression levels of *Klf4*, *Runx2* and *Col1A*, and confirmed that expression of both *Klf4* and *Col1A* were significantly decreased, on the other hand, *Runx2* level had no significant difference between Rhy-treated BM-hMSCs and control cells (Figure 3; $P = 0.177$), emphasizing the need to further investigate alternative signaling pathway(s). NANOG interacts with stemness genes *Oct4* and *Sox2*, which has lineage with multiple downstream genes that control cellular stemness and terminal differentiation. For example, low stem cell expression of NANOG advances cell differentiation by providing an intermediate environment that moderates the obstruction of transition. *Nanog* expression in Rhy-treated BM-hMSCs resulted in extremely low levels in comparison with control cells (15% versus control), but the expression of core stem cell-specific genes (*Oct4* and *Sox2*) were not altered (Figure 3). A possible explanation for these contradictory findings was that the number of NANOG-expressing cells is not associated with the proliferative and

differentiative capabilities of the BM-hMSC population [44]. PPAR γ indirectly prevents functionality of RUNX2, which impairs differentiation into pre-osteoblasts [53]. In addition to adipogenesis [47], PPAR γ regulates a variety of physiological processes, including lipid metabolism, angiogenesis and osteoclastogenesis, while also playing a pivotal role in modulating both neuronal stem cell proliferation and neuronal differentiation [53]. Osteoblast differentiation primarily depends on activation of enhancers in BM-MSCs, whereas adipogenesis involves major chromatin remodeling and *de novo* enhancer establishment, implying that the undifferentiated BM-MSC has a tendency to differentiate into osteoblasts as opposed to adipocytes [4,54]. RUNX3 expression is essential for neurogenesis, and functions as a tumor suppressor via multiple mechanisms. Rhy significantly upregulated the *Runx3* and *Ppar γ* expression, and did not affect the expression of proto-oncogene *cMyc* in BM-hMSCs, indicating that Rhy shifts the differentiative priority of BM-hMSCs.

Rhy impedes EphA4 signal transduction, which is an upstream pathway of NMDAR. We predicted that the expression of EphA4 and NMDAR subunits would be altered by Rhy administration. However, Rhy did not affect the expression levels of EphA4, GluN2A and GluN3B, and additionally promoted expression of GluN1, GluN2B and GluN3A (Figure 3). This suggests that EphA4 signaling pathways are interrelated with the expression of GluN1, GluN2B and GluN3A in BM-hMSCs. The combination of GluN1, GluN3A and GluN3B constitutes the glycine-gate cation channel, and, in contrast, the combination of the GluN1 and GluN2 subunits, the GluN3 subunit decreases the channel [22,23]. NMDAR signaling transduction is critical for stimulating differentiation into osteogenesis/chondrogenesis from BM-MSCs [55]. The NMDAR is composed of GluN1/GluN3A subunits, which promotes RUNX2-mediated transcription, and induces differentiation into chondrocytes [56]. qRT-PCR results exhibited that Rhy remarkably reduces *Col1A* expression and does not modulate *Runx2* expression levels (Figure 3). To explain this discrepancy, we posit

that because BM-MSCs are a heterogeneous population of multipotent progenitor cells, cell processing has variances between undifferentiated progenitors and differentiating cells. Although molecular mechanisms by which EphA4 regulates the signal transduction pathway in BM-hMSCs are not fully understood, we sought to determine how EphA4 signaling interacted with NMDAR subunit expression in the cell.

The BM-MSC aging process has a critical impact on the biological regulative efficiency of BM cells, which plays a large role in determining hematopoietic stem cells' maximal longitudinal lifespan and differentiation potential [5]. Glycolytic metabolism attenuates the detrimental effect elicited by aged stem cells, by reducing regenerative capacity of aged cells, decreasing quiescence and diminishing the stem cell pool [5]. Compared with differentiated progeny, hMSCs express higher levels of glycolytic enzymes, whereas OXPHOS proteins are lower; therefore, ATP production of MSCs contributes more to glycolysis than their differentiated cells. An increase of aerobic glycolysis is dependent on an upregulation of glycolytic key enzymes and glucose influx, whereas the downregulation of enzymes is linked to mitochondrial OXPHOS. A fundamental mathematical analysis is required to provide a dynamic and mechanistic understanding of physical processes [57] that underlie the glycolytic metabolism response to Rhy treatment. We explored the enzyme kinetic reaction by which Rhy could regulate glycolysis-associated enzyme activity and the levels of glucose and lactate. The levels of glucose uptake between Rhy-treated BM-hMSCs and the control cells were not significantly altered (Table 1; $P = 0.443$), which were consistent with the results of GLUT1 ($K_m^{\text{D-Glucose}} \approx 2 \text{ mmol/L}$) and GLUT3 ($K_m^{\text{D-Glucose}} < 1 \text{ mmol/L}$) expression level, although Rhy upregulated the expression level of GLUT4 ($K_m^{\text{D-Glucose}} \approx 5 \text{ mmol/L}$; Supplemental Figure 2B and 2C). GLUT4 is an insulin-sensitive transporter protein that is mainly expressed in skeletal muscle, adipose tissue and heart, and its upregulation and translocation to the cell membranes are attributed to be

Fig. 5. Schematic diagram illustrating the theory in the current study. Rhy stimulates the proliferation of BM-hMSC by modulating the glucose metabolism pathway. Note that the metabolic pathways described are not stoichiometric. The arrow and red color indicate significant upregulation, blue color means significant downregulation and green designates no significant differences between Rhy-treated BM-hMSCs and the control cells. (Color version of figure is available online).

critical steps of the insulin-responsive increment of glucose uptake [58]. These results support that Rhy adapts BM-hMSCs in preparation for different nutritional circumstances, termed as “metabolic flexibility”. As illustrated in Table 1, the kinetic parameters of HK (intracellular glucose captured enzyme and converted glucose to G6P) and PFK1 (regulator of rate determining glycolytic step and irreversible reaction) [48,59] significantly increased, although PK (the final step of ATP production) demonstrated no difference between Rhy-treated BM-hMSCs and control cells. An increase in HK activity leads to a high glycolytic rate, and amplified PFK1 activity further upregulated the maximal velocity in glycolysis [48,59]. Pyruvate (the final production of glycolysis reaction) was converted into acetyl-CoA and lactate by PDH and LDH, respectively. The reaction of these enzymes demonstrates a reciprocal relationship for allocating OXPHOS signaling or causing glycolysis [60]. Activation of mitochondrial enzyme complexes, such as PDH, enhances the catalyzation of pyruvate in the TCA cycle, resulting in increased OXPHOS [61]. LDH reversibly converts pyruvate to lactate, depending on the redox of nicotinamide adenine dinucleotide (NAD⁺/NADH) ratio. Inhibiting LDH activity impairs cell proliferation because the production of lactate reinforces NAD⁺ regeneration, which catalyzes the G3P/1,3BPG reaction as a “redox shuttle” during production of pyruvate from glucose (Figure 5), while secreted lactate is tightly associated with cell proliferation [60]. Rhy significantly diminished PDH activity and amplified both LDH activity (Table 1) and ATP production (Figure 2D), but did not change the $\Delta\psi_m$ comparison with control cells (Figure 2E). These results prompted us to speculate that Rhy induced aerobic glycolysis for the acceleration of BM-hMSC proliferation.

The activity of G6PD (the first and rate-limiting enzyme of the pentose phosphate pathway [PPP] [62]) was significantly increased (Table 1) and the increased protein expression was observed (Supplemental Figure 2B and 2C). Enhanced G6PD activity coincides with an increased ratio of 6PGL to R5P during late G1 and S-phase. Notably, nascent protein synthesis during S-phase is dramatically decreased [40], and aerobic glycolysis metabolism in mitogen-stimulated cells undergoes changes throughout the cell cycle to reach a peak in S-phase [48]. The PPP (a branch pathway of glycolysis) regenerates the reduced form of glutathione, which protects proteins, fatty acids and genomic DNA replication against reactive oxygen species [38], and synthesizes lipid and R5P (an essential precursor for biosynthesis of nucleotides), suggesting a potential function for PPP in proliferative signaling. The accumulation of intracellular lactate (pKa = 3.86) suppresses the activity of G6PD (optimum pH = 8.7), which is vulnerable to acidic circumstance [62]. Levels of lactate between Rhy-treated BM-hMSCs and the control were similar (Table 1; $P = 0.090$), which implies that intracellular lactate was transported across the cell membrane by monocarboxylic transporters, that in which serves as a symporter for lactate and protons. These results indicate that Rhy enhances glucose flux into PPP, evidenced by the enhancement of cell proliferation, BrdU activity and active protein synthesis (OPP).

The present results provide novel data demonstrating Rhy-induced modulation of macromolecule biosynthesis, which is primarily achieved through a network of cells that mainly comprise cellular metabolic pathways directing both the acquisition and utilization of ATP for facilitating BM-hMSC proliferation, and conversion of epigenomic networks to control divergent lineage determination of multipotent BM-hMSCs. These findings support the use of Rhy-based compounds in improving cell metabolic activity, which may enhance the cell transplantation approach in the selection of therapeutic stem cells.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript or in the decision to publish the results. The authors declare no competing interests.

Author Contributions

Y.K., M.J. and C.V.B. conceptualized this study. Y.K. and A.B.C. performed the experiments and analyzed the data. Y.K., A.B.C., J.P.T., M.J. and C.V.B. evaluated the results, then drafted and finalized the manuscript.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2019.12.008.

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