Iran. J. Biotechnol. April 2024;22(2): e3863 DOI: 10.30498/ijb.2024.435096.3863

Research Article

DNA Methyltransferase Inhibition by RG108 Improves Stemness and Multipotential Differentiation of Human Adipose Tissue-derived Stem Cells

Razieh Asgharian,[1,2♯] Afrooz Hashemi,[1,3♯] Arash Javeri,[1] Masoumeh Fakhr Taha[1*]

[1]Department of Stem Cells and Regenerative Medicine, Institute for Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.
[2] Department of Biology, Islamic Azad University, Gorgan Branch, Gorgan, Iran.
[3] Department of Biology, Islamic Azad University, Damghan Branch, Damghan, Iran.

[*]*Corresponding author*: Masoumeh Fakhr Taha. Department of Stem Cells and Regenerative Medicine, Institute for Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran, Tel: +98- 2144580381, Fax: +98-2144787390, E-mail: mftaha@nigeb.ac.ir

♯ These two authors contributed to the manuscript equally.

Received: 2024/01/13 ; **Accepted:** 2024/03/09

Background: DNA methylation plays important roles in regulating various biological processes, including self-renewal, differentiation and regenerative capacity of stem cells. Previous studies have demonstrated that lineage-specific differentiation of mesenchymal stem cells can be promoted using nontoxic chromatin-modifying drugs.

Objectives: Here we evaluated the impact of RG108, a known DNA methyltransferase inhibitor, on the expression of pluripotency genes in human adipose tissue-derived stem cells (hADSCs) and their proliferation and differentiation.

Materials and Methods: Human ADSCs were isolated by collagenase treatment and characterized. Then, ADSCs were treated with 5 µM RG108 for four days. The control and RG108-treated cells were analyzed for the cell cycle progression, apoptosis and the expression of pluripotency genes. Also, ADSCs were cultured in adipogenic and osteogenic differentiation media for three weeks and were assessed by Oil Red O and Alizarin Red S staining and qPCR analysis.

Results: We showed that RG108 treatment increased proliferation of hADSCs and upregulated the expression of pluripotency-related genes. Additionally, RG108 had a positive impact on the differentiation capability of ADSCs. This was evident through elevated levels of Oil Red O staining in the RG108 treatment group. Also, qPCR analysis showed the upregulation of some adipogenic and osteogenic markers by RG108.

Conclusion: These findings indicate that pretreatment with RG108 improves the differentiation potential of ADSCs, probably making these cells more beneficial for cell therapy applications.

Keywords: Adipocyte, Adipose tissue-derived stem Cells (ADSCs), Differentiation, Osteocyte, Pluripotency, RG108.

1. Background

DNA methylation is an epigenetic process that involves addition of a methyl group (CH3) covalently to the carbon 5 of cytosine at CpG dinucleotides. Non-CpG methylation has also been observed in mammalian cells, including embryonic stem cells (ESCs) and other somatic cells (1, 2). DNA methylation is a necessary process for mammalian development. It plays important roles in regulation of gene expression, transposon silencing, inactivation of X chromosome, silencing of

one allele of imprinted genes, cell specification, and maintenance of cellular identity (3-5).

The pluripotent embryonic stem cells (ESCs) exhibit global hypomethylation, which is essential for activating the pluripotency network and maintaining a high level of plasticity until exposure to proper differentiation stimuli (4). Additionally, global DNA hypomethylation, along with the demethylation of genes associated with aging and pluripotency, occurs during reprogramming into induced pluripotent stem cells (iPSCs) (6, 7). Evidence also shows that changes in the pattern of DNA methylation play key roles in the self-renewal, differentiation and fate specification of adult stem cells (8). During differentiation, there is a global increase in DNA methylation, which silences pluripotency-associated genes and other genes related to non-specific cell type differentiation. Conversely, the loss of methylation in specific loci regulates gene expression in a cell type- and stage-specific manner (9). As shown, *in vitro* and *in vivo* aging of adult stem cells is associated with increased epigenetic alterations, including modifications in DNA methylation. These alterations can affect gene expression and fate specification but can be reversed by reprogramming factors during iPSC production (10).

Studies on the epigenetic control of stem cell differentiation have revealed that lineage-specific differentiation of mesenchymal stem cells can be promoted using nontoxic chromatin-modifying drugs which alter the state of CpG methylation and histone acetylation (11). In the past decade, the use of small molecules has emerged as a novel strategy for reprogramming and transdifferentiation of somatic cells (12). Small molecules have the advantages of being cell-permeable and non-immunogenic (13). Moreover, they are relatively cost-effective and can be conveniently synthesized, standardized, and used (14).

To date, a wide range of small chemical compounds have been identified to target different signalling pathways, metabolic processes or epigenetic modifiers. Among the various small molecules that can epigenetically reprogram somatic cells, 5-azacytidine (5-aza), RG108 and 3-deazaneplanocin A (DZNep) are known to reduce DNA methylation levels through inhibition of DNA methyltransferase (12).

2. Objective

In this study, our aim was to reprogram adipose tissue-derived stem cells (ADSCs) using RG108, a non-nucleoside inhibitor that blocks the active site of DNA methyltransferase (15). RG108 has been used to enhance reprogramming of human and mouse somatic cells, as well as to improve the generation of induced pluripotent stem cells (16). In 2018, Assis *et al*. (17) showed that RG108 induces genetic changes in human bone marrow-derived mesenchymal stem cells (hBM-MSCs).

In that study, RG108 reduced DNMT activity and decreased the overall methylation of DNA. As a result, the expression of NANOG and OCT4 was upregulated, while the viability of BM-MSCs remained unaffected. Similarly, we demonstrated here that treatment of hADSCs with RG108 upregulated the expression of some pluripotency-associated markers and also modified the adipogenic and osteogenic differentiation of human ADSCs.

3. Materials and Methods

3.1. Isolation and Characterization of hADSCs

Adipose tissue was harvested during elective abdominoplasty from women aged 40 to 50 at Erphan Hospital, Tehran, Iran. The study was approved by the Ethics Committee of the *National Institute of Genetic Engineering and Biotechnology* (IR.NIGEB.EC.1400.2.26.C), and all donors provided their informed consent. The ADSCs were isolated and characterized as previously described (20). In brief, adipose tissue was minced and digested using 2 $mg.mL^{-1}$ collagenase I (Thermo Fisher Scientific, Massachusetts, USA) in phosphate-buffered saline (PBS) with 2% bovine serum albumin. The stromal vascular fraction (SVF) was sedimented by centrifugation and resuspended in a growth medium consisted of Dulbecco's Modified Eagles Medium (DMEM), 20% fetal bovine serum (FBS) and 1% penicillin and streptomycin (all from Gibco, Thermo Fisher Scientific). The cells were then plated in tissue culture flasks and passaged after reaching 80-90% confluence.

To confirm the mesenchymal nature of the ADSCs, third-passage cells were fixed in 70% cold ethanol and immunostained with primary antibodies against CD73, CD90, CD105 and CD45 proteins (all from Abcam, Cambridge, UK). Subsequently, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, St.

Louis, MO, USA). The cells were examined using a BD FACSCaliburTM (BD Biosciences) and analyzed with FlowJo 7.6.1 (TreeStar Inc., Ashland, OR, USA).

3.2. RG108 Treatment of hADSCs

The fourth-passage hADSCs were seeded in tissue culture plates at a concentration of 10^4 cells. mL^{-1}. The following day, the ADSCs were subjected to treatment with 5 µM RG108 for a duration of 4 days. Some untreated cells were considered as the control group.

3.3. Adipogenic and Osteogenic Differentiation of the ADSCs

The control and RG108-treated ADSCs were cultured in 0.1% gelatin-coated tissue culture plates at a density of $5x10^4$ cells. mL^{-1}. The following day, the growth medium was replaced either by adipogenic or osteogenic differentiation medium. The adipogenic medium consisted of DMEM, 10% FBS, 1 µM dexamethasone, 100 µM indomethacin, 5 µg. mL^{-1} insulin and 500 µM isobutylmethylxanthine (IBMX). After three weeks of differentiation, Oil Red O staining was used to detect adipogenesis. To quantify intracellular lipid accumulation, after washing the unbounded dye, the stained lipid droplets were eluted by isopropanol for 10 min, and the optical density was measured on a microplate reader (BioTek Instrument) at an absorbance of 500 nm. The data obtained from four biologic replicates were analyzed using GraphPad Prism 8 (GraphPad Software Inc).

For osteogenic differentiation, the cells were cultured in a medium consisted of DMEM, 10% FBS, 10^{-8} M dexamethasone, 10 mM glycerol phosphate, 3.7 g. L^{-1}sodium bicarbonate and 0.05 g. L^{-1} ascorbic acid (all reagents were obtained from Sigma) for three weeks. Then, Alizarin Red S staining was used to detect osteogenic differentiation. For quantification of staining degree, the dye was extracted using a solution of 10% acetic acid for 20 min. The cells were harvested by scraping, heated at 85 ºC for 10 min, cooled on ice for 5 min and centrifuged to remove any cell debris. Then, supernatant was neutralized with 10% ammonium hydroxide solution, and optical density was measured on a microplate reader (BioTek Instrument) at an absorbance of 405 nm. The data obtained from four biologic replicates were analyzed using GraphPad Prism 8.

Table 1. Primers used for RT-PCR and quantitative real-time PCR.

Target	Forward	Reverse	Size
B2M	5'-TCCAGCGTACTCCAAAGATTCA-3'	5'-GTCAACTTCAATGTCGGATGGAT-3'	113
GAPDH	5'-CAAATGAGCCCCAGCCTTCT-3'	5'-TCACCATCTTCCAGGAGCGA-3'	116
OCT4	5'-GATGTGGTCCGAGTGTGGTT-3'	5'-AAGGGACCGAGGAGTACAGT-3'	202
NANOG	5'-GAAGCATCCGACTGTAAAGAATC-3'	5'-TGGTGGAAGAATCAGGGCTGT-3'	169
SOX2	5'-AGAACCCCAAGATGCACAACT-3'	5'-TCCTTCTTCATGAGCGTCTTG-3'	184
KLF4	5'-ATTACCAAGAGCTCATGCCACC-3'	5'-GTGTGCCTTGAGATGGGAACT-3'	158
LIN28	5'-TTCCATGTGCAGCTTACTCT-3'	5'-CAGCAGTTTGCAGGTGGC-3'	228
ADIPOQ	5'-CATACC AGAGGAGACGGG ATT-3'	5'-CTT GAG TCG TGGTTT CCT GGT-3'	165
FABP	5'-ATGAAAGAAGTAGGAGTGGGCT-3'	5'-GGTTATGGTGCTCTTGACTTTC-3'	162
RUNX2	5'-CAGAGTCAGATTACAGACCCC-3'	5'-CAGAGGTGGCAGTGTCATCAT-3'	200
ALP	5'-TATCTTTGGTCTGGCCCCCA -3'	5'-CGCCTGGTAGTTGTTGTGAG-3'	108

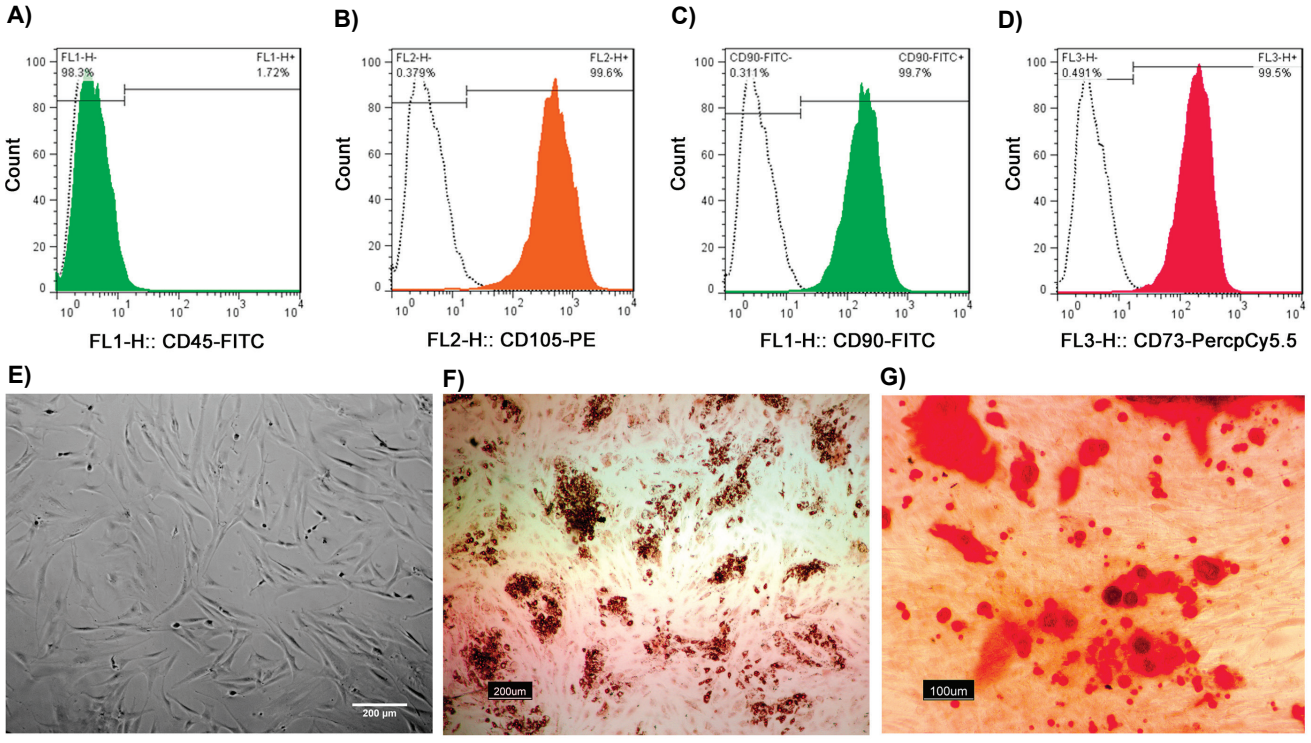

Figure 1. Characterization of hADSCs. **A-D)** Flowcytometry analysis on the fourth-passage hADSCs for the expression of hematopoietic marker CD45 and mesenchymal stem cell markers CD105, CD90 and CD73, respectively. **E)** The undifferentiated ADSCs. **F)** Oil Red O staining of hADSCs after three-week differentiation in adipogenic medium. **G)** Alizarin Red S staining of hADSCs after three-week differentiation in osteogenic medium.

3.4. Cell Cycle Analysis by Flow Cytometry

To investigate the impact of RG108 on the cell cycle progression of hADSCs, the control and RG108-treated cells were isolated by trypsin-EDTA, washed with cold PBS and fixed using 70% ethanol at 4 ºC overnight. The cells were then stained with propidium iodide (PI) staining solution containing 0.1% Triton X-100, 10 µg.L^{-1} PI, and 100 µg.L^{-1} DNase-free RNase A in phosphate-buffered saline for 30 min at room temperature. The analysis of DNA content was performed on a BD FACSCalibur™ flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star Inc.).

3.5. RT-PCR and qPCR Analyses

Total RNA of the cells was extracted using High Pure RNA Isolation Kit (Roche Applied Science, Germany). 1 µg of total RNA was transcribed into cDNA using cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA samples were then subjected to PCR amplification with a panel of specific primers, as described in **Table 1**. The PCR products were size fractionated by electrophoresis on 2% agarose gel. Quantitative assessment of gene expression was performed using the RealQ PCR Master (Ampliqon A/S, Denmark) with a green dye on a Rotor-Gene™ 6000 (Corbett Research, Qiagen) real-time analyzer. Relative quantification was performed using REST 2009 (Relative Expression Software Tool, Qiagen) based on the Pair Wise Fixed Reallocation Randomization Test® (Pfaffl *et al*. 2002). Four biological replicates of each group were included in the qPCR experiments, and β2 microglobulin (*B2M*) and *GAPDH* were used to normalize the quantitative data.

4. Results

4.1. Characterization of hADSCs

Based on flow cytometry analysis, the mesenchymal stem cell markers CD105, CD90 and CD73 were

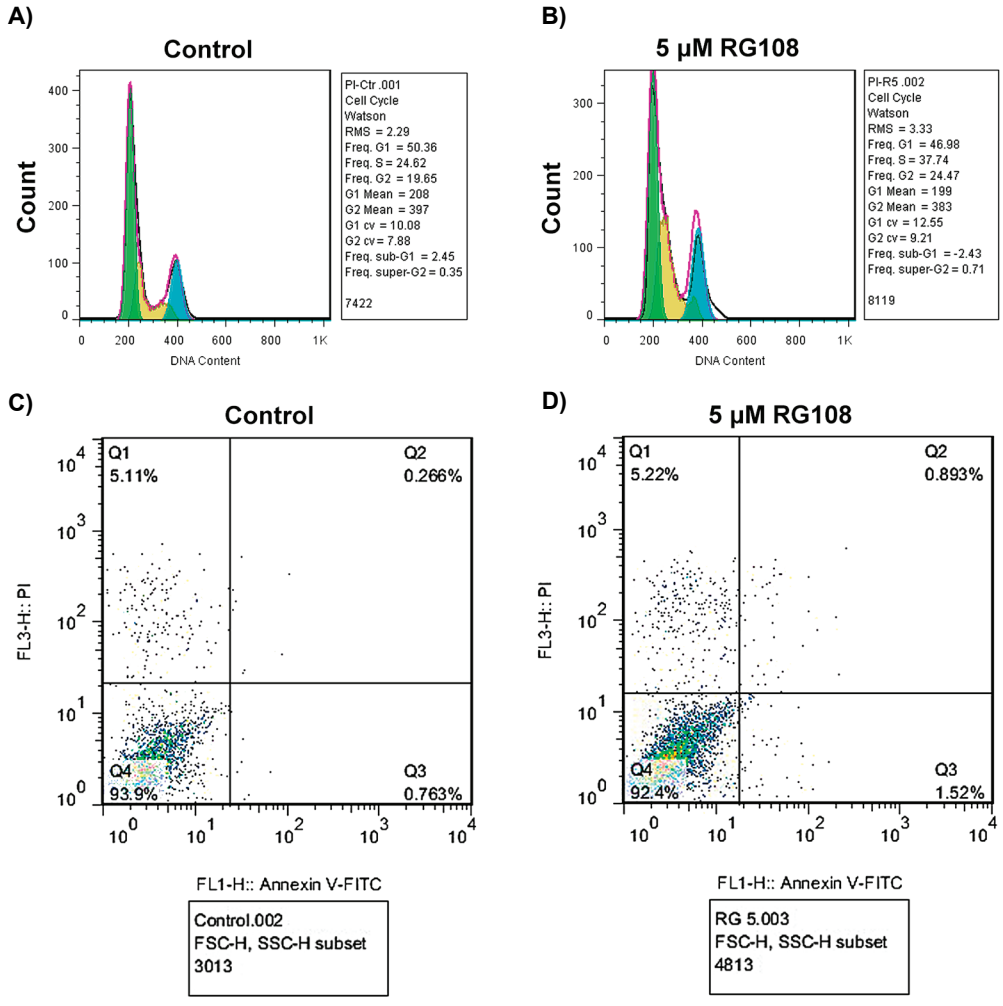

Figure 2. Effect of RG108 on the cell cycle and apoptosis in hADSCs. **A, B)** Cell cycle analysis by flow cytometry on PI-stained ADSCs of the control and RG108 treatment groups, respectively. **C, D)** Annexin V/PI apoptosis assay on the control and RG108-treated ADSCs, respectively.

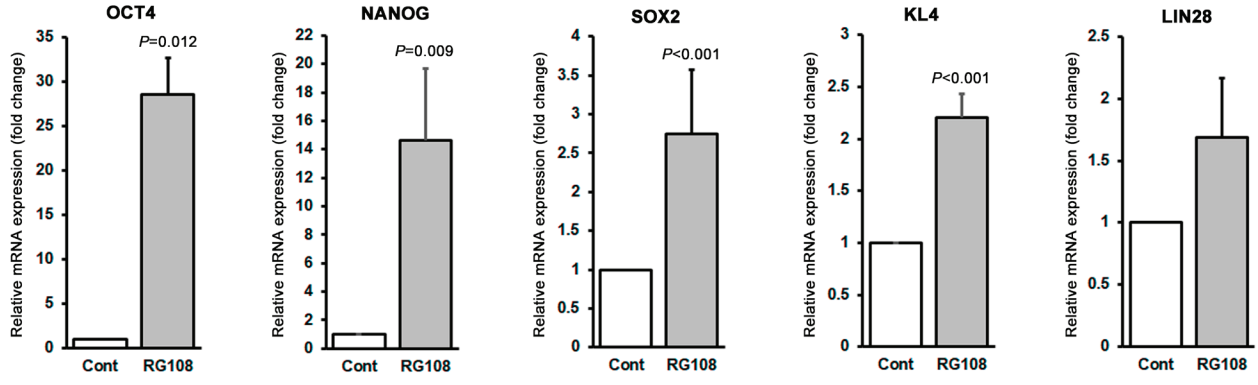

Figure 3. Quantitative real-time PCR analysis for the expression of pluripotency associated genes in the control and RG108-treated hADSCs. Significant differences were indicated by P values (PairWise Fixed Reallocation Randomization Test® performed by REST 2009 software).

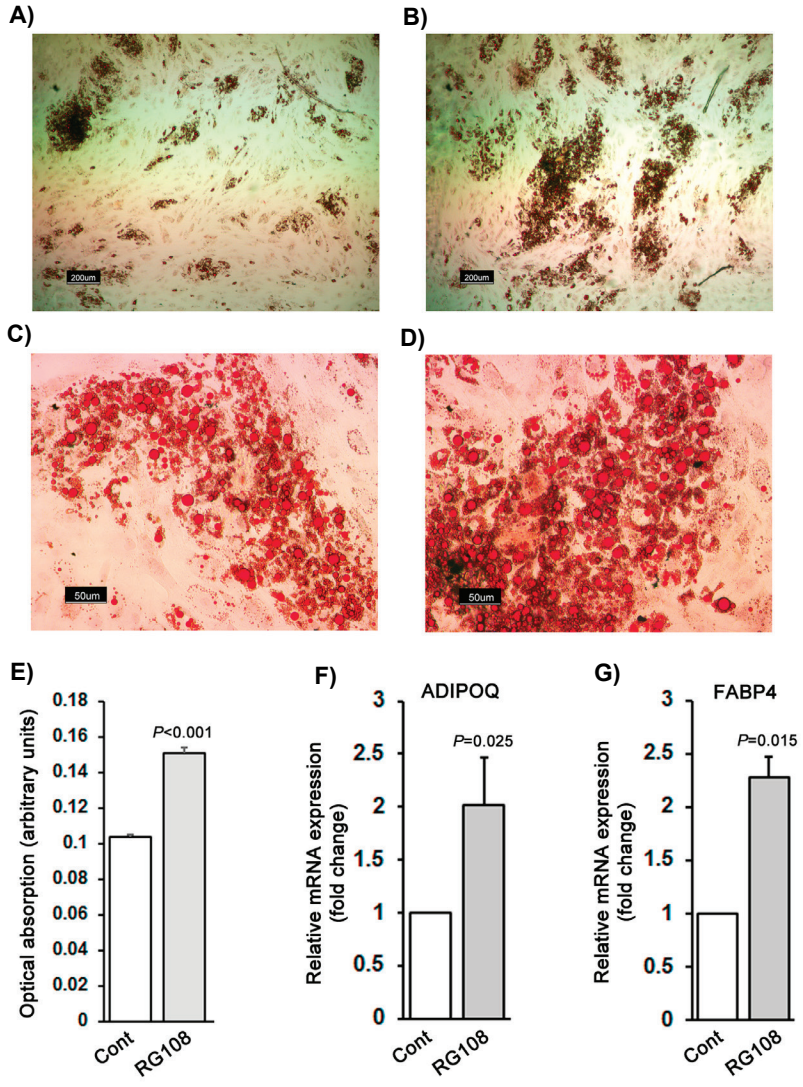

Figure 4. Adipogenic differentiation of the control and RG108-treated ADSCs. **A,B)** Oil red O staining showed accumulation of intracellular lipid droplets in the three-week differentiated ADSCs of the control and RG108 treatment groups, respectively, and **C,D)** demonstrate higher magnification of the same groups**. E)** Quantifying the lipid accumulation by eluting the Oil Red O deposit with isopropanol and measuring the absorbance at 518 nm. **F,G)** QPCR analysis for the expression of *ADIPOQ* and *FABP4* in three-week differentiated ADSCs. *Significant differences were indicated by P values (PairWise Fixed Reallocation Randomization Test® performed by REST 2009 software).

expressed in 99.6%, 99.7% and 99.5% of the fourth-passage ADSCs, respectively. However, CD45 was only expressed in 1.72% of the cells (**Fig. 1A -1D**). The third-passage ADSCs showed a fibroblast-like morphology (**Fig. 1E**). After three weeks of differentiation in adipogenic and osteogenic media, the cells showed positive staining for Oil Red O (**Fig. 1F**) and Alizarin Red S, respectively (**Fig. 1G**).

4.2. Effects of RG108 on Proliferation and Apoptosis in Human ADSCs

Based on the cell cycle analysis by flow cytometry, the frequency of the S-phase and G2-phase subpopulations increased from 24.6% and 19.6% in the control group to 37.7% and 24.4% in the RG108 treatment group, respectively. In contrast, the frequency of G1-phase subpopulation decreased from 50.3% in the control

group to 46.9% in the RG108 treatment group (**Fig. 2A**). The Annexin V/PI apoptosis assay demonstrated that RG108 treatment of the ADSCs did not induce cell death through apoptosis (**Fig. 2B**).

4.3. The Impact of RG108 on the Expression of Pluripotency Genes in hADSCs

Treatment with 5 µM RG108 upregulated the expression of *OCT4* by 28.6-fold (*P*=0.012), *SOX2* by 2.7-fold (P<0.001), *NANOG* by 14.7-fold (*P*=0.009) and *KLF4* by 2.2-fold (*P*<0.001). The expression of *LIN28* was not significantly different between the control and RG108 treatment groups (1.7-fold; *P*=0.062) (**Fig. 3**).

4.4. The Effect of RG108 on Adipogenic Differentiation of hADSCs

The control and RG108-treated cells were cultured in adipogenic differentiation medium for a duration

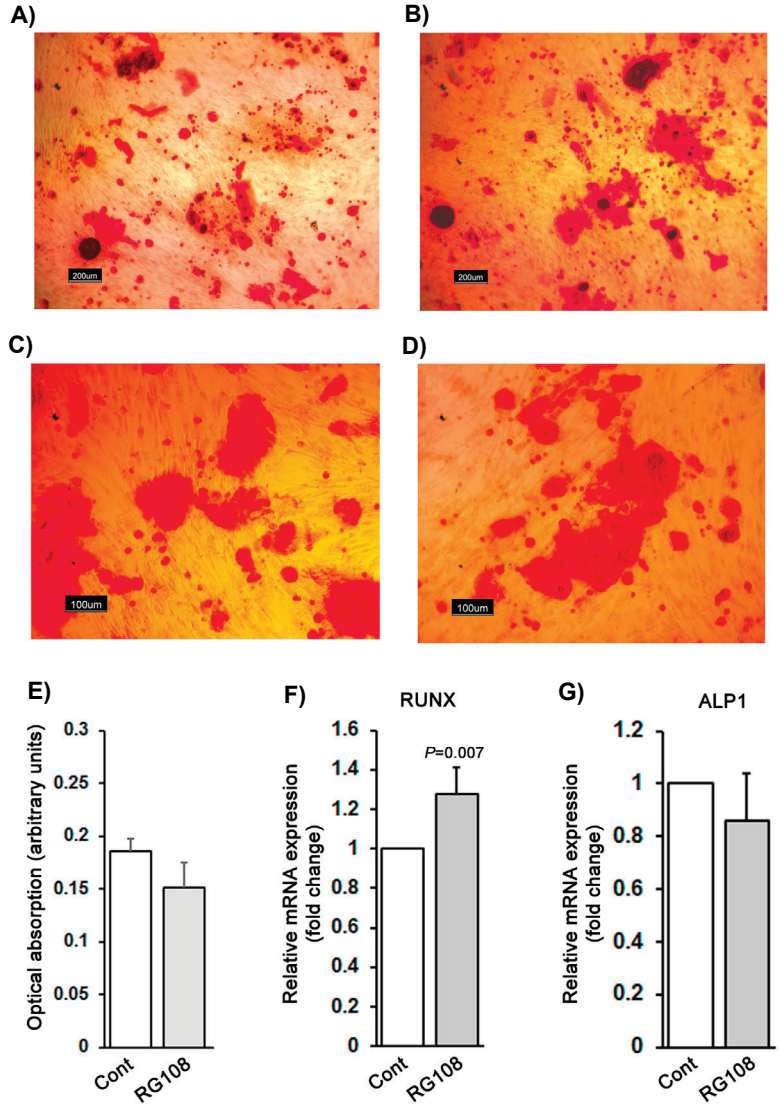

Figure 5. Osteogenic differentiation of the control and RG108-treated ADSCs. A-B) Alizarin Red S staining showed the extracellular calcium deposits in three-week differentiated ADSCs, and **C-D)** demonstrate higher magnification of the same groups**. E)** Quantifying the lipid accumulation by eluting the Alizarin Red S staining using 10% acetic acid and measuring the absorbance at 570 nm. **F ,G)** QPCR analysis for the expression of *ALP1* and *RUNX* in three-week differentiated ADSCs. *Significant differences were indicated by P values (PairWise Fixed Reallocation Randomization Test® performed by REST 2009 software).

of three weeks. Fat droplets appeared from day 3 of the differentiation and gradually increased in size and quantity. Oil red O staining confirmed the accumulation of intracellular lipid droplets in three-week differentiated cells (**Fig. 4A-4D**). Quantifying the staining degree showed that the lipid accumulation in the differentiated cells of 5 μM RG108 treatment group was significantly higher than the control group (**Fig. 4E**).

QPCR analysis showed that the expression of *ADIPOQ* in three-week differentiated cells of RG108 treatment group was 2.02-fold (P=0.025) higher than the control group (**Fig. 4F**). Also, the expression of *FABP4* was upregulated by 2.27-fold (*P*=0.015) in the RG108 treatment group compared to the control group (**Fig. 4G**).

4.5. Osteogenic Differentiation of the ADSCs

After three weeks of osteogenic differentiation, the extracellular calcium deposits were confirmed by Alizarin Red S staining (**Fig. 5A-5D**). As depicted in **Figure 5E**, the staining degree of the differentiated cells in the RG108 treatment groups was not significantly different to the control group. Also, qPCR analysis indicated no significant difference in the expression of *ALP1* gene between the control and RG108 treatment groups (**Fig. 5F**). However, *RUNX* gene expression exhibited an upregulation of 1.27-fold (*P*=0.005) in the RG108 treatment group compared to the control group (**Fig. 5G**).

5. Discussion

Over the last decades, ADSCs have been identified as a promising candidate for regenerative medicine, mainly due to their secretion of numerous cytokines, chemokines and growth factors which induce angiogenesis, modulate immune responses and promote tissue repair (Kilroy, Foster et al. 2007, Zhao, Johnson *et al*. 2017). The undifferentiated ADSCs express several genes characteristic of all three germ layers and have the ability to transdifferentiate into various mesodermal, endodermal, and ectodermal cells under specific culture conditions (18-25). However, the differentiation potential of the ADSCs is more limited than pluripotent stem cells, including ESCs and iPSCs. This potential is further influenced by different factors, including the age, gender, and metabolic index of the donors (26). Furthermore, it has been shown that extended culture of MSCs induces cellular senescence, leading to diminished proliferation, differentiation and regenerative properties (27-30). So, different strategies to overcome senescence and to enhance the overall stemness and differentiation capability of MSCs are crucial for regenerative medicine.

Senescence and exhaustion of MSCs happens as a result of increased epigenetic modifications (31) which results in the formation of senescence-associated heterochromatin foci (32), and particularly can be triggered by DNA methylation (33). Consequently, modifying the epigenetic landscape of the aging MSCs using various DNA methylation inhibitors can rejuvenate them for cell therapy purposes. Thus far, various factors have been investigated for this purpose, among them small chemical molecules, like RG108, hold great importance in this area. Previous studies have investigated the effects of RG108 on different types of stem cells, including porcine (33) and human BM-MSCs (17, 34), as well as human periodontal ligament-derived stem cells (35). These studies have demonstrated that RG108 reduces the global methylation of MSCs, leading to an increased expression of pluripotency-related markers and reversal of the senescent phenotype. However, except for the last study, none of these investigations have focused on the impact of RG108 on the differentiation potential of MSCs. In our current study, we aimed to examine the impact of RG108 on both the stemness and multipotential differentiation capability of hADSCs.

According to qPCR analysis, treatment of hADSCs with 5 μM RG108 resulted in a significant upregulation in the expression of pluripotency-associated genes, including *OCT4*, *SOX2*, *NANOG* and *KLF4*. This change in gene expression aligns with a previous study on BM-MSCs (Oh YS *et al*. 2015; Li Q *et al*. 2020), suggesting that it is likely caused by a decrease in methylation and hydroxymethylation levels at the regulatory elements of pluripotency genes.

Recently, a study conducted by Li *et al*. (36) demonstrated a feedback loop between DNMT1 and OCT4/NANOG in MSCs. This study suggested that the proliferation of MSCs may be regulated by the opposing functions of DNMT1 and OCT4/NANOG; DNMT1 inhibits proliferation (37), while OCT4 and NANOG promote it (38). Therefore, it is expected that RG108 would enhance cell proliferation by inhibiting DNMT1 and inducing pluripotency genes. Our findings support

this conclusion, as treating the ADSCs with RG108 increased the frequency of the S-phase and G2-phase subpopulations. This is consistent with previous studies on BM-MSCs (17, 34, 39) but in contrast to previous reports on human prostate cancer cells (40) and human endometrial carcinoma cells (41). It seems that RG108 affects normal stem cells and cancer cells differently.

As demonstrated previously, RG108 treatment of BM-MSCs induced the expression of genes associated with anti-senescence and anti-apoptosis while decreased the expression of apoptosis-related genes (33, 34). Here we did not assess the expression of anti-apoptotic or pro-apoptotic genes but showed by the Annexin V/PI apoptosis assay that RG108 treatment did not induce apoptosis in hADSCs.

To investigate the impact of RG108 on the multipotential differentiation capability of hADSCs, the control and RG108-treated cells were cultured in adipogenic or osteogenic differentiation medium for three weeks. Our findings demonstrated that pretreatment with 5 μM RG108 enhanced the adipogenic differentiation of hADSCs. This was confirmed by measuring the degree of Oil Red O staining of lipid droplets and analyzing the expression of the *ADIPOQ* and *FABP4* genes in the differentiated cells. However, the impact of RG108 on the osteogenic differentiation of hADSCs was not as pronounced as its impact on adipogenesis, as revealed by Alizarin Red S staining of extracellular calcium deposits, as well as qPCR analysis for the expression of *ALP1* and *RUNX* genes. A different concentration of RG108 may be more effective in promoting osteogenic differentiation.

6. Conclusion

Our findings demonstrated that treatment of hADSCs with the DNA methyltransferase inhibitor, RG108, can alter their gene expression profile and improve their stemness and differentiation potential. These alterations suggest the possibility of enhancing the regenerative efficacy of the ADSCs simply by exposing them to non-toxic small molecules that modify chromatin structure without genetic manipulation.

Acknowledgements

This work was supported by a research grant (No. 980901-I-744) from the *National Institute of Genetic Engineering and Biotechnology* (NIGEB). All the authors declare no conflict of interest.

References

1. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(10):5237-5242. doi: 10.1073/pnas.97.10.5237.
2. Ziller MJ, Muller F, Liao J, Zhang Y, Gu H, Bock C, *et al*. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. *PLoS Gen*. 2011;**7**(12):e1002389. doi: 10.1371/journal.pgen.1002389.
3. Smith ZD, Meissner A. DNA methylation: roles in mammalian development. *Nature Rev Gen*. 2013;**14**(3):204-220. doi: 10.1038/nrg3354.
4. Suelves M, Carrio E, Nunez-Alvarez Y, Peinado MA. DNA methylation dynamics in cellular commitment and differentiation. *Briefings in functional genomics*. 2016;**15**(6):443-453. doi: 10.1093/bfgp/elw017.
5. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nature Rev Gen*. 2008;**9**(6):465-476. doi: 10.1038/nrg2341.
6. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;**126**(4):663-676. doi: 10.1016/j.cell.2006.07.024.
7. Puri D, Wagner W. Epigenetic rejuvenation by partial reprogramming. *Bioessays*. 2023;**45**(4):e2200208. doi: 10.1002/bies.202200208.
8. Cheng Y, Xie N, Jin P, Wang T. DNA methylation and hydroxymethylation in stem cells. *Cell biochem function*. 2015;**33**(4):161-173. doi: 10.1002/cbf.3101.
9. Nagae G, Isagawa T, Shiraki N, Fujita T, Yamamoto S, Tsutsumi S, *et al*. Tissue-specific demethylation in CpG-poor promoters during cellular differentiation. *Human Mol Gen*. 2011;**20**(14):2710-2721. doi: 10.1093/hmg/ddr170.
10. Franzen J. WW, Fernandez-Rebollo E. Epigenetic Modifications upon Senescence of Mesenchymal Stem Cells. *Curr Stem Cell Rep*. 2016;**2**:248-254. doi: 10.1007/s40778-016-0051-7.
11. Cho YD, Yoon WJ, Kim WJ, Woo KM, Baek JH, Lee G, *et al*. Epigenetic modifications and canonical wingless/int-1 class (WNT) signaling enable trans-differentiation of nonosteogenic cells into osteoblasts. *J Biolog Chem*. 2014;**289**(29):20120-20128. doi: 10.1074/jbc.M114.558064.
12. Qin H, Zhao A, Fu X. Small molecules for reprogramming and transdifferentiation. *Cell Mol Life Sci* : CMLS. 2017;**74**(19):3553-3575. doi: 10.1007/s00018-017-2586-x.
13. Zentelyte A, Zukauskaite D, Jaceryte I, Borutinskaite VV, Navakauskiene R. Small Molecule Treatments Improve Differentiation Potential of Human Amniotic Fluid Stem Cells. *Front Bioeng Biotechnol*. 2021;**9**:623886. doi: 10.3389/fbioe.2021.623886.
14. Wang L, Wang N, Zhang W, Cheng X, Yan Z, Shao G, *et al*. Therapeutic peptides: current applications and future directions. *Signal Transduct Target Ther*. 2022;**7**(1):48. doi: 10.1038/s41392-022-00904-4.
15. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, *et al*. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res*. 2005;**65**(14):6305-6311. doi: 10.1158/0008-5472.CAN-04-2957.
16. Shi Y, Desponts C, Do JT, Hahm HS, Scholer HR, Ding S.

Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell*. 2008;**3**(5):568-574. doi: 10.1016/j.stem.2008.10.004.

17. Assis RIF, Wiench M, Silverio KG, da Silva RA, Feltran GDS, Sallum EA, *et al*. RG108 increases NANOG and OCT4 in bone marrow-derived mesenchymal cells through global changes in DNA modifications and epigenetic activation. *PloS One*. 2018;**13**(12):e0207873. doi: 10.1371/journal.pone.0207873.
18. Faghih H, Javeri A, Amini H, Taha MF. Directed differentiation of human adipose tissue-derived stem cells to dopaminergic neurons in low-serum and serum-free conditions. *Neurosci Lett*. 2019;**708**:134353. doi: 10.1016/j.neulet.2019.134353.
19. Faghih H, Javeri A, Taha MF. Impact of early subcultures on stemness, migration and angiogenic potential of adipose tissue-derived stem cells and their resistance to *in vitro* ischemic condition. *Cytotechnol*. 2017;**69**(6):885-900. doi: 10.1007/s10616-017-0104-5.
20. Hasani S, Javeri A, Asadi A, Fakhr Taha M. Cardiac Differentiation of Adipose Tissue-Derived Stem Cells Is Driven by BMP4 and bFGF but Counteracted by 5-Azacytidine and Valproic Acid. *Cell J*. 2020;**22**(3):273-282. doi: 10.22074/cellj.2020.6582.
21. Radtke C, Schmitz B, Spies M, Kocsis JD, Vogt PM. Peripheral glial cell differentiation from neurospheres derived from adipose mesenchymal stem cells. *Int J Dev Neurosci*. 2009;**27**(8):817-823. doi: 10.1016/j.ijdevneu.2009.08.006.
22. Soheilifar MH, Javeri A, Amini H, Taha MF. Generation of Dopamine-Secreting Cells from Human Adipose Tissue-Derived Stem Cells *In Vitro*. *Rejuvenation Res*. 2018;**21**(4):360-368. doi: 10.1089/rej.2017.1994.
23. Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, *et al*. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem Biophys Res Commun*. 2006;**341**(4):1135-1140. doi: 10.1016/j.bbrc.2006.01.072.
24. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002;**13**(12):4279-4295. doi: 10.1091/mbc.e02-02-0105.
25. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. 2001;**7**(2):211-228. doi: 10.1089/107632701300062859.
26. Zhang J, Liu Y, Chen Y, Yuan L, Liu H, Wang J, *et al*. Adipose-Derived Stem Cells: Current Applications and Future Directions in the Regeneration of Multiple Tissues. *Stem Cells Int*. 2020;**2020**:8810813. doi: 10.1155/2020/8810813.
27. de Witte SFH, Lambert EE, Merino A, Strini T, Douben H, O'Flynn L, *et al*. Aging of bone marrow- and umbilical cord-derived mesenchymal stromal cells during expansion. *Cytotherapy*. 2017;**19**(7):798-807. doi: 10.1016/j.jcyt.2017.03.071.
28. Gharibi B, Farzadi S, Ghuman M, Hughes FJ. Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells. *Stem Cells*. 2014;**32**(8):2256-2266. doi: 10.1002/stem.1709.
29. 29. Sethe S, Scutt A, Stolzing A. Aging of mesenchymal stem cells. *Ageing Res Rev*. 2006;**5**(1):91-116. doi: 10.1016/j.arr.2005.10.001.
30. Yang Y, Zhang W, Wang X, Yang J, Cui Y, Song H, *et al*. A passage-dependent network for estimating the *in vitro* senescence of mesenchymal stromal/stem cells using microarray, bulk and single cell RNA sequencing. *Front Cell Dev Biol*. 2023;**11**:998666. doi: 10.3389/fcell.2023.998666.
31. Sun Y, Zhang H, Qiu T, Liao L, Su X. Epigenetic regulation of mesenchymal stem cell aging through histone modifications. *Genes Dis*. 2023;**10**(6):2443-2456. doi: 10.1016/j.gendis.2022.10.030.
32. Johnson AA, Akman K, Calimport SR, Wuttke D, Stolzing A, de Magalhaes JP. The role of DNA methylation in aging, rejuvenation, and age-related disease. *Rejuvenation Res*. 2012;**15**(5):483-494. doi: 10.1089/rej.2012.1324.
33. Li Q, Zhai Y, Man X, Zhang S, An X. Inhibition of DNA Methyltransferase by RG108 Promotes Pluripotency-Related Character of Porcine Bone Marrow Mesenchymal Stem Cells. *Cell Reprogram*. 2020;**22**(2):82-89. doi: 10.1089/cell.2019.0060.
34. Oh YS, Jeong SG, Cho GW. Anti-senescence effects of DNA methyltransferase inhibitor RG108 in human bone marrow mesenchymal stromal cells. *Biotechnol Appl Biochem*. 2015;**62**(5):583-590. doi: 10.1002/bab.1393.
35. Roato I, Baima G, Orrico C, Mosca Balma A, Alotto D, Romano F, *et al*. Senescent Markers Expressed by Periodontal Ligament-Derived Stem Cells (PDLSCs) Harvested from Patients with Periodontitis Can Be Rejuvenated by RG108. *Biomedicines*. 2023;**11**(9). doi: 10.3390/biomedicines11092535.
36. Li K, Fan L, Lin J, Heng BC, Deng Z, Zheng Q, *et al*. Nanosecond pulsed electric fields prime mesenchymal stem cells to peptide ghrelin and enhance chondrogenesis and osteochondral defect repair in vivo. *Sci China Life Sci*. 2022;**65**(5):927-939. doi: 10.1007/s11427-021-1983-y.
37. Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. *Nature*. 2010;**463**(7280):563-567. doi: 10.1038/nature08683.
38. Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, *et al*. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet*. 2006;**38**(4):431-440. doi: 10.1038/ng1760.
39. Oh YS, Kim SH, Cho GW. Functional Restoration of Amyotrophic Lateral Sclerosis Patient-Derived Mesenchymal Stromal Cells Through Inhibition of DNA Methyltransferase. *Cell Mol Neurobiol*. 2016;**36**(4):613-620. doi: 10.1007/s10571-015-0242-2.
40. Graca I, Sousa EJ, Baptista T, Almeida M, Ramalho-Carvalho J, Palmeira C, *et al*. Anti-tumoral effect of the non-nucleoside DNMT inhibitor RG108 in human prostate cancer cells. *Curr Pharm Des*. 2014;**20**(11):1803-1811. doi: 10.2174/13816128113199990516.
41. Wang X, Li B. DNMT1 regulates human endometrial carcinoma cell proliferation. *Onco Targets Ther*. 2017;**10**:1865-1873. doi: 10.2147/OTT.S130022.