Original Article

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Increased mir33 Expression in Expanded Hematopoietic Stem Cells Cultured on Adipose Stem Cells Feeder layer

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Abstract

Bachgroun: Hematopoietic stem cell derived from umbilical cord blood (UCB) has been used for regenerative medicine in hematological abnormalities. MicroRNAs are important regulators of gene expression that control both physiological and pathological processes such as development of tissue and cancer. Some studies have shown that miR-33, has a critical role in control of self-renewal cells. Here to understand the role of adipose derived mesenchymal stem cells (ASCs) as a feeder layer on expansion of hematpoietic stem cells (HSCs), the expression of p53 and miR-33a were evaluated.

Material and methods: Isolated human ASCs in passage 3 were cultured as a feeder layer. Ex vivo cultures ofcord bloodCD34+ cells that expand in three culture conditions for 7 days were performed in following groups: fresh CD34+ cells, in the present of cytokines, directly cultured on feeder layer,indirectly cultured on feeder layer(cultured on thincert plate with 0.4 µm pore size). Expression of genes p53 and Mir-33a were analyzed by real-time PCR.

Result: The expression of p53 was significantly ($p<0.05$) down regulated in HSCs directly cultured on ADSCs feeder layer compared to without feeder layer. The expression of miR-33a was significantly ($p>0.05$) up regulated in HSCs directly cultured on feeder layer compare to without feeder layer.

Conclusion: Defining the role of ADSCs in controlling the HSC self-renewal through mir33 and p53may lead to the prevention and treatment of hematopoietic disorders.

Keywords: mir33, Expression, Hematopoietic Stem Cells, Adipose

Introduction

Umbilical cord blood (UCB) has been used for transplantation in regenerative medicine of hematological disorders. The use of hematopoietic stem cells for regenerative medicine have been largely unsuccessful due to the inability to create sufficient stem cell numbers and also to excessive differentiation of them (1). In vitro studies showed that control of HSCs self-renewal in culture is difficult. Hematopoietic cytokines have failed to support reliable amplification of HSCs in culture; it seems additional factors to be required. Recently factors such as feeder layer have been reported to effect to HSC expansion. Mesenchymal stem cells as a feeder layer have been able to prevent apoptosis of expanded hematopoietic stem cells derived from cord blood (2).

Adipose stem cells (ASCs) show properties similar to that observed in bone marrow mesenchymal stem cells. Due to easy accessibility, human adipose-derived mesenchymal stem cells (hADSC) are an attractive source for regenerative medicine (3). ASCs have been shown to be immuno privileged, to prevent severe graft-versus-host disease in vitro and in vivo and to be genetically stable in long-term culture. They also act as providing hematopoietic support and gene transfer (4). Adipose-derived stem cells exhibit high intrinsic expression of self-renewal supporting factors compare to bone marrow mesenchymal stem cells (5). Since ADSCs produce various factors to support stem cells maintenance and cell growth, in this paper we have we used them as a feeder

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layer for expansion of HSCs. MicroRNAs as regulators gene expression involves in physiological and pathological processes such as development and cancer (5). MicroRNAs are short non-coding RNAs, usually 18–25 nucleotides in length, which repress translation and cleave mRNA by base pairing to the 3′untranslated region of the target genes (7). It has been demonstrated that miRNAs play important roles in developmental biology, cellular differentiation programs and oncogenesis(8). They regulate various cellular processes of tumor, including cell proliferation, differentiation, progression, apoptosis and invasion (9-10). Alterations in the miRNA expression have emerged as an important mechanism for the development and progression of cancers (11-12).Various number of miRNAs have been studied in HSCs. The role of miR-33 in regulating cell proliferation and cell cycle progression is the subject of many investigations. It has been shown that miR-33 family members modulate the expression of genes involved in cell cycle regulation and cell proliferation (13). Various functions have been defined for miR-33 such as reduction of cell proliferation and cell cycle progression and impairing the p53 tumor suppressor gene function.

The function of mir33 is associated with genes such as p53 that is a cell cycle inhibitor gene. p53 activates the transcription of genes that induce cell cycle arrest, apoptosis and senescence in response to several stress conditions including DNA damage13.In this study with regard to the roles for p53 and mir33 in self renewal of HSCs, the effects of ADSCs as a feeder layer on HSCs cultures was investigated.

Material and Methods

Cell culture

Human subcutaneous adipose tissue samples were obtained from donors during abdominoplasty Erfan hospital. The tissue samples were processed using a modified procedure by Zhu et al (14). Subcutaneous adipose tissue samples were incubated with 0.075% collagenase II (sigma-Aldrich, St.Louis,MO) for 30 min, centrifuged at 150g for 5 min. The pellet was washed three- time in phosphate buffered saline. The cells seeded in 1×105 cells/dish and cultured in DMEM (Dulbeccos Modified Eagle's Medium) with 10% FBS (fetal bovine serum), 100U/ml penicillin/stereptomycin. Human HSCs were taken from Royan institute.

Proliferative and phenotypic analysis

Surface markers of ASCs were analyzed by flow cytometry, and monoclonal antibodies were used against for CD73, CD90 and CD105 markers. The adipogenic medium was ready by DMEM supplied with 3-isobutyl-1-methylxanthine (0.5 mM, IBMX), Dex (1 μM), insulin (10 μg/mL) and indomethacin (100 mM, Indo). To differentiate into osteoblast cells a medium of DMEM, with 10% fetal bovine serum and 10 nM dexamethasone, 35mg/mL of ascorbic acid, and 1 mM β-glycerophosphate was used. Cells were incubated in %5 CO2, at 37˚C for 21 days. To demonstrate the differentiation into adipocyte and osteoblast cells, alizarin red and oil red were used.

CD34+ cells derived from umbilical cord blood

After obtaining written consent, mononuclear cells with ficoll (Sigma, 1.077 ± 0.001) were separated. Then, these cells using anti-CD34 antibody with nanoparticles of Fe (America Milton Biotech) were incubated and CD34+ cells using column MACs (America Milton Biotech) were separated. To confirm CD34 marker for cells isolated from umbilical cord blood, the antibody anti-CD34 and anti-CD38 were used.

Culture of CD34+ cells

After feeder layer preparation with mitomycin c, CD34+ cells was cultured for 7 days with 100 ng/ml cytokines such as Stem Cell Factor(SCF), Thrombopoietin

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(TPO), and fetal liver tyrosine kinase 3 ligand (Flt-3L), and stem span medium as follows:

1) Only in the presence of mentioned cytokines.

2) Directly in contact with ASCs feeder layer.

3) Indirectly cultured on ASCs feeder layer (cultured on Thincert plate with 0.4 µm pore size).

Addition to this groups, one group of cells that were not cultured and immediately after extraction were analyzed and considered as a control.

MTT Assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to assess cell viability of all of group. This assay measures amount or ratio of cell proliferation and is a colorimetric assay dependent on the reduction of the tetrazolium salt, to form blue formazan crystals. After completion of incubation, the overlying culture medium was removed. After the addition of MTT, cells were incubated for 4 hours in an incubator containing CO2 at 37°C. Isopropanol acid was added and the optical density (OD) of the obtained solution was read at 630nm as the reference wavelength and at 570nm as the measurement wavelength using the ELISA reader. One-way ANOVA was used for data analysis.

Apoptosis Analysis by Annexin V and Propidium Iodide

Apoptosis kit (Bioscience, USA) was used for apop¬tosis analysis. On 14th day of culture, 1×104 cells resuspended in 1x binding buffer were treated with fluorochrome- conjugated Annexin V for 10 min and then washed and resuspended in 1x binding buffer. Next, propidium iodide solution was added and flu¬orescence of the stained cells was analyzed by flow cytometry.

RT-PCR

After RNA extracted from cells by Trizol, for reverse transcription of mRNA, cDNA was synthesized from 2µg of total RNA. For the synthesis of cDNA Random Hexamer and Oligo dt primers were used. GAPDH and U48 were used as loading controls for quantitation of mRNA and miRNAs.

The sequences of GAPDH, p53 and mir 33 are as follows:

P53

Forward:

5′-TCCTCAGCATCTTATCCGAGTG- 3′

Reverse:

5′- AGGACAGGCACAAACACGCACC-3′

GAPDH

Forward:

5′- ATGGGGAAGGTGAAGGTCG-3′

Reverse:

5′-GGGGTCATTGATGGCAACAATA-3′

Mir33a

Forward:

5′ GTGCATTGTAGTTGCATTGCA3′

Reverse:

5′ TGACCCCAGGTAACTCTGAGTG 3′

Real-time PCR

Real–time PCR was performed with an Evagreen and data were analyzed using the formula 2-ΔΔct..

Statistical analysis

Data are presented as mean standard deviation (SD). Two-way ANOVA and Duncan test were performed. Differences were considered significant at $P<0.05$.

Results

Surface antigen markers of ASCs were analyzed by flow cytometry. Our results showed that ASCs are positive for mesenchymal stem cells markers such as CD105, CD90, and CD73 and negative for hematopoietic stem cells markers such as CD34, and CD45 (Figure1, 2).

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Differentiation potential of ASCs to adipocyte and osteoblast cells were analyzed by Oil Red and Alizarin Red staining. Differentiated cells were positive for these staining ($p<0.01$) (Figure 3). Separated CD34+ HSCs analyzed by flow cytometry were 88.8%. 22.2% of them were positive for CD38 marker (Figure 4). Ex-vivo expansion of human cord blood enriched CD34+cells in serum-free medium supplemented with SCF, TPO and FLT3L was evaluated either with or without feeder layer using flow cytometry analysis (fig 4). Annexin V and PI staining was performed for apoptosis analysis of expanded cells. The percentage of apoptotic cells for PI and Annexin in co-culture with and without feeder layer was shown in Figure 4. The data of MTT assay showed that proliferation rate of CD34+ cells directly cultured on a ADSCs feeder layer group were higher than other group ($p<0.05$) (figure 5).

Results of RT-PCR showed expression of mir-33a in HSCs directly cultured on ASCs feeder layer group was higher than the other groups. Moreover, the expression level of p53 in this group was lower than the other groups (Figure 6, 7). Results showed that mir-33a and p53 negatively regulates each other. RT-PCR analysis showed that expression of mir-33a in groups that HSCs indirectly cultured on feeder layer and groups that HSCs only cultured in the presence of mentioned cytokines not different to each other (Figure 4). The results showed that the expression of mir-33a and p53 gene in thincert plate with 0.4 µm pore sized groups were lower than HSCs cultured directly on ASCs feeder layer group ($p<0.05$). The direct contact between HSCs and feeder layer was prevented by microporous membrane and consequently the expression of P53 increased compared to direct contact of feeder layer with hHSCs group (Figure 7) results of real time PCR analysis as same as RT-PCR analysis. These results showed that expression of mir-33a in groups that HSCs directly cultured on feeder layer was higher than other groups ($p<0.05$) (Figure 8). In control group, expression of mir-33a was lower because HSCs in these group were quiescence. The highest expression level of P53 gene was observed in CD34+ hHSCs ($P < 0.05$). Expression of p53 in the present of feeder layer was lower compare to the other experiments ($p<0.05$).

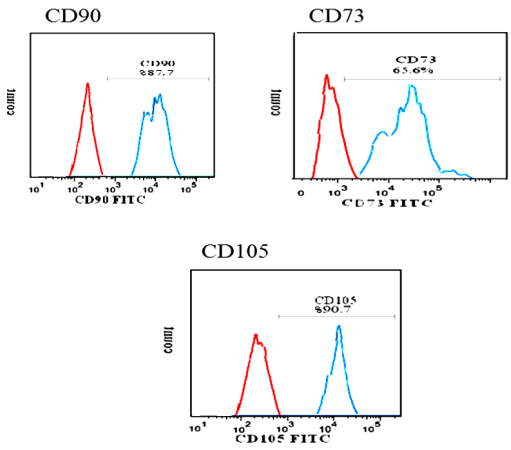


Figure1. Analysis of ADSCs markers with flow cytometry. %87.7 of ADSCs is positive for CD90, %65.6 of cells is positive for CD73. And, %90.7 of ADSCs is positive for CD105.

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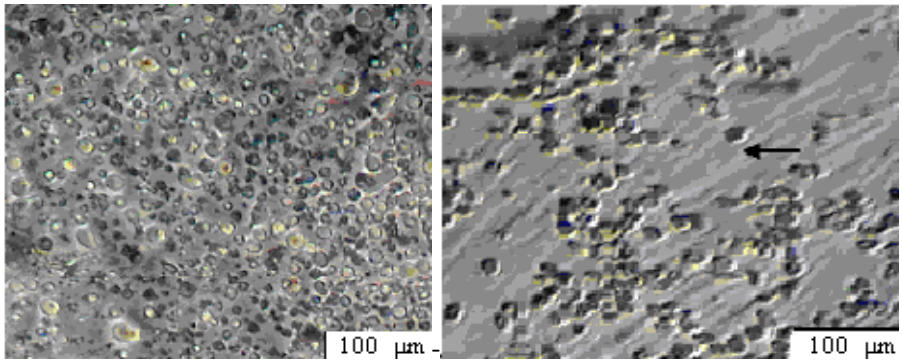


Figure 2. HSCs was cultured on ASCs feeder layer A) After 2 day B) After 7 day

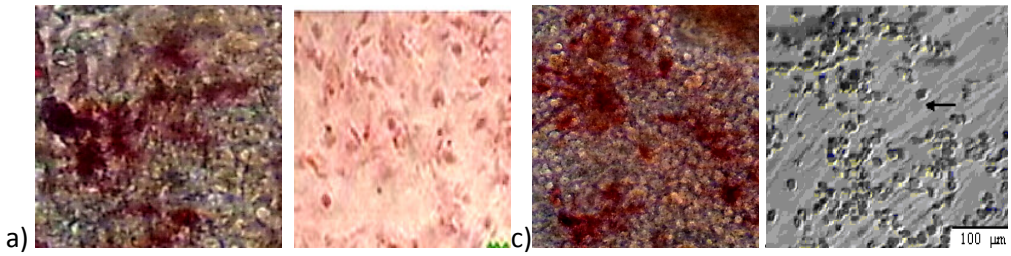


Figure 3. Osteogenic differentiation of adipose derived mesenchymal stem cells (×200). A: Positive reaction in osteoblastic differentiated cells with alizarin red staining; B) Undifferentiated cells; C) Osteoblastic differentiated cells with increased alkaline phosphatase activity; D) Undifferentiated cells.

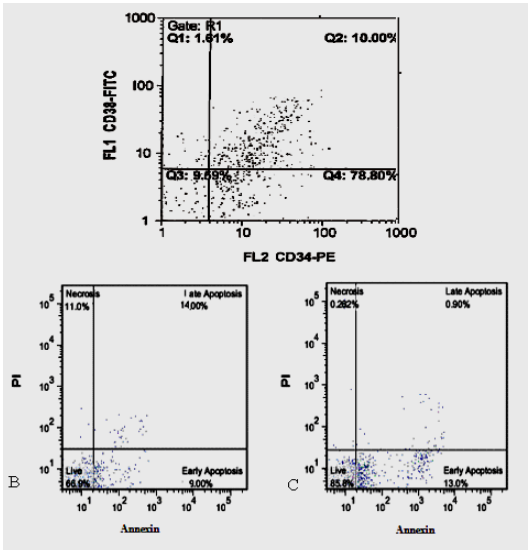


Figure 4. Flow cytometric analysis of cord blood HSCs: **A)** Flow cytometric analysis of fresh CD34+ enriched cells. Specific staining was performed with anti-CD34 FITC and anti-CD38 PE antibodies. Flow cytometric analysis of apoptosis at day 14 in different culture condition of CD34+ with and without ADSCs feeder layer by PI and Annexin V staining,. B) Cord blood CD34+ in co-culture with ADSCs, and C) Cord blood CD34+ without ADSCs feeder layer.

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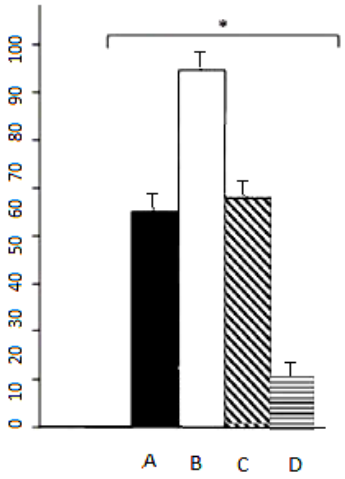


Figure5. MTT analysis in different culture condition. A: CD34+ cells that indirectly cultured on feeder layer. B) CD34+cells directly cultured on a ADSCs feeder layer. C) CD34+ cells in the present of cytokines. D) Fresh CD34+ cells before culture ($p<0.05$).

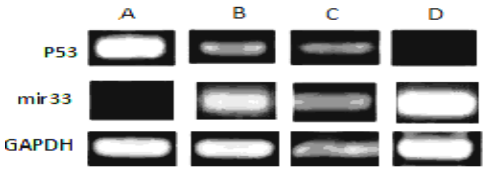


Figure 6. Results of RT-PCR. A) CD34+cells that considered as a control group, In these group Expression of p53 was higher and miR-33a expression was lower than the other groups. B) CD34+cells that only cultured with cytokines C) CD34+ cells cultured indirectly on a feeder layer. D) CD34+ cells directly cultured on a ADSCs feeder layer, in this group expression of miR-33awas higher than other groups.

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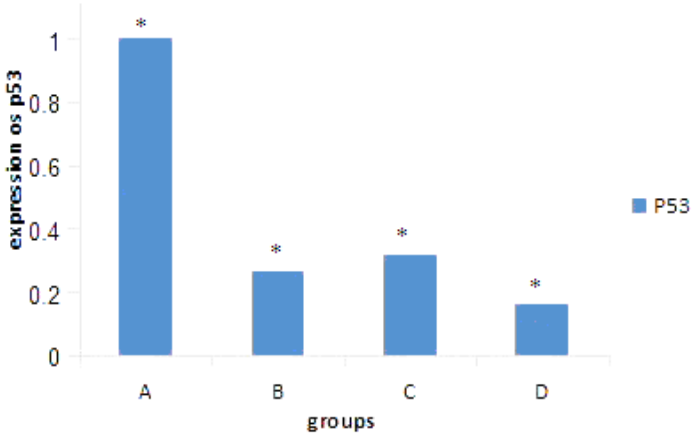


Figure7. A) Analysis of p53 gene expression in fresh CD34+ cells by real-time PCR. In this group expression of p53 was higher than other groups. B) Expression of p53 in CD34+ cells in the present of cytokines. C) Expression of p53 in CD34+ cells that indirectly cultured on feeder layer expression of p53 in B and C not different to each other. D) expression of p53 in CD34+ cells that directly cultured on feeder layer, in these group expression of p53 was lower than other groups ($p<0.05$).

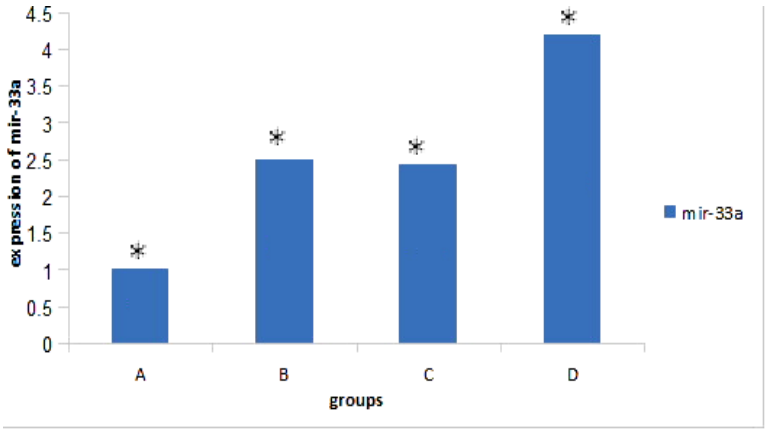


Figure 8. A) Analysis of miR-33a expression in fresh CD34+ cells by real-time PCR. B) Expression of mirR-33a in CD34+ cells in the present of cytokines.C) expression of miR-33a in CD34+ cells that indirectly culturd on feeder layer, expression of miR-33a in B and C groups not different to each other. D) expression of miR-33a in CD34+ cells that directly cultured on feeder layer, in these group expression of miR-33a was higher than other groups ($p<0.05$).

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Discussion

The results showed that in the presence of ADSCs feeder layer self-renewal of HSCs was higher than the other groups. Because of insufficient number of HSCs, the expansion of these cells is important for clinical application. Recently reported that bone marrow mesenchymal stem cells are as feeder layers and cytokines such as SCF and TPO increase proliferation of HSCs2. Glettig et al. showed that different feeder layers for HSCs limit the differentiation of these cells (15). The data revealed that the expression of p53 as a self-renewal inhibitor gene in HSCs cultured on ASCs feeder layer was lower than the other groups. The results showed that the expression of mir-33a gene in thincert plate with 0.4 µm pore sized groups were decreased compare to the HSCs cultured directly on ASCs feeder layer group. It has been reported that direct contact between HSCs and feeder layer was critical for expansion of cells. Silva et al. reported that direct contact of HSCs and feeder layer can be affect on increased self-renewal of HSCs (16). Alakel et al. showed that direct contact between HSCs and bone marrow mesenchymal stem cells feeder layer could improve the self-renewal of HSCs and could effective on the migratory behavior of HSCs (17). Based on the results of the present study, HSCs direct cultured on ADSCs showed higher level of mir33a expression than indirect contact group (thincert plate mediate). These results showed that high expression of miR-33a in the presence of ASCs feeder layer could down regulate the p53 and enhance the expansion of HSCs. . It seems mir-33 is mediated down regulation of p53. However, the other research resulted that miR-33 reduces cell proliferation and cell cycle progression and impairing the p53 tumor suppressor gene function (18). Perhaps it explained that the expression level of mir-33 was dependent on cell type. The findings of this study contribute to current understanding of the functions of miR-33a in HSCs cultured on ADSCs as a down regulator of p53. It seems factors secreted by adipose stem cells feeder layer target mir-33 -mediated down regulation of p53 in expansion of hematopoietic stem cells.

Conclusion

It seems that mir 33 can increase the proliferation of HSC cultured on ADSCs by impairing the p53 function. Defining the role of ADSCs in controlling the HSC self-renewal through increased mir33 and reduced p53 may lead to the treatment and prevention of hematopoietic disorders.

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