

Research Article



## NF- $\kappa$ B Activation in U266 Cells on Mesenchymal Stem Cells

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### Article info

#### Article History:

Received: 4 June 2016  
Revised: 27 August 2016  
Accepted: 30 August 2016  
ePublished: 25 September 2016

#### Keywords:

- NF- $\kappa$ B signaling pathway
- CD54
- U266 cell line
- Mesenchymal Stem Cell
- IL-6
- p65

### Abstract

**Purpose:** Mesenchymal Stem Cells (MSCs) are one of the essential members of Bone Marrow (BM) microenvironment and the cells affect normal and malignant cells in BM milieu. One of the most important hematological malignancies is Multiple Myeloma (MM). Numerous studies reported various effects of MSCs on myeloma cells. MSCs initiate various signaling pathways in myeloma cells, particularly NF- $\kappa$ B. NF- $\kappa$ B signaling pathway plays pivotal role in the survival, proliferation and resistance of myeloma cells to the anticancer drugs, therefore this pathway can be said to be a vital target for cancer therapy. This study examined the relationship between U266 cells and MSCs.

**Methods:** U266 cells were cultured with Umbilical Cord Blood derived-MSCs (UCB-MSCs) and Conditioned Medium (C.M). Effect of UCB-MSCs and C.M on proliferation rate and CD54 expression of U266 cells were examined with MTT assay and Flowcytometry respectively. Furthermore, expression of CXCL1, PECAM-1, JUNB, CCL2, CD44, CCL4, IL-6, and IL-8 were analyzed by Real Time-PCR (RT-PCR). Moreover, status of p65 protein in NF- $\kappa$ B pathway assessed by western blotting.

**Results:** Our findings confirm that UCB-MSCs support U266 cells proliferation and they increase CD54 expression. In addition, we demonstrate that UCB-MSCs alter the expression of CCL4, IL-6, IL-8, CXCL1 and the levels of phosphorylated p65 in U266 cells.

**Conclusion:** Our study provides a novel sight to the role of MSCs in the activation of NF- $\kappa$ B signaling pathway. So, NF- $\kappa$ B signaling pathway will be targeted in future therapies against MM.

### Introduction

Multiple myeloma is an important hematological malignancy,<sup>1</sup> and is defined by the clonal proliferation of plasma cells. MM is responsible for ~1% of all cancers and ~2% of cancer deaths.<sup>2</sup> Some diagnostic criterion of multiple myeloma are monoclonal proteins, plasma cells with light chains secretion, and organ damages related to myeloma.<sup>3</sup> Some examples of end-organ damages in multiple myeloma are: hypercalcemia, renal failure, anemia, osteolytic lesions or osteoporosis, reduction of hematopoietic function and reduction of polyclonal immunoglobulin production.<sup>4,7</sup> The bone marrow microenvironment known as niche and this microenvironment includes two components: cellular and non-cellular components.<sup>8-11</sup> Stroma cells, endothelial cells, hematopoietic stem cells and pluripotent precursors, adipocytes, osteoblasts and osteoclasts constitute the cellular component. Mesenchymal stem cells (MSCs) are among these cells. These cells are multipotent and they can be differentiated into some mesodermal lineages such as osteocytes, adipocytes and chondrocytes. MSCs can be isolated from several tissues including Umbilical Cord Blood, Bone Marrow, Placenta and Adipose tissue, Synovial fluid, Fetal

pancreas, Lung, Liver, Amniotic fluid.<sup>12</sup> Several studies have demonstrated the supportive effects of MSCs for normal and malignant cells.<sup>13,14</sup> The bone marrow stromal cells (BMSC) in MM cells microenvironment have supportive effects through direct or indirect contacts. When MM cells are localized in the bone marrow, via the interaction of cell surface adhesion molecules, they bind to the BMSCs and as a result, they trigger signaling pathways such as IL-6, SDF-1, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ ) and they are involved in tumor cells proliferation.<sup>9,15</sup> One of the main signaling pathways in MM cells is the NF- $\kappa$ B pathway. BM cells secrete different factors, such as VEGF and IGF1, can indirectly activate the NF- $\kappa$ B pathway in MM cells.<sup>3,10</sup> Also, in myeloma tumors, this signaling pathway supports cell survival, proliferation and resistance to anticancer drugs, however, the inhibition of NF- $\kappa$ B is a vital option for anti-cancer therapies.<sup>16</sup> NF- $\kappa$ B proteins consist of two great subfamilies: NF- $\kappa$ B and 'Rel' molecules.<sup>17</sup> One of the most important proteins that plays a key role in this

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pathway is p65. This protein forms a dimer with p50, then they attach to Ikappa B alpha (I $\kappa$ B $\alpha$ ). This event results in their translocation to the nucleus and gene transcription, so the NF- $\kappa$ B transcription activity is dependent on p65 phosphorylation.<sup>18</sup>

Previous studies have shown that this pathway can be very helpful in MM therapies. In this study, the effects of derived Umbilical Cord Blood-MSCs (UCB-MSCs) on the NF- $\kappa$ B pathway activation in U266 cell lines were investigated. As well documented, Bone Marrow-MSCs derived from MM patients displayed multiple aberrant characteristics such as the production of certain cytokines, abnormal proliferative capacity, and distinctive genes expression. Moreover, in a variety of cancers, MSCs have been shown to exhibit tropism for migrating to tumor sites. Also, the vital role of MSCs in MM pathogenesis is now well established.<sup>19</sup> These findings led to the suggestion that activation of the NF- $\kappa$ B pathway is essential for supporting roles of UCB-MSCs in proliferation rate of U266. Therefore, this idea was examined using western blotting technique, in order to determine if p65 and phosphorylated p65 are activated through co-culturing that involves cell to cell interaction of UCB-MSCs with U266 cells, or not.

## Materials and Methods

### Cell Culture and Conditioned Medium (C.M) preparation

The U266 myeloma cell line was obtained from the Pasteur Institute of Iran. The cells were cultured and incubated in RPMI-1640 medium (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS, Gibco, UK), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Gibco, UK). U266 cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C. In all steps of the experiments, cell viability was checked by trypan-blue staining. It was found that cell viability exceeded 86% in all experiments.

UCB-derived MSCs were purchased from the Pasteur Institute of Iran, then cultured and incubated in Low Glucose DMEM medium (Sigma-Aldrich, USA) with 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Gibco, UK).

In some steps of our experiments, we designed co-culture models by U266 cells and UCB-MSCs co-culturing. To this purpose, 2 $\times$ 10<sup>3</sup> UCB-MSCs were seeded in each well of 6 well plates and U266 cells (2 $\times$ 10<sup>4</sup> cell/well) were laid down on UCB-MSCs. DMEM was replaced with RPMI-1640 for co-culturing.

For some tests, the C.M was required, so UCB-MSCs were incubated with RPMI-1640, without FBS, for 24 hrs. After incubation, C.M was collected and was used for tests.

### MTT - assay

The proliferative effect of UCB-MSCs on U266 cells was determined by the MTT dye uptake method. In summary, the UCB-MSCs were cultured in 6 well plates and then U266 cells were added to UCB-MSCs with different concentrations (500, 1000, 2000, 5000, 10000

cell/well) and were incubated at 37°C for 48 hrs. Thereafter, 10  $\mu$ L MTT (2.5 mg/mL in phosphate-buffered saline) was added to each well. After incubation, the U266 were collected and centrifuged. After centrifugation, the supernatant was removed and 200  $\mu$ L RPMI-1640 with 10  $\mu$ L 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) solution (0.4 mg/ml) were added and incubated for 2.5 hrs. Then, Isopropanol/HCl (0.04M) was added and incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C overnight. Finally, the optical density of solutions was measured by Picodrop (Picodrop, UK) at 580 and 650 nm.

### Flow cytometry

In our experiments, we harvested U266 cells and washed them with PBS. In the next step, 20  $\mu$ L of phycoerythrin-conjugated anti-CD54 (Becton Dickinson, San Jose, CA, USA) was added to the cells for 30 min at 4°C. In addition, forward and side scattering were used to distinguish live and apoptotic cells by flow cytometry (FACS Calibur, USA).

### Western blot

At first, co-cultured U266 cells with UCB-MSCs were harvested by centrifugation and the resulting pellet was suspended in lysis buffer (10 Mm Tris-HCl, 1 mM EDTA, 0.1% (v/v) Triton-X100, PH 7.4) and sonicated at 4°C. The supernatants were cleared by centrifugation and protein concentrations were analyzed by the Bradford assay. Total cell lysates for western blots were prepared after lysing of the cell pellets in radioimmuno-precipitation assay buffer.

In this study, lysates were separated by 15% SDS-PAGE and were transferred to Immobilon™-P nitrocellulose membranes. Equal amounts of proteins were resolved on 15% (w/v) acrylamide gels by SDS-PAGE and were transferred onto a nitrocellulose membrane. After the separation of proteins in the sample by gel electrophoresis, the transfer buffer (25 mM Tris, 192 mM glycine, pH 8.0, 20% methanol) was prepared. The proteins were transferred from the gel to a membrane. In the next step, the membrane was incubated for 1 h in blocking buffer (phosphate-buffered saline (PBS), 5% (w/v) nonfat dry milk or PBS, 0.1% (v/v) Tween-20 (PBS-T), 5% (w/v) nonfat dry milk), the membrane was immunoblotted with a 0.2-10  $\mu$ g/ml primary antibody overnight at 4°C. It was thereafter washed in PBS or PBS-T, then nitrocellulose sheets were incubated with a secondary antibody (67-1,000 ng/ml) for 3-4 hrs at room temperature with gentle agitation. The blot was washed to remove excess secondary antibody. More so, the blot was incubated using Pierce ECL Scientific kit that allowed the substrate to react with the blot for 2 min and this was carried out with gentle agitation in a tray. At the end, excess reagent was drained and Image blot which detects luminescent signal with film was utilized. The bands were detected on a cool camera BIO IMAGER film.

**Real-time PCR (RT-PCR)**

Total RNA containing genes were detected by RT-PCR. RNAs of U266 were isolated by QIAzol (QIAGEN), thereafter, the quality of isolated RNA was checked by Pico drop (Picodrop, UK). cDNA was produced by cDNA synthesis kit (Applied bio system) and RT-PCR was performed by RT-PCR kit (Applied bio system) on StepOne Plus instrument (Applied Biosystems) using

standard protocols. The threshold cycle (Ct) value for each gene was normalized by the Ct number of housekeeping gene (β-actin). In this study, 8 genes with real-time PCR were analyzed (Primers shown in Table 1). The expression of 4 genes (CXCL1, CCL4, IL-6, and IL-8) was increased. Four of them (PECAM-1, JUNB, CCL2, CD44) were not changed.

**Table 1.** List of primers for real time-PCR.

Genes	Forward Primer	Reverse Primer
IL-6	5'-AGACAGCCACTCACCTTTCAG-3'	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
IL-8	5'-GAGAGTGATTGAGAGTGACCAC-3'	5'-CACAAACCTCTGCACCCAGTTT-3'
CXCL1	5'-AGCTTGCTCAATCCTGCATCC-3'	5'-TCCTTCAGGAACAGCCACCAGT-3'
CCL4	5'-GCTTCTCGCAACTTTGTGGTAG-3'	5'-GGTCATACACGTACTCCTGGAC-3'
PECAM-1	5'-AAGTGGAGTCCAGCCGCATATC-3'	5'-ATGGAGCAGGACAGGTTTCAGTC-3'
CCL2	5'-AGAATCACCAGCAGCAAGTGTC-3'	5'-TCCTGAACCACTTCTGCTTGG-3'
JUNB	5'-CGATCTGCACAAGATGAACCACG-3'	5'-CTGCTGAGTTGGTGTAAACGG-3'
CD44	5'-CCAGAAGGAACAGTGGTTTGGC-3'	5'-ACTGTCCTCTGGGCTTGGTGT-3'

**Statistical analysis**

Data are shown as means ± SD from three separate experiments. The data were evaluated using a GraphPad Prism v 5.00 (GraphPad Software, Inc., La Jolla, CA). Student's t-test (for single comparison) or two-way ANOVA (for multigroup comparisons) with Tukey post hoc test were used as appropriate. P < 0.05 was regarded as the statistical significance.

**Results**

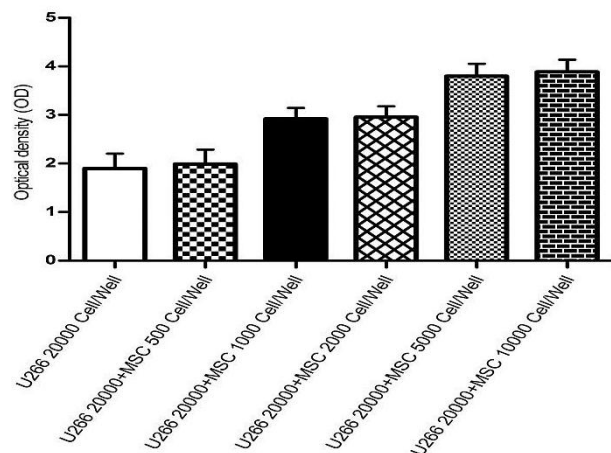
**UCB-MSCs supported proliferation rate of U266 cells**

The UCB-MSCs (at the density of 2×10<sup>3</sup> cell/well) were cultured to 60% confluency in 6-wells plate. After that, U266 (2×10<sup>4</sup> cell/well) was laid down to UCB-MSCs and cells were incubated for 48 hrs. After incubation, U266 were collected and MTT assay performed. The results were obtained from the MTT assay indicated that U266 proliferation was markedly higher in the co-culture with UCB-MSC, as compared with the control. In contrast, U266 proliferation cultured in C.M was unchanged, as compared with the control group (Figure 1).

**UCB-MSCs increased expression of CD54 on U266 cell line**

CD54 expression is depend on NF-κβ signaling pathway activity.<sup>20</sup> In this regard, to determine the effect of UCB-MSCs on CD54 expression, U266 cells were co-cultured with UCB-MSCs and incubated for 24 hrs. U266 cells in RPMI-1640 were referred to as the control group. After incubation, the cells were washed with PBS by centrifugation and mixed with 20 μl of phycoerythrin-conjugated anti-CD54 (Becton Dickinson, San Jose, CA, USA). In addition, the forward and side scattering were used to distinguish live and dead cells by flow cytometry

(FACS Calibur, USA). Sorting showed that (10<sup>6</sup> cells in 100 μl), CD54 expression increased in co-culture with UCB-MSCs (FACS Calibur, USA), whereas no increase was observed in the control group (Figure 2).



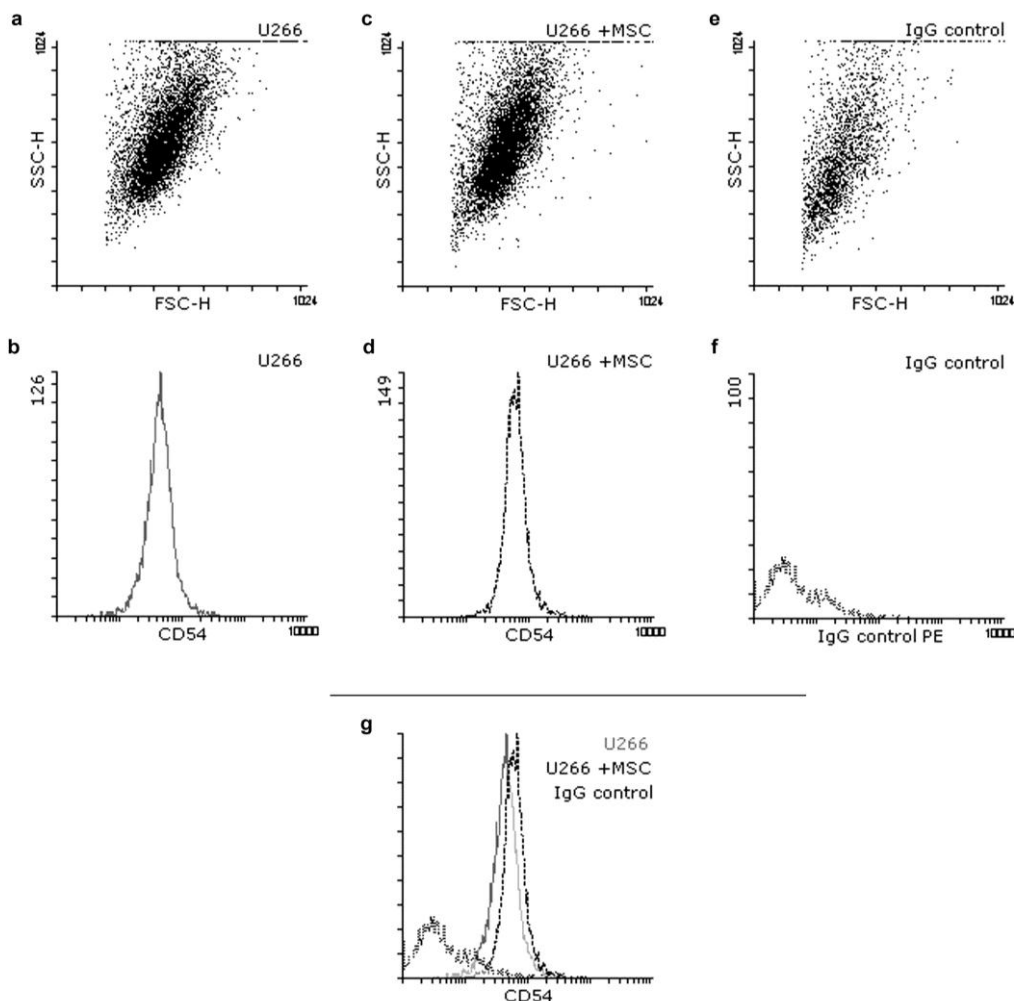
**Figure 1.** The evaluation proliferation rate of U266 cells after 48 hrs incubation. The proliferation rate was analyzed using MTT assay. UCB-MSCs, Umbilical Cord Blood-Mesenchymal stem cells; C.M, conditioned medium. (\*P<0.05, using student's t-test).

**UCB-MSCs affected the NF-κβ signaling pathway in U266 cells**

NF-κβ signaling pathway was analyzed by western blotting. Western blot performed to assess the protein levels of p65 and phosphorylated p65. Figure 3 shows the bands of p65 and phosphorylated p65 proteins. Phosphorylated p65 protein status is equivalent of NF-κβ activity, while p65 shows that NF-κβ is in inactive status.<sup>21</sup> According to our data, phosphorylated p65 increased markedly in presence of UCB-MSCs compare

with the control group, while C.M did not show strong bands (Figure 3a). Although, in the evaluation of p65

bands, no significant difference was observed between the control sample and the other conditions (Figure 3b).



**Figure 2.** Assessment of CD54 expression in U266 cells after 24 hrs incubation with UCB-MSCs by mono-color flow cytometry. CD54 expression is shown using (a) dot plot and (b) histogram of U266 cells. CD54 expression is similarly illustrated by (c) dot plot and (d) histogram of co-cultured U266 cells with UCB-MSCs. (e) Dot plot and (f) histogram of isotype control to identify and control for the nonspecific antibody binding. (g) Combination of CD54 expression histograms by U266 cells, co-cultured U266 cells with UCB-MSCs and IgG control. UCB-MSCs, Umbilical Cord Blood-Mesenchymal stem cells. (\*P<0.05, using student's t-test).



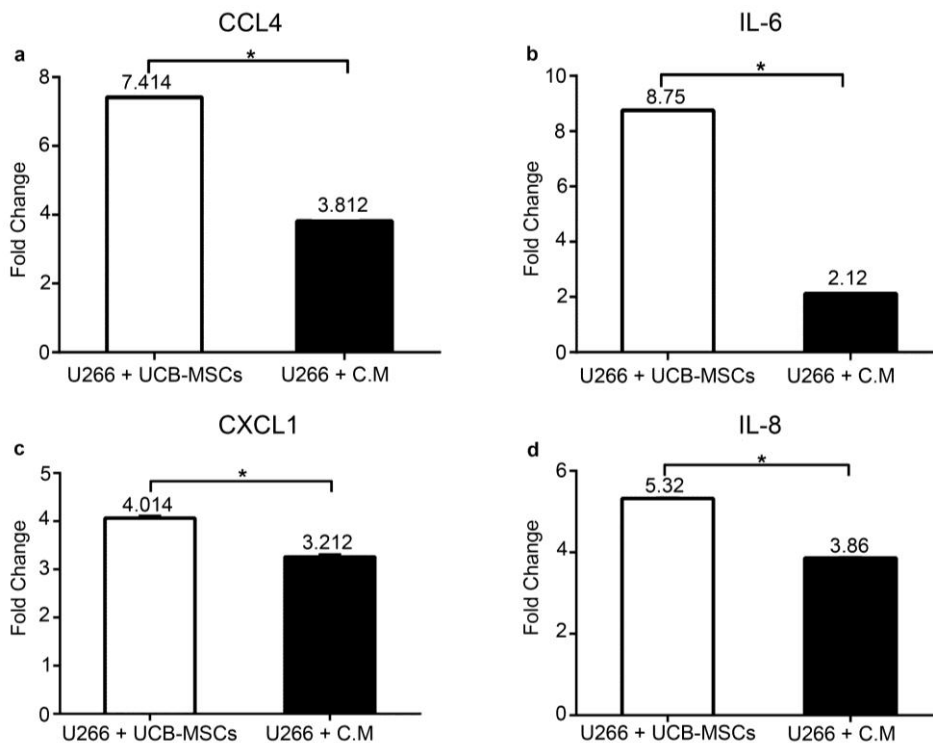
**Figure 3.** Comparison of phosphorylated p65 and p65 proteins expression in U266 cells. (a) Analyzing of phosphorylated p65 protein. Lane 1. Cultured U266 cells with UCB-MSCs. Lane 2. Cultured U266 cells in C.M. Lane 3. Control group. (b) p65 protein evaluation in U266 cell line. Lane 4. Cultured U266 cells with UCB-MSCs. Lane 5. Cultured U266 cells in C.M. Lane 6. Control group. UCB-MSCs, Umbilical Cord Blood-Mesenchymal stem cells; C.M, conditioned medium. (\*P<0.05, using student's t-test)

**UCB-MSCs up-regulated IL-8, IL-6, CXCL1 and CCL4 in U266 cells**

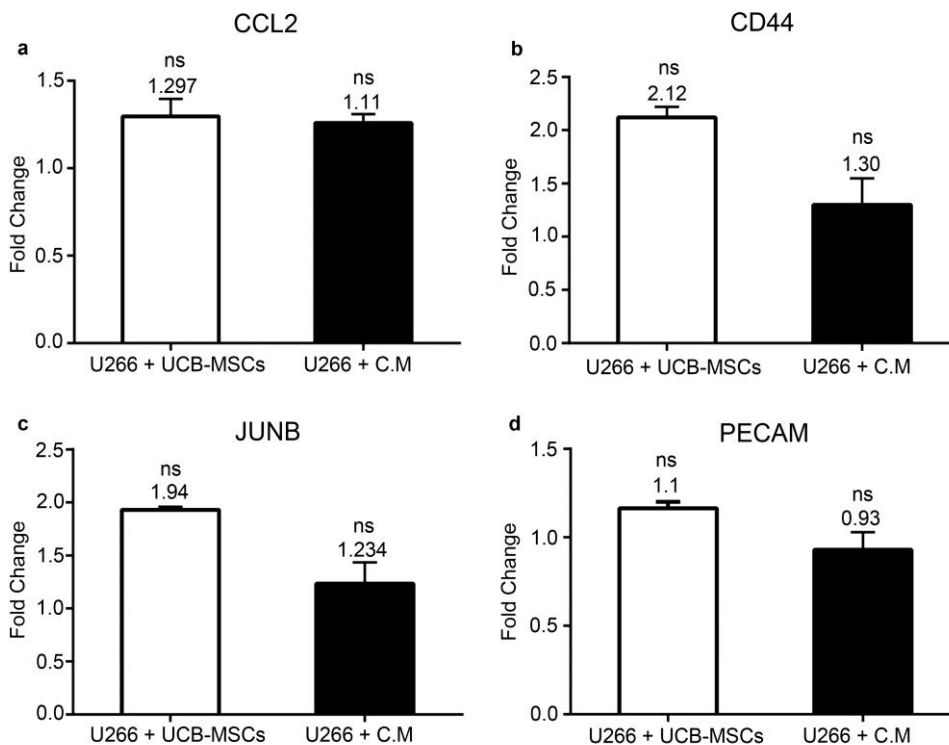
RT-PCR was used to analyze the expression rates of target genes of NF-κβ signaling pathway. Although, the expression of 8 genes including CCL2, CCL4, CD44, IL-6, IL-8, JUNB, PCAM1, and CXCL1 was assessed. To achieve this purpose, 2 groups in triplicate were

compared. After that, U266 cells were cultured with UCB-MSCs for 48 hrs and RT-PCR was performed.

Changes in the mean amounts of IL-8(5.32±0.053), IL-6(8.75±0.027), CXCL1 (4.014±0.46) and CCL4 (7.414±0.033) expression was depended on the presence of UCB-MSCs. Further, C.M didn't show meaningful changes in IL-8 (3.862±0.087), CCL4 (3.812±0.042) and CXCL1 (3.212±0.075) in comparison with the control group (p<0.05, n=3, Figure 4). In this study, other markers were analyzed in the presence of UCB-MSCs and C.M [In presence of UCB-MSCs: PECAM (1.1±0.048), JUNB (1.94±0.036), CD44 (2.12±0.242), and CCL2 (1.297±0.076). In presence of C.M: PECAM (0.93±0.05), JUNB (1.234±0.014), CD44 (1.3±0.392), and CCL2 (1.11±0.092)] and according to our finding UCB-MSCs and C.M hadn't any meaningful effects on expression rates of PECAM, JUNB, CD44 and CCL2, when compared with the control group (p<0.05, n=3, Figure 5).



**Figure 4.** Expression of CCL4, IL-6, CXCL1 and IL-8 by U266 cell line were analyzed by using real time-PCR technique during co-culture with UCB-MSCs and C.M after 48 hrs incubation. **(a)** CCL4 gene expression, **(b)** IL-6 gene expression, **(c)** CXCL1 gene expression, **(d)** IL-8 gene expression. U266 cells in RPMI-1640 used as a control. UCB-MSCs, Umbilical Cord Blood-Mesenchymal stem cells; C.M, conditioned medium. (\*P<0.05, using student's t-test).



**Figure 5.** Diagrams shows expression of CCL2, CD44, JUNB and PECAM on U266 cell lines by using real time-PCR technique in co-culture with UCB-MSCs and C.M after 48 hours incubation. **(a)** CCL2 gene expression, **(b)** CD44 gene expression, **(c)** JUNB gene expression, **(d)** PECAM gene expression. U266 cells in RPMI-1640 used as a control. UCB-MSCs, Umbilical Cord Blood-Mesenchymal stem cells; C.M, conditioned medium. (\*P<0.05, using student's t-test).

## Discussion

It has been shown that the bone marrow (BM) microenvironment influences the myeloma cells.<sup>22</sup> Also, myeloma cells have been connected to stromal cells and endothelial cells within the BM through direct cell-to-cell contacts or indirectly, through soluble factors.<sup>23-25</sup>

Importantly, in numerous researches, the survival and proliferation of malignant plasma cells rely on their interactions with nonmalignant stromal cells, in particular MSCs, in the BM microenvironment.

On the other hand, previous studies have confirmed that MSCs produce soluble autocrine and paracrine factors such as IL-6, IGF-1, SDF-1 $\alpha$ , and CXCL12, and have been shown to play an important role in the proliferation and survival of malignant cells.<sup>26</sup> Reagan et al. reported that malignant plasma cells induced IL-6 production in MSCs. As previously reported, IL-6 is one of the main growth and survival factors for MM cells, which can activate some signaling pathways; for instance, MAPK and the (PI-3K)/Akt signaling cascades mediate IL-6-induced MM cell growth and survival.<sup>27-32</sup>

Most recently, some studies have shown that NF- $\kappa$ B activation in the regulation of IL-6 transcription is produced in the BMSCs after MM cell adhesion. Furthermore, stromal cells can induce some anti-apoptotic pathways.<sup>33</sup> Several studies suggest that, the proliferation rate of MM cell lines in the presence of MSCs was promoted despite the control group.<sup>34</sup> Consist with this notion, the findings of the present study showed the supportive role of MSCs on U266 cells proliferation. CD54 (ICAM-1) is a surface antigen of plasma cells that is in close relation with NF- $\kappa$ B signaling pathway and it makes difference between normal plasma cells and mature myeloma cells.<sup>35</sup> Our previous data showed that the activation of NF- $\kappa$ B, increase the expression of CD54 in myeloma cells. Furthermore, P53 activates CD54 expression in an NF- $\kappa$ B -independent manner.<sup>36</sup> In this study, flow cytometry data showed that the expression of CD54 on U266 surface increased after the cells were co-cultured with MSCs. NF- $\kappa$ B signaling cascade lead to a change in the expression of target genes, in which expression of CD54 was closely correlated with the activity of this pathway. Also, this study demonstrated that up-regulation of CD54 is parallel to NF- $\kappa$ B activation in myeloma cells were co-cultured with MSC cells.

Different studies have shown that human bone marrow derived mesenchymal stem cells regulate leukocyte-endothelial interactions and activate the NF- $\kappa$ B transcription factor.<sup>37</sup> In addition, several studies have shown that the NF- $\kappa$ B pathway is vital to increase the survival and proliferation of normal plasma cells, MGUS and also obvious myeloma cells, can be inhibited by NF- $\kappa$ B restriction drugs such as bortezomib that target this pathway. Mitsiades et al. documented that the classical pathway of NF- $\kappa$ B was activated in MM cell lines and NF- $\kappa$ B plays an anti-apoptotic function in MM cell lines through the production of some proteins.<sup>33</sup> Moreover, NF- $\kappa$ B controls a huge number of genes expressions,

which play critical roles in the control of cell main functions such as cell survival, proliferation, apoptosis, adhesion and differentiation. The active NF- $\kappa$ B transcription factor promotes the expression of over 150 target genes.<sup>38-41</sup>

The finding of the western blotting showed that phosphorylated p65 increased through the co-culturing of U266 cells with UCB-MSCs, although amount of p65 in U266 cells and U266 cells on UCB-MSC did not indicate significant change. Studies have documented that IKKB is a crucial component in the phosphorylation of p65 and may ignite the NF- $\kappa$ B pathway activity, modulate the entry of phosphorylated p65 into the nucleus and activate gene transcription.<sup>42,43</sup>

Previous studies have demonstrated the presence of CXCL1 on MM cell lines and malignant plasma cells.<sup>40</sup> Bone marrow stromal cells and endothelial cells from myeloma patients secrete IL-8. Recently, Pellegrino et al. have shown that IL-8 can induce proliferation and play important roles in the chemo taxis of MM cell lines and tumor progression.<sup>1,44</sup> CCL2 was expressed by myeloma and BM stromal cells highly and western blot analysis confirmed that CCL2 promoted growth and survival signaling in macrophages in multiple myeloma patients via PI3K/Akt and ERK MAPK pathways activating.<sup>45</sup> CD44v10 and CD44v6 are effective in power selective homing of myeloma cells in the bone marrow. The level of IL-6 production seems dependend on expression of the CD44v9 isoforms in myeloma cells through cell-to-cell contact, and some isoforms of CD44 were essential to induce growth factors such as IGF-1, FGF, HGF or HB-EGF.<sup>46</sup>

## Conclusion

The findings of this study showed that stromal cells, such as MSCs, have supportive effects on proliferation rate of U266 cells. In addition, this study analyzed the molecular mechanisms of the supportive roles of MSCs on U266 cells through NF- $\kappa$ B signaling pathway activity analyzing. In line with this, the up-regulation of the CD54 expression, which relates to NF- $\kappa$ B signaling pathway activity, was observed and it could be a sign of NF- $\kappa$ B activation in cells. On the other hand, increasing in amount of phosphorylated p65 protein confirmed NF- $\kappa$ B activation. Furthermore, we demonstrated that MSCs changed the expression of CCL4, IL-6, IL-8, and CXCL1 in U266 cells, noticeably.

Our result confirmed that MSCs paracrine activities in experiments and these observations provide evidences for important roles of MSCs, as a key component of the BM microenvironment, in facilitating the growth of malignant plasma cells in MM. Therefore, future studies are required to examine the effects of MSCs on the activation of NF- $\kappa$ B signaling pathway in *in vivo* samples such as animals or multiple myeloma patients.

## Acknowledgments

Authors would like to thank Tabriz Blood Transfusion Research Center for supporting this project.

**Ethical Issues**

Not applicable.

**Conflict of Interest**

The authors report no conflicts of interest.

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