# The Effects of Hif-1 Gene on the Expression of IL-10, TGF-β and Versican Genes in Human Bone Marrow and Adipose Tissue Mesenchymal Stem

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# Abstract

Mesenchymal stem cells (MSCs) derived from bone marrow and adipose tissues are often hypoxic. These cells are important in tissue repair as well as in the secretion of anti-inflammatory factors and reduction of inflammation. IL-10 and TGF-β are anti-inflammatory cytokines and play an important role in the immune system by modulating inflammation. Versican is a protein that plays an important role in repairing damaged tissues. In this study, the effect of hypoxia on expression of IL-10, TGF-β and versican genes in MSCs was studied in comparison with normoxia. Flow cytometry for surface markers (CD105, CD73 and CD90) was used to confirm MSCs. Desferrioxamine and cobalt chloride (200 μM and 300 μM final concentrations respectively) were added to the cell culture media to induce hypoxia-mimetic conditions. Western Blotting was used to detect HIF-1α protein and confirm the hypoxia-mimetic conditions in MSCs. The expression of VEGF, IL-10, TGF-β and versican genes in MSCs was evaluated using RT-PCR. Microscopic examination and results of flow cytometry confirmed the mesenchymal nature of the cells. Western blotting results also showed that the expression of HIF-1α gene in MSCs increased significantly under hypoxia-mimetic conditions. RT-PCR analyses showed that the expression of VEGF, IL-10, TGF-β and versican genes in hypoxic conditions increased significantly compared to normoxia in both mesenchymal cells derived from bone marrow and from adipose tissues. The results of this study showed that MSCs in hypoxia can repair tissue and inhibit inflammation by expressing anti-inflammatory cytokines and versican restorative factor.


# Introduction

Mesenchymal stem cells (MSCs) are present in various tissues such as bone marrow, umbilical cord blood, adipose tissue, amniotic fluid, etc. (Herzog, Chai, & Krause, 2003; Prockop, Gregory, & Spees, 2003). These cells are similar to fibroblasts and have the ability to adhere to plastic during cultivation (Friedenstein, Chailakhyan, & Gerasimov, 1987). Mesenchymal stem cells are able to differentiate to mesenchymal and non-mesenchymal cell types (Reyes *et al.*, 2001). Because of the unique potential for tissue regeneration, these cells are used in regeneration and repair of damaged tissues (Shi, Liu, & Wang, 2011).

IL-10 is a potent anti-inflammatory cytokine that plays an important and often fundamental role in preventing autoimmune disease and inflammation (Sabat *et al.*, 2010). IL-10 plays an important role in mediating the host's anti-inflammatory response. Identifying the cells produce IL-10 cellular as well as molecular mechanisms regulating IL-10 expression are essential to developing treatment strategies (Iyer & Cheng, 2012). TGF-β is cytokine with strong regulatory and anti-inflammatory activity (Li & Flavell, 2008). TGF-β is produced by many immune system and non-immune system cells, and almost all cell types respond to this polytropic cytokine (Li, Wan, Sanjabi, Robertson, & Flavell, 2006). TGF-β is helpful in the treatment of many autoimmune and inflammatory conditions, in cancer, and also in Alzheimer's disease (Sanjabi, Zenewicz, Kamanaka, & Flavell, 2009). Versican is a versatile molecule that plays an important role in cell-matrix interactions during adhesion, migration, and inflammatory responses.

Versican has been shown to be expressed by fibroblasts and is involved in tissue regeneration and reduction of inflammation (Andersson-Sjöland *et al.*, 2015).

By activating HIF-1, hypoxia initiates a wide range of responses in mesenchymal stem cells. Activation of this transcription factor alters the gene expression profile in MSC through a series of complex signals (Ejtehadifar *et al.*, 2015). Hypoxia is also a strong physiological and pathological stimulus that stimulates angiogenesis (Pastukh *et al.*, 2015). Forsythe *et al.* in 1996 showed that transcription of the VEGF gene under hypoxia in vascular endothelial cells increases due to the persistence of HIF-1α (Forsythe *et al.*, 1996), so the VEGF gene is used to confirm hypoxia in MSCs. Given the anti-inflammatory and therapeutic role of IL-10 and TGF-β and tissue repair of versican, in this study the effect of the hypoxia mimetics desferrioxamine and cobalt chloride, which induce HIF-1 (REFS), on the regulation of IL-10, TGF-β and versican genes in mesenchymal cells was investigated.

## Material And Methods

# Materials

FBS, αMEM, DMEM, Penicillin-Streptomycin, Trypsin/EDTA solution, PBS and FICOLL were purchased from GIBCO, Germany. Desferrioxamine and DMSO were obtained from Sigma-Aldrich, USA. Cobalt chloride was purchased from Merck, Germany.

# Cell culture

Bone marrow and adipose tissue mesenchymal stem cells were purchased from the ROYAN stem cell bank, Iran, Tehran. Cells were grown in 75cm$^2$ plastic flasks in αMEM medium supplemented with 10% foetal bovine serum, and 100 units/mL of penicillin and 100 µg/mL of streptomycin, and incubated in a $CO_2$ incubator (37°C, 5% $CO_2$ and humidified atmosphere). Cells were passaged at 80% confluence after 5-min treatment with Trypsin/EDTA solution at 37°C, and centrifugation at 400 g for 10 min. Old medium was exchanged with fresh medium and old medium was centrifuged at 400 g for 5 min at 37°C and stored at -70°C.

# Immunophenotype analysis

Bone marrow and adipose tissue mesenchymal stem cells were trypsinized and suspended in αMEM at a concentration of 2×10$^6$cells/ml, then 100µl were incubated for 60 min at 4°C with mouse anti-human monoclonal antibodies against CD73, CD90, CD105, and CD34 (as a control negative), all conjugated with fluorescein isothiocyanate (FITC). The cells were washed twice with PBS and then resuspended and analyzed by flow cytometry (FACS-Calibur, USA). The data were analyzed using FlowJo software.

# Hypoxia-mimetic treatment

MSCs were treated with $CoCl_2$ (300 µM) or deferoxamine (DFO; 200 µM) (which mimic hypoxia by inducing HIF-1 in the normoxic environment REFS), for 24 hours. You really need to make it clear for each

figure EWHICH mimetic was used. WHY were both mimetics used? Were they used at random? Why not just use DFO as you know this induces all the genes including versican? Your own PLOS ONE paper showed that $CoCl_2$ does NOT induce versican. You should reference this paper.

# Evaluation of HIF-1α expression using western blot analysis

Total cellular proteins were isolated from MSCs and 25 or 50µg total protein from cell lysates was loaded onto each lane and the proteins were separated by SDS-PAGE. The resolved proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk powder (Anchor, Kowloon, Hong Kong) in PBS-Tween (PBS containing 0.1% Tween-20) for 1 hour and probed for 1 hour with a HIF-1α monoclonal antibody (Abcam, 1:1000), or anti-β–actin (Millipore; 1:10,000) antibody in PBS-Tween. The membranes were washed twice with PBS-Tween and mouse HRP-conjugated secondary antibody (Thermo Fisher Scientific, USA; 1:100,000 in PBS-Tween) was added and washed again. Then 300µl substrate containing 150µl Supersignal West Femto luminal /enhancer solution (Thermo Fisher Scientific, USA) and 150µl Supersignal West Femto stable peroxide buffer was added until the bands appeared. The β -actin band was used as control and band densities were analyzed using Image-J software.

# RNA isolation and cDNA synthesis

Total cellular RNA was prepared by a single-step method using the Trizol reagent kit (Life Technologies). The RNA extracted from peripheral blood mononuclear cells was resuspended in DEPC (diethyl pyrocarbonate) treated water and quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific, USA). To synthesize the cDNA, 4µl (1 µg) of the extracted RNA was mixed with 1 µl of a random hexamer primer (50 µM) and the volume of the reaction was adjusted to 13.5 µl using $ddH_2O$. The reaction was incubated at 70°C for 5 min. Then, 5 µl of 5x buffer, 1 µl of dNTPs (10 mM each), 0.5 µl RNasin (40U/µl) and 1 µl of M-MLV enzyme (200U/µl) (Promega, USA) (final volume 20µl) were mixed in a microtube. The reaction was then incubated at 42°C for 30 min and then terminated by incubation for 5 min at 70°C.

# Quantitative real-time PCR

Quanti□cation of *VEGF* and *Versican* mRNA was performed by quantitative qRT-PCR. PCR reactions were performed by combining 2 µl of cDNA with 6 µl of $H_2O$, 10 µl SYBR Green master mix (Takara, Korea), and 1 µl (10pM) of sense and antisense primers (Sina Clone) (table 1) for *Versican* and *VEGF* cDNAs. The PCR reaction involved an initial denaturation at 95°C for 10 min, followed by 40 cycles of annealing for 20 sec at 60°C, extension for 20 sec at 72°C, and denaturation for 20 sec at 95°C, with a final 20-min extension at 72°C. β-2M (β-2 microglobulin) gene was selected as the housekeeping gene (Staples *et al.*, 2011).

# Statistical analysis

Data are generated as mean±SEM of at least three independent experiments. Data were analyzed by t-test (GraphPad Prism 8 software, San Diego, USA) to assess differences among the groups.

## Results

# Immunophenotype analysis

In this study, the phenotype of human mesenchymal cells isolated from bone marrow and adipose tissue was confirmed by examining the expression of CD105, CD73 and CD90 markers and lack of expression of CD34 marker. The results of flow cytometry showed that the expression of CD105, CD90, CD73 and CD73 markers in human mesenchymal cells isolated from bone marrow tissue was 88.7%, 99.2% and 98.5%, respectively. In adipose tissue-derived human mesenchymal cells, the expression of these markers were 78.8%, 76.8% and 50.8% respectively. The expression of the hematopoietic marker CD34 (negative control) for mesenchymal stem cells was measured as 0.11%.

# Evaluation of HIF-1α expression

The results (figure 3) show that the levels of HIF-1α protein in hypoxia-mimetic conditions in mesenchymal cells isolated from bone marrow and adipose tissues increased significantly as compared to normoxia. As shown in Fig. 3, a HIF-1α protein band (130kDa ) appeared under hypoxia-mimetic conditions.

# Determination of the effects of hypoxia-mimetic conditions versus normoxia on IL-10 mRNA expression

Results showed that the expression of IL-10 mRNA significantly increased in hypoxia-mimetic conditions compared to normoxia and the expression of this gene in adipose MSCs was significantly higher than bone marrow MSCs($p<0.05$).

# Determination of the effects of hypoxia-mimetic conditions versus normoxia on TGF-b mRNA expression

The results of figure 5 showed that the expression of the TGF-β gene was significantly increased in hypoxia-mimetic conditions ($p<0.001$) but the expression of this gene was higher in bone marrow MSCs as compared by adipose MSCs.

# Expression of versican gene in hypoxia-mimetic and normoxia using Real Time PCR

The same results was observed about versican gene and results showed that in bone marrow MSCs, the expression of this gene was significantly higher than adipose tissue MScs ($p<0.01$).

# Expression of VEGF mRNA in hypoxia and normoxia using Real Time PCR

VEGF was used as positive control and the results showed that the expression of this gene was increased significantly in hypoxia-mimetic conditions and the expression of this gene was higher in bone marrow

MSCs as compared by adipose MSCs ($p<0.001$).

# Discussion

Adult mesenchymal cells are multipotent cells and can be differentiated into different types of specialized mesenchymal cells such as osteoblasts, chondrocytes, adipocytes, and other cells (Caplan, 1991). Mesenchymal stem cells have been shown to be actively involved in tissue repair and transplantation. These cells perform this role through their potential to regulate immune cells (Aggarwal & Pittenger, 2005). Another important feature of mesenchymal cells is their ability to inhibit immune responses by inhibiting dendritic cells, and B and T lymphocytes (Jiang *et al.*, 2005). This has made mesenchymal stem cells attractive candidates for tissue repair in medicine (S.-H. Yang *et al.*, 2009). Some studies have shown the role of MSCs in the treatment of certain types of diseases in both *in vivo* and *in vitro* conditions. For example, Lei Chen *et al.* in 2014 referred to the role of mesenchymal cells in hypoxic conditions for wound healing and stated that the factors secreted by mesenchymal stem cells, especially VEGF-A and bFGF, could play an effective role in wound healing (Chen *et al.*, 2014a).

One of the most common elements of tissue injury is the presence of hypoxia. It is known that reduction in oxygen tension in a variety of tissues leads to increased levels of the hypoxia inducible factor (HIF-1), which induces transcription of a wide variety of genes including angiogenic genes such as vascular endothelial growth factor (VEGF) (Ahluwalia & S Tarnawski, 2012; Berniakovich & Giorgio, 2013), as well as the MSC chemoattractant stromal cell-derived factor 1 (SDF-1) (Youn *et al.*, 2011).

HIF-1 is a transcription factor that is present in almost all cell types and is regulated by oxygen levels, causing hundreds of genes to be up-regulated. HIF-1 is a heterodimer and consists of α and β subunits. HIF-1β constitutively expressed in both normoxia and hypoxia, whereas HIF-1α is present in very small amounts under normoxia. HIF-1 acts by binding to regulatory elements called Hypoxia Responsive Elements (HREs) within target genes. To do this, HIF-1α must first dimerise with HIF-1β. When oxygen levels are high, levels of HIF-1α are limiting as it is degraded in a process involving oxygen-dependent hydroxylation and subsequent ubiquitination by the Von Hippel-Lindau protein (VHL) and therefore cannot bind to HIF-1β (Lofstedt & Nilsson, 2006). The results of the present study showed the HIF-1α protein was abundant in MSC in hypoxia-mimetic conditions, but was present at very low levels in normoxia. Research by Ying-Wei Lan *et al.* has shown similar results (Lan *et al.*, 2015) .

Several groups have demonstrated the relevance of hypoxia to MSC growth factor production *in vitro*. For example, exposure of bone marrow (BM)-MSC to 24 hours of hypoxia (1% oxygen) resulted in marked induction of VEGF, Fibroblast growth factor 2 (FGF-2), Hepatocyte growth factor (HGF), and Insulin like growth factor 1 (IGF-1) production, in an NF-kappa β dependent manner (Crisostomo *et al.*, 2008). The stimulation of growth factor production by hypoxia is not specific to BM-MSC and has also been demonstrated in MSC derived from adipose tissue (Rasmussen *et al.*, 2011), placenta (Yust-Katz *et al.*, 2012), and dental pulp (Iida *et al.*, 2010). Furthermore, hypoxic stimulation of angiogenic and anti-

apoptotic factors such as VEGF, FGF-2, HGF and IGF-1 has been reported to also occur in MSC from aged animals, supporting clinical utility (Efimenko, Starostina, Kalinina, & Stolzing, 2011).

The results of the present study showed that the expression of IL-10, TGF-β, VEGF and versican genes in hypoxia-mimetic conditions was significantly higher than normoxia. Our study also found that the fold change of IL-10 in mesenchymal cells derived from adipose tissue was significantly higher than in bone marrow, but the fold change of TGF-β, VEGF and versican in bone marrow MSCs was significantly higher. Nasef *et al.* have shown that the production and secretion of IL-10 by mesenchymal stem cells can inhibit the proliferation and activity of T lymphocyte cells (Nasef *et al.*, 2006). In 2017, Volchenkov *et al.* showed that Th17 cells increase the expression of IL-10 gene under hypoxia (Volchenkov, Nygaard, Sener, & Skålhegg, 2017). In 2014, Lei Chen *et al.* reported that the production of anti-inflammatory factors such as IL-10 and TGF-β in MSCs under hypoxia was significantly higher than innormoxia, and these factors could activate regulatory T cells (Treg), which leads to a reduction of inflammation and cytotoxic T lymphocyte activity (Chen *et al.*, 2014b). Rehman J *et al.* in 2004 showed that adipose tissue-derived mesenchymal cells expressed factors such as HGF, VEGF, TGF-β, fibroblast growth factor (bFGF and FGF2) and macrophage-granulocyte colony stimulating factor (GM-CSF) and the expression of these molecules increases under hypoxia. In particular, the increase in VEGF expression in hypoxia is higher than other factors (Rehman *et al.*, 2004). Studies conducted by Tischer *et al.* in 1991 showed that the VEGF promoter has a binding site for the HIF-1 transcription factor (Wang, Jiang, Rue, & Semenza, 1995). Forsythe *et al.* showed that transcription of the VEGF gene under hypoxia was increased due to the stabilization of HIF-1α (Forsythe *et al.*, 1996). The VEGF gene is one of the genes regulated by the HIF-1 transcription factor. Interestingly, various studies have shown that VEGF can increase the expression of the TGF-β gene in a variety of cells (Shih & Claffey, 2001).

We also investigated the regulation of versican in MSCs by hypoxia-mimetic conditions which induce HIF-1. Versican is an extracellular matrix proteoglycan and is one of the genes that respond to hypoxia (Sotoodehnejadnematalahi *et al.*, 2015). Research showed that versican can play a role in the repair of various tissues, including lung, skin ,and other tissues (Andersson-Sjöland *et al.*, 2014; W. Yang & Yee, 2014). Several reports highlighted the role of versican in wound healing (Theocharis, 2002) and in vascular disease, especially atherosclerosis (Rahmani, McDonald, Wong, & McManus, 2004; Seidelmann *et al.*, 2008). Versican binds low-density lipoprotein particles, and accumulation of versican in blood vessel walls is believed to promote extracellular lipoprotein retention and uptake leading to foam cell formation (Wight & Merrilees, 2004). In one study hypoxic induction of Versican was reported and suggested to be regulated, at least in part, by HIF-1 (Asplund *et al.*, 2010).

# Conclusion

In general, the results showed that in bone marrow and adipose tissue stem cells, the expression of IL-10, TGF-β, VEGF and versican genes in hypoxia-mimetic conditions increased significantly as compared to normoxia and in bone marrow MSCs, the expression of TGF-β and versican was higher than adipose tissue MSCs, while the fold change of IL-10 in adipose tissue MSCs was significantly higher than bone

marrow MSCs. Based on the above results, it is recommended to use both cells to reduce inflammatory factors.

# Declarations

**Ethical approval:** This article does not contain any studies with animals performed by any of the authors.

**Conflict of Interest:** Authors declares that they have no conflict of interest.

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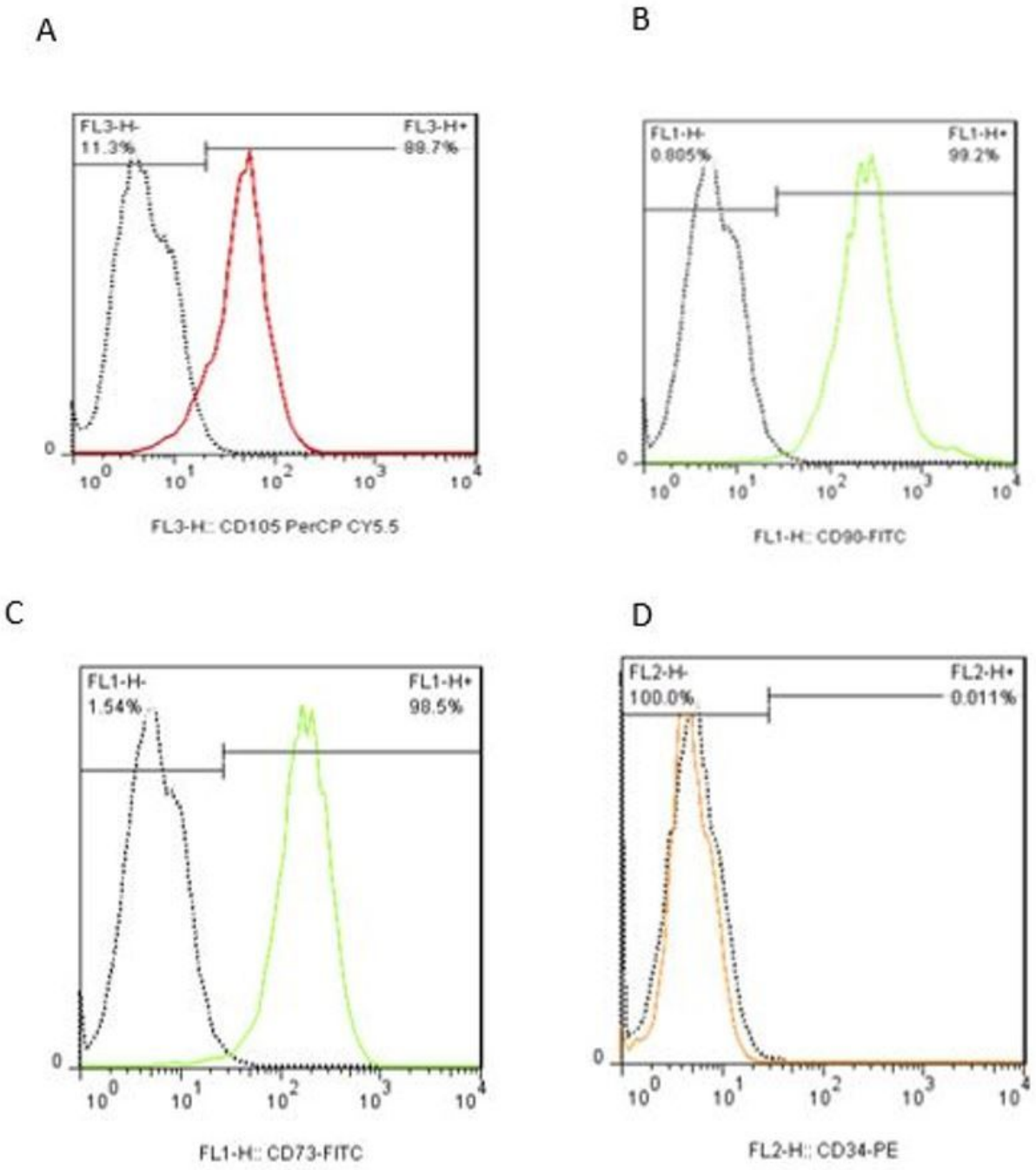
# Tables

Table1. List of primer pairs used.

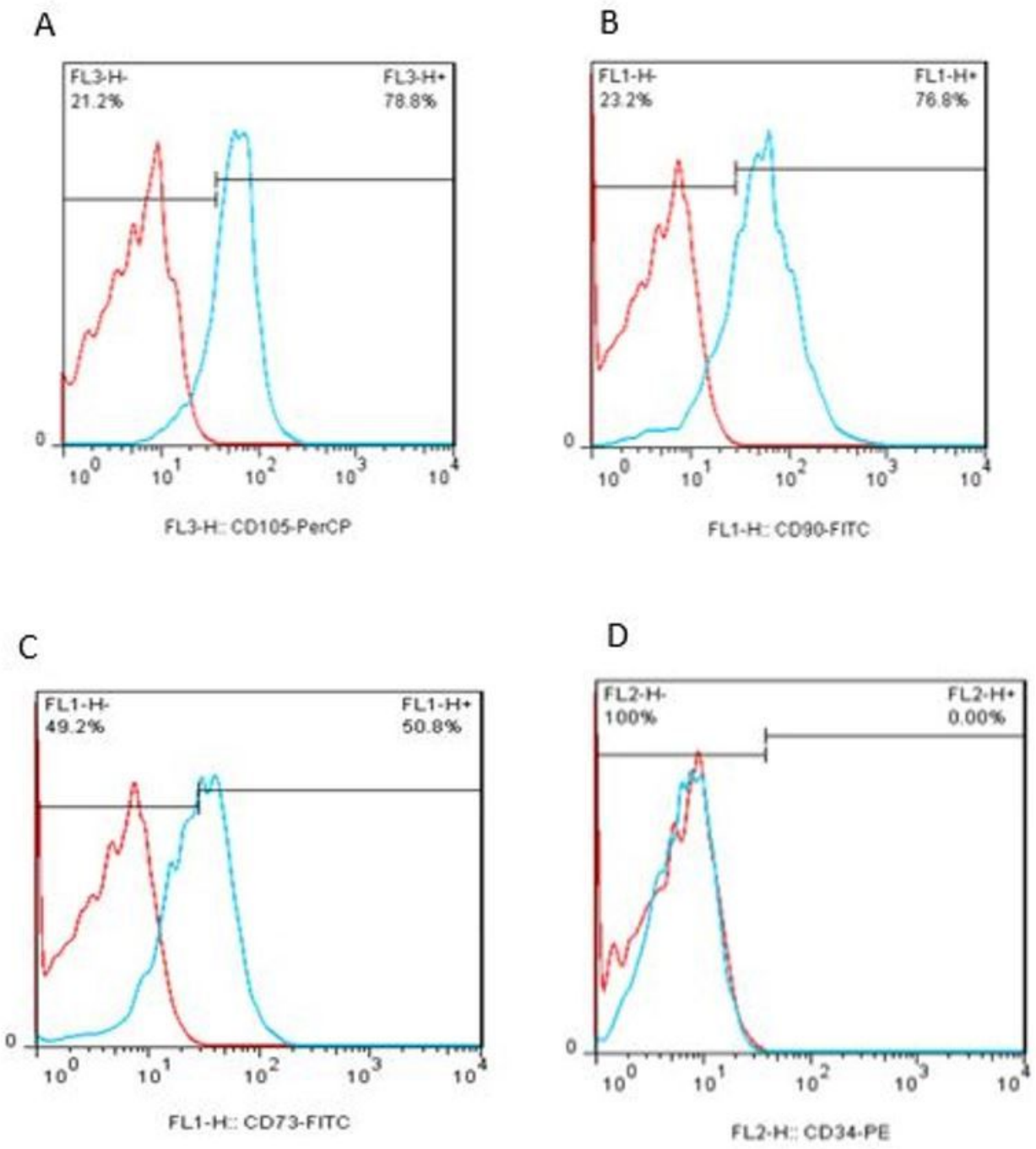
| gene | primer | Sequence |
|---|---|---|
| *β2M* | 5’ Forward | 5’- GACTGTCTTTCAGCAAGGA-3 |
| | 3 ‘reverse | 5’-ACAAAGTCACATGGTTCACA-3’ |
| *VEGF* | 5’ Forward | 5’-CTGTGGCGTGTTCTCTGCT-3’ |
| | 3 ’reverse | 5’-CTCCAGATCTTTGCTTGCAT-3’ |
| *IL-10* | 5’ Forward | 5’-CTTTAAGGGTTACCTGGGTTGC-3’ |
| | 3 ’reverse | 5’-CTCACTCATGGCTTTGTAGACAC-3’ |
| *TGF-β* | 5’ Forward | 5’-AACATGATCGTGCGCTGCAAGTGCAGC -3’ |
| | 3 ’reverse | 5’-AGGACGGACAGACGTGATAAGGAA-3’ |
| *Versican* | 5’ Forward | 5’-ACAAGCATCCTGTCTCACG-3’ |
| | 3 ’reverse | 5’-TGAAACCATCTTTGCAGTGG-3’ |

# Figures



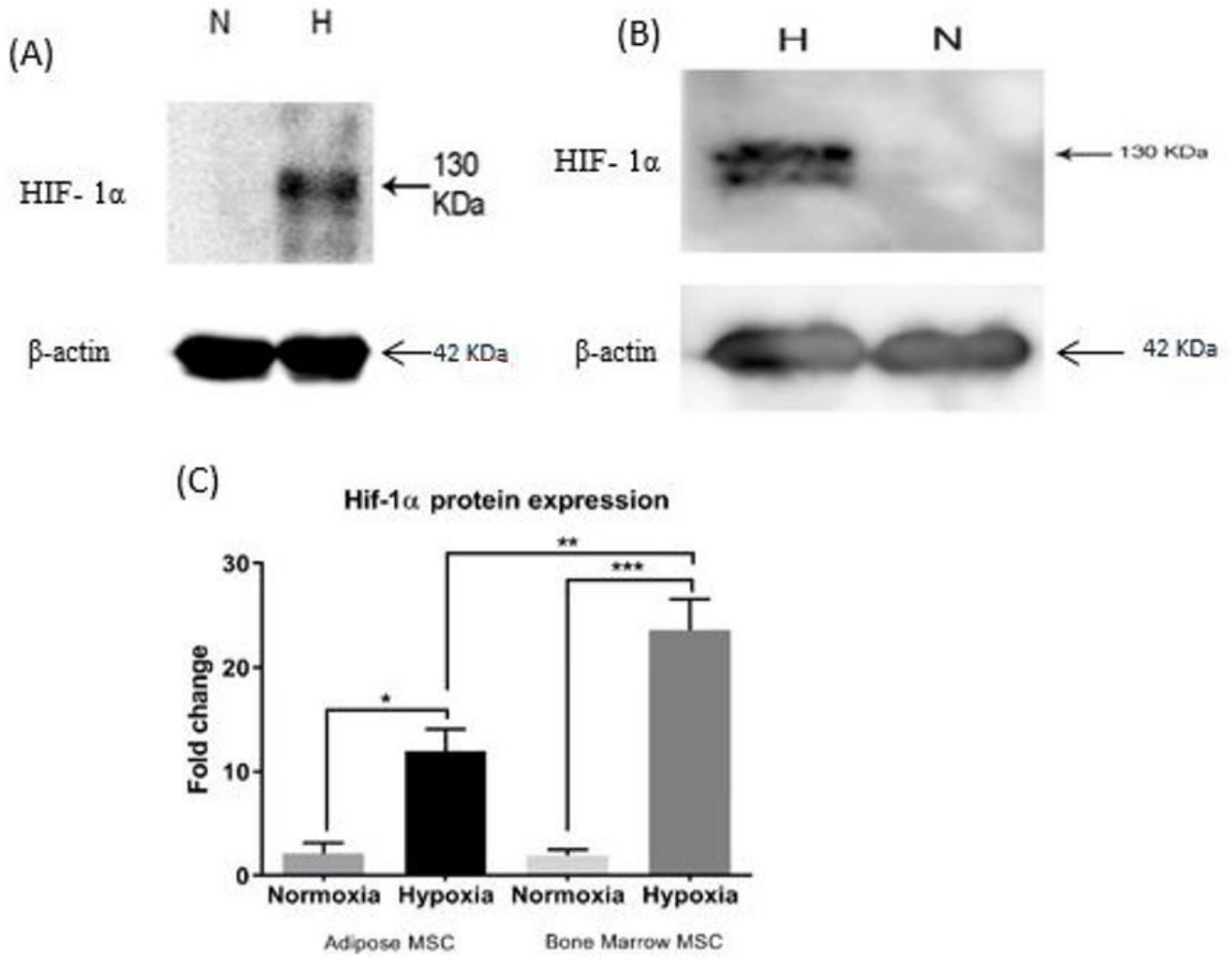

## Figure 1

Examination of bone marrow mesenchymal cell phenotypes using flow cytometry. The expression markers of CD105, CD90, CD73 are 88.7% (A), 99.2% (B) and 98.5% (C), respectively. D) The expression of hematopoietic CD34 marker (negative control) for mesenchymal stem cells is 0.011%. The dotted lines represent control-MSCP and the undotted lines represent stained cells.




## Figure 2

Investigation of adipose tissue mesenchymal cell phenotype using flow cytometry method. The expression markers of CD105, CD90, CD73 are 78.8% (A), 76.8% (B) and 50.8% (C), respectively. D) The expression of hematopoietic CD34 marker (negative control) for mesenchymal stem cells is 0.001%. The red graphs represents the control unstained samples and the blue graphs represent the stained test samples.




## Figure 3

Western Blotting of HIF-1α and β-actin proteins under hypoxia-mimetic conditions and normoxia in human mesenchymal stem cells isolated from adipose tissue (A) and bone marrow (B). Results showed that (C) hypoxia-mimetic conditions are well induced in both mesenchymal cells, and a statistically significant difference between hypoxia-mimetic and normoxic conditions is evident in both mesenchymal cells. Also HIF-1α expression levels in bone marrow mesenchymal cells were significantly higher ($P<0.01$) than in adipose derived mesenchymal cells.

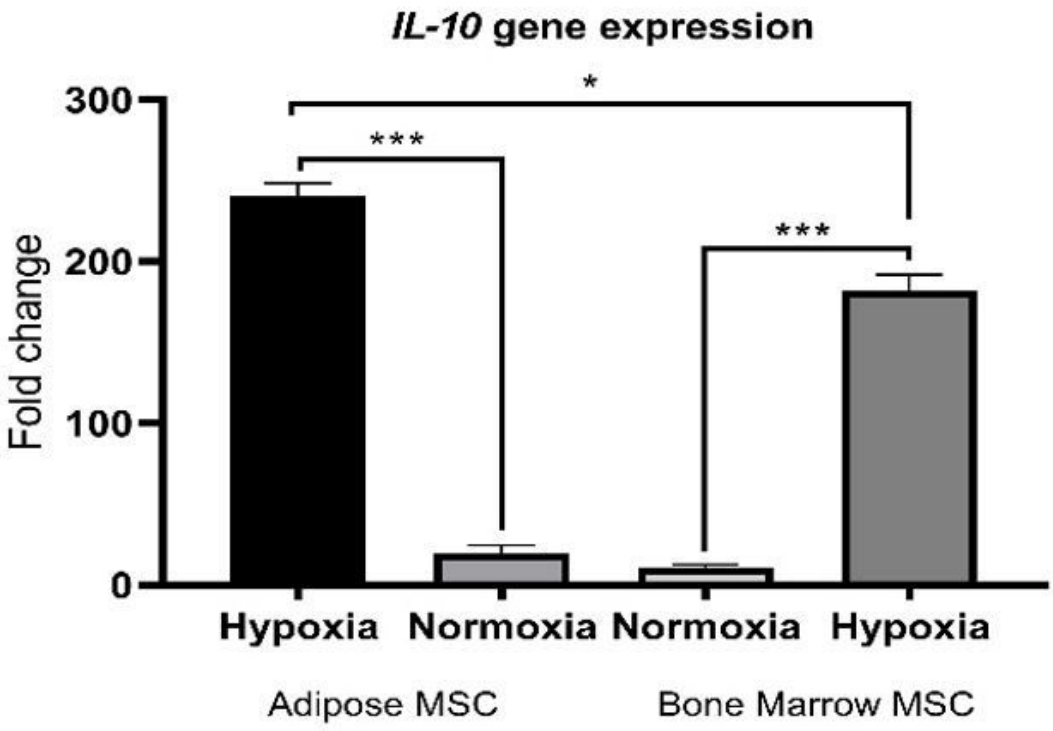



**Figure 4**

Expression of IL-10 mRNA in both hypoxia-mimetic and noromoxic conditions. As shown, the expression of this gene under hypoxia-mimetic conditions in both bone marrow and adipose mesenchymal cells increased significantly as compared to normoxia.

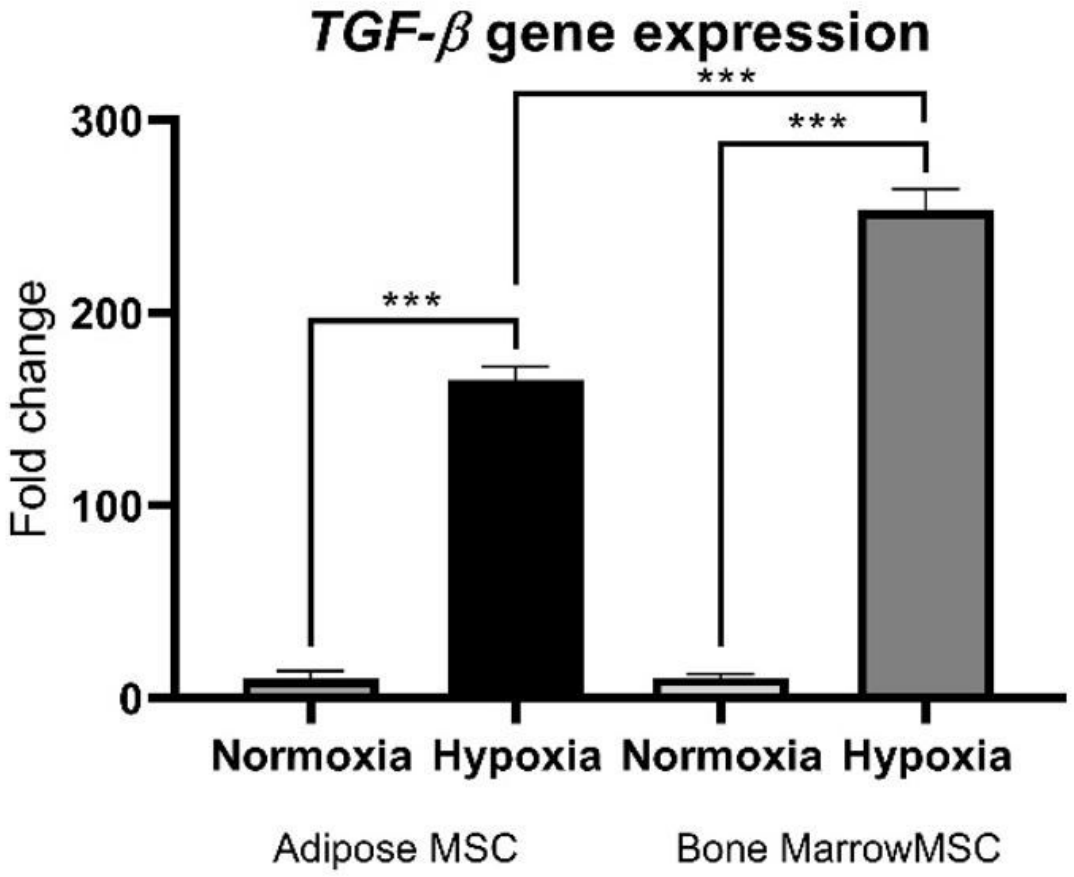



**Figure 5**

Expression of TGF-β gene in both hypoxia-mimetic and normoxia. The expression of TGF-β gene under hypoxia conditions in both bone marrow and adipose mesenchymal cells increased significantly.

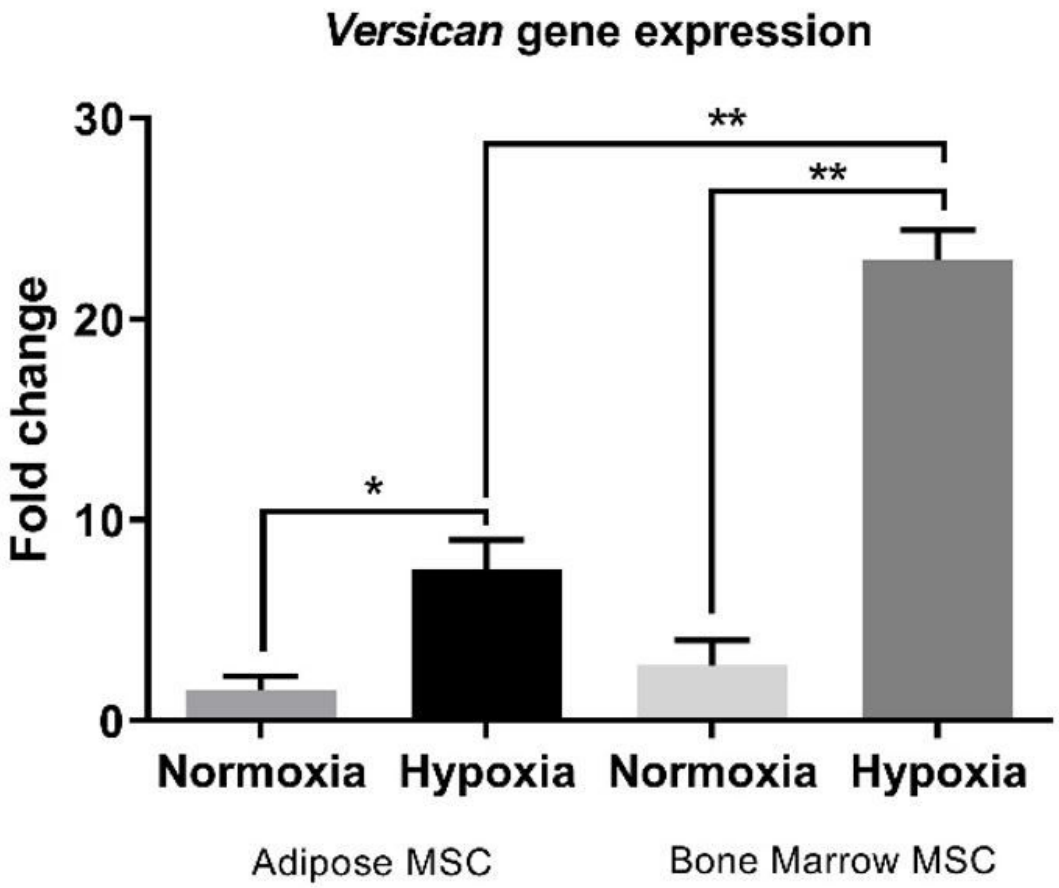
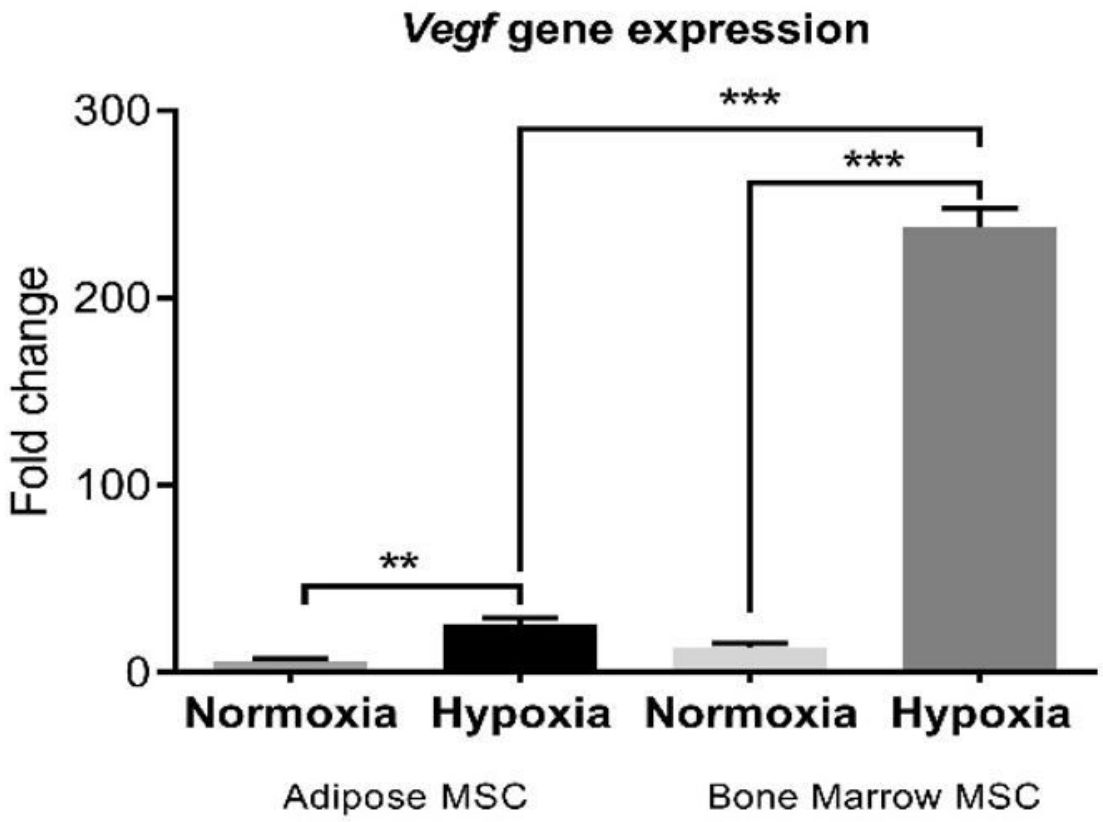



**Figure 6**

Expression of versican gene in both hypoxia-mimetic conditions (200uM DFO) and normoxia. Results showed that the expression of this gene under hypoxia-mimetic conditions in both bone marrow and adipose mesenchymal cells increased significantly.




## Figure 7

Expression of VEGF gene in both hypoxia-mimetic and normoxia. The expression of this gene was increased significantly in hypoxia and the expression of this gene was higher in bone marrow MSCs as compared by adipose MSCs.