Pak. J. Biochem. Mol. Biol., 2013; 46(1): 16-21

Effect of Rap1 GTPase activation and hypoxia on the gene expression pattern of cell adhesion molecules in bone marrow derived Mesenchymal stem cells

*Irfan Khan, Nazia Ahmed, Muhammad Aleem Akhter, Anwar Ali, Kanwal Haneef, Nadia Naeem and Asmat Salim**

Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan

Abstract: Cellular therapy is considered as a better option for the treatment of degenerative disorders. Different cell types are being employed depending upon the nature of damaged tissue. Despite extensive research in this field, there are several issues that are yet to be addressed with regard to cell transplantation. One of these issues is the survival of administered cells in the injured tissue that depends upon the adherent property of these cells. For enhancing the cell adherence and survival, Rap1 GTPase was activated in mesenchymal stem cells (MSCs) by using 8-pCPT-2'-O-Me-cAMP in different concentrations and effect on gene expression was determined through reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Rap1 activation and hypoxia resulted in up regulation of connexin-43 (C-43) and TIMP metallopeptidase inhibitor 3 (TIMP3) cell adhesion genes. Treatment of stem cells prior to transplantation with chemicals that lead to Rap1a activation and hypoxia might help to enhance the regeneration potential of stem cells for cellular regeneration.

Keywords: GTPase activiation, hypoxia, cell adhesion, bone marrow, Mesenchymal stem cells.
Received: September 10, 2012 **Accepted:** December 20, 2012
***Author for Correspondence:** asmat.salim@iccs.edu

INTRODUCTION

Stem cell research is one of the most attractive fields of biological research today. Regenerative medicine is the phenomena of regenerating or replacing the injured tissues or organs so that the normal physiological function of these organs can be restored[1]. During this strategy, damaged tissues can be replaced directly or via the stimulation of various repair processes intrinsic to that tissue. Resident adult stem cells repair the damage either by differentiating into a particular cell phenotype, secreting cytokines and other factors to enhance recovery of indigenous cells or undergoing cell fusion, a process that may ultimately lead to stem cell trans-differentiation[2]. MSCs are advantageous over other stem cell types for several reasons. First, they avoid the ethical issues that surround embryonic stem cell research. Second, they are immuno-privileged, and therefore, represent an advantageous cell type for allogenic transplantation, reducing the risks of rejection and complications of transplantation[3]. Recently, there have also been significant advances in the use of autologous MSCs to regenerate human tissues, including cartilage, tendons, bone and heart[4].

Rap1 is a member of the Ras superfamily of GTPases and is similar in structure to Ras but is different from them in that it is a small GTPase. It was described by Kitayama et al., as Krev-1 which is a 21 kDa protein having anti-oncogenic property[5]. Bourne and colleagues subsequently identified this protein as a Ras related protein[6]. In higher organisms, multiple forms of the Rap protein are present. These forms are coded by a number of genes. Rap1a and 1b (Rap1) share 90% homology with each other. Rap2a, 2b, and 2c (or Rap2) are 65-70% homologous to Rap1[7]. These monomeric proteins cycle between an active GTP and an inactive GDP bound state and act as molecular switches in the signaling pathways pertaining to them[8]. Guanosine exchange factors (GEFs) facilitates the switching with release of GDP and binding of GTP. It has also been established that different subtypes of Rap proteins are not functionally unanimous, and have distinct cellular functions[9,10]. The activated Rap1 is involved in inside-out signaling to integrins, and helps in the formation of focal adhesion, leading to increased cell adhesion[11-13]. Rap1 is primarily localized into the intracellular vesicles; but there is also some evidence of its localization in the cell membrane[14-15].

Exploring the molecular mechanisms that underlie cell signals and cell-to-cell communication may help in increasing the regeneration capability of these stem cells. In this respect, the role of small GTP-binding proteins is also worth investigating. In this study, we have analyzed the effect of six cell adhesion genes after treating MSCs with Rap1a activator and DNP for hypoxia induction. These genes include activated leukocyte cell adhesion molecule (Alcam), intracellular adhesion molecule 1 (Icam1), vascular cell adhesion molecule 1 (Vcam1), tissue inhibitor of metallopeptidase 3 (Timp3), a disintegrin and metalloproteinase with a thrombospondin type 1 motif (Adamts 1) and connexin-43 (C-43).

MATERIALS AND METHODS

Animals

All animal procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals and approval of the local ethical committee. NMRI mice weighing 25-30 grams were allowed for a period of 3-4 days acclimatization prior to the start of the experiment. The animals were housed in groups of five per cage on sawdust bedding and provided with sterile water and food *ad libitum*, in a temperature controlled room (25±1ºC) and humidity (55±5%) with 12 hours light and 12 hours dark cycle and kept in the animal house of PCMD, University of Karachi.

Isolation of Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) were isolated from tibia and femur of NMRI mice. Whole bone marrow was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100µg/mL streptomycin. This medium is referred to as complete medium. Culture was maintained at 37^0C in a humidified atmosphere containing 5% CO_2 in T-75 tissue culture flask. Non-adherent hematopoietic cells were removed from the culture. Medium was changed on every 3rd day. The cells were sub-cultured using 0.25% trypsin when they reached 70-80% confluence. The dissociated cell suspension was centrifuged at 1000rpm for 8 min. The cell pellet was re-suspended in DMEM and distributed evenly to the two new flasks. Passage 1-2 bone marrow MSCs were used in all experiments.

Characterization of cells: Immunocytochemistry

Cultured mesenchymal stem cells were analyzed for the presence of specific surface markers by immuno-staining using antibodies against stem cell growth factor receptor (SCFR), also known as proto-oncogene, c-Kit or CD117, homing associated cell adhesion molecule (CD44), glycosylphosphatidylinositol-anchored glycoprotein, Thy-1 (CD90), and hematopoietic progenitor cell antigen (CD34). Cells were fixed in 4% paraformaldehyde, and blocked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), 2% normal goat serum and 0.1% tween 20 followed by incubation with primary antibodies at 1:200 dilution in blocking solution overnight at 4°C. It was followed by incubation with Alexa fluor 546 goat anti mouse secondary antibody at a dilution of 1:200 for 1 hour at room temperature. The cells were counter-stained with 0.5 µg/ml DAPI (4', 6-diamidino-2-phenylindole) and examined under fluorescent microscope.

Rap1 activation in MSCs

Rap1 was activated with 100ng/ml and 200ng/ml 8-pCPT-2'-O-Me-cAMP for 10 and 20 minutes in complete medium kept at 37ºC in humidified incubator. Cells were washed with PBS to remove traces of activator, and supplemented with fresh complete medium.

Hypoxia induction to MSCs

Prior to induction of hypoxia, MSCs were washed with glucose free DMEM. 2, 4-dinitrophenol (DNP) was used to induce hypoxia chemically. The concentration and time period of DNP treatment was optimized such that the cells remain viable. The optimized dose of 0.25mM was used for 20 min throughout the experiments. Hypoxic medium was replaced with high glucose DMEM. The cells were then incubated for 2 hours at 37°C in a humidified atmosphere containing 5% CO_2 for re-oxygenation.

Gene expression analysis

Total RNA from control (untreated) and DNP treated groups were isolated using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. RNA concentration was measured at 260 nm. One microgram of RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) and amplified using oligonucleotide primers. Some important cell adhesion molecules were selected after review of the literature. Forward and reverse primers were designed accordingly using primer3 online software (v.0.4.0; http://frodo.wi.mit.edu). Details of primers used in this study along with their sequences, expected product sizes and annealing temperatures are listed in Table 1. Rat GAPDH gene was used as standard.

Table 1: Primer sequences, annealing temperatures and product sizes.

Primer	Sequence	Annealing Temperature (°C)	Product size (bps)
Rap1a	ATGCTGGAGATCCTGGACAC TCTTTGCCAACTACCCGTTC	57 ºC	233
Alcam	CTTGCACAGCAGAAAACCAA TAGACGACACCAGCAACGAG	56 ºC	190
Icam1	CCTGTTTCCTGCCTCTGAAG GCTCAGAAGAACCACCTTCG	58 ºC	310
Vcam1	ATTTTCTGGGGCAGGAAGTT ACGTCAGAACAACCGAATCC	58 ºC	238
Timp3	AGATCCCTGAACCCAGACCT CAGAGATCCCCAGTTGGAAA	57 ºC	235
Adampts	GACAGGGGAGGTGGTGTCTA GAATGTGCCCTTCACCACTT	58 ºC	199
C-43	GCGTGAAGGGAAGAAGCGAT GCTAAGGGCTGGAGTTCGTG	54 ºC	355
GAPDH	GGAAAGCTGTGGCGTGATGG GTAGGCCATGAGGTCCACCA	60ºC	414

The products of reverse transcription reactions were denatured for 2 min at 94°C, followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 51 - 62°C for 1 min and extension at 72°C for 1 min) and a final extension of at 72°C for 10 min. Each PCR product was electrophoretically resolved on 1% agarose gel. Flourchem AlphaEaseFC software was used to measure the gel density of the specific primer bands. Density of each band was adjusted relative to GAPDH density in all samples.

RESULTS

Characterization of Mesenchymal stem cells

Immunocytochemical analysis of rat bone marrow MSCs showed that cells were positive for specific surface markers against CD 117, CD 29 and CD90. MSCs did not express hematopoietic marker, CD45 (Figure 1). These result confirmed that a majority of cells in culture contains mesenchymal stem cells.

Hypoxia induction

The optimized concentration of DNP was 0.25nM for 20 min. Immediately after DNP treatment, MSCs experienced shock and were shrunken but regained normal morphology when normal conditions were restored during re-oxygenation for 2 hours.

Rap1 activation

Rap1 activation was confirmed by RT-PCR Expression increased gradually from normal MSCs to activated MSCs. Highest expression was observed in activated and hypoxic MSCs (Figure 2).

Gene expression in normal, activated and hypoxic activated MSCs through RT-PCR

Figure 3 shows the gene expression pattern of cell adhesion molecules (CAMs), extracellular matrix proteins and cell signaling molecule used in this study. These genes were analyzed in normal, activated (100ng and 200ng) and activated hypoxic MSCs after 2 hours re-oxygenation. Rap1a expression was increased gradually when MSCs were activated and in case of hypoxic activated MSCs while Rap1b expression was decreased in all conditions as compared to normal MSCs. Alcam Icam Vcam and Adamts expression was also decreased in all conditions. Timp3 expression was increased when Rap1 was activated with 200ng concentration. C-43 showed increase in expression in hypoxic and activated MSCs.

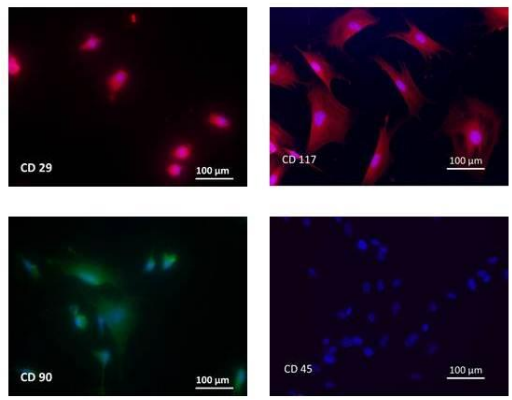

Figure 1: Immunocytochemical analysis of mesenchymal stem cells (MSCs) for the presence of cell surface markers, CD29, CD117, CD90 and CD45. Alexa fluor goat anti mouse 546 or 488 was used as the secondary antibody. Nuclei were stained with DAPI. MSCs were positive for CD29, CD117, CD90 and negative for CD45.

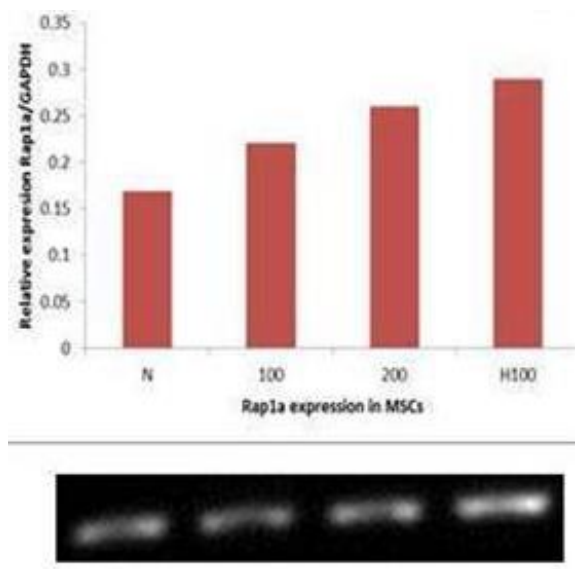

Figure 2: Gene expression level of Rap1a in normal, activated (100 and 200ng) and activated and hypoxic MSCs. Gene expression was analyzed by RT-PCR.

DISCUSSION

We have analyzed the gene expression pattern of some cell adhesion molecules (CAMs) and extracellular matrix degradation enzymes in MSCs. MSCs express cell adhesion molecules in response to various stimuli, which play an important role in different biological phenomena.

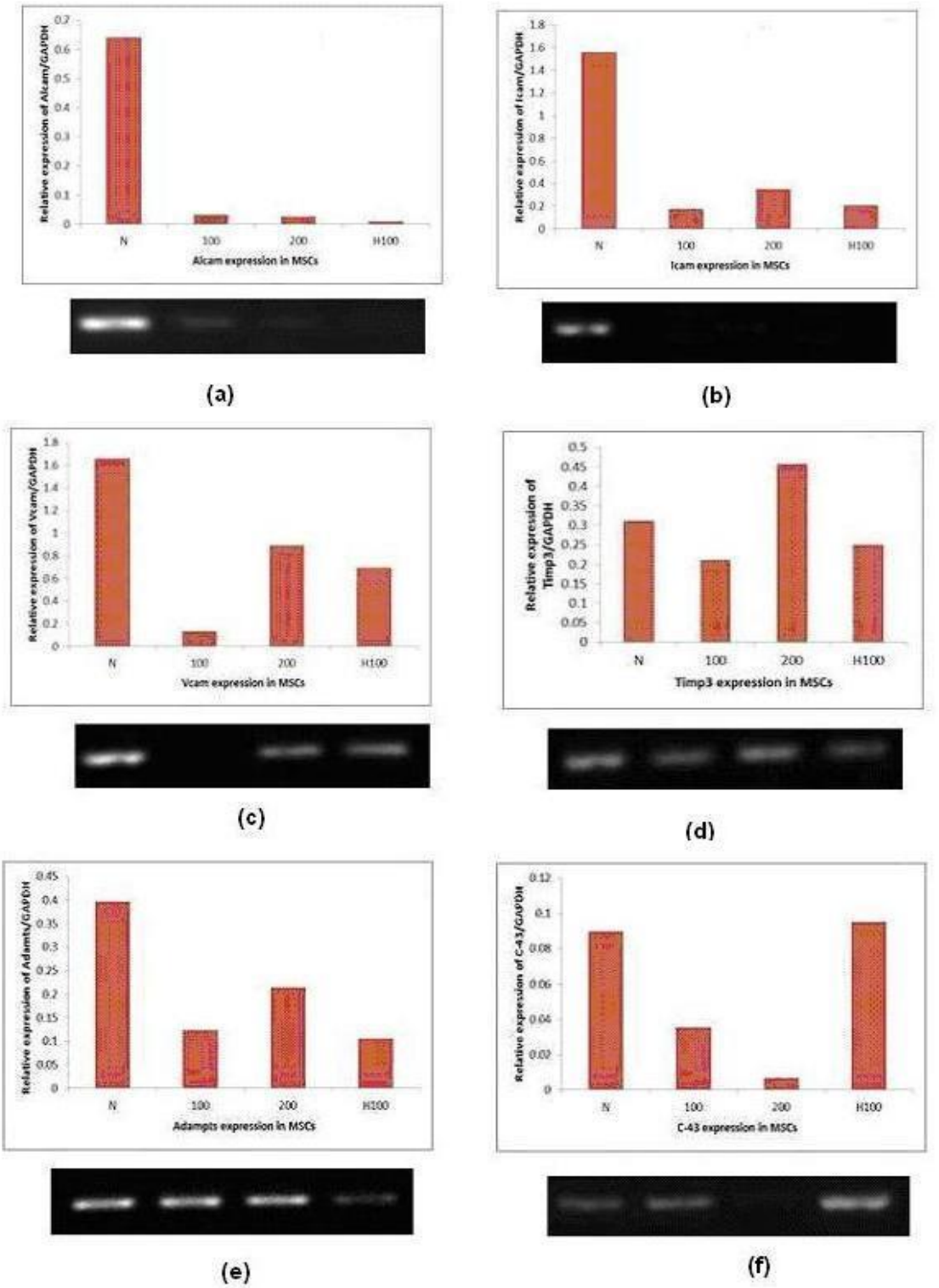

Figure 3: Gene expression levels of (a) Alcam, (b) Icam, (c) Vcam, (d) Timp3, (e) Adamts, and (f) C-43 in normal, activated (100 and 200ng) and activated and hypoxic MSCs. Gene expression was analyzed by RT-PCR.

We have used chemical activation and chemical hypoxia methods to activate Rap1 and induce hypoxia in MSCs. Rap1 activation was done by an activator, EPAC/8-pCPT-2-*O*-Me-cAMP while chemical hypoxia was induced with optimized concentration of 2, 4-dintrophenol[16]. Gene expression profile of normal MSCs, 100ng activated MSCs, 200ng activated MSCs, and 100ng activated and hypoxic MSCs were analyzed by RT-PCR. These genes include Alcam, Icam, Vcam, Timp3, Adamts and C-43.

We have observed that out of six genes studied, Timp3 and C-43 showed increased expression as a result of Rap1 activation alone and both Rap1 activation and hypoxia respectively. Expressions of other cell adhesion molecules, Alcam, Icam, Vcam, as well as Adamts were not increased by either Rap1 activation or hypoxia. It may be possible that higher concentration of Rap1 activator is required for their increased expression or these molecules are not affected by Rap1 at all. Previously, it was reported that activation of Rap1 also plays a role in integrin mediated inside-out signaling, increases cell adhesion and is involved in the formation of focal adhesions, proliferation and cell junction formation[7,11,12]. Rap1 localization in cell is responsible for activation; it is also localized to the plasma membrane[14,15]. In fibroblasts, Rap1 is responsible for the regulation of extracellular matrix[17], cell proliferation[18] and cell migration[19]. In hind limb ischemia, EPAC-Rap1 conditioned EPCs did better homing and increased neovascularization in remodeling tissue[20]. Rap1 also plays an important role in cell junction formation[21]. We found that expression of connexin-43 is not directly related to Rap1 activation but it required the hypoxic condition as well.

Various cell adhesion molecules are expressed at different levels depending on the cellular event they participate into. It remains to be elucidated whether these differences contribute differently to paracrine actions. Taken together, the difference in gene expression profiles under normal, activated and hypoxic and activated conditions, difference in gene expression at various times, could cause their distinctive paracrine effects in terms of cell adhesion, including cell survival. The study should be further extended to delineate the role of cell adhesion molecules at various time points after Rap1 activation and hypoxia induction and thus would aid in designing the therapeutic strategy in which different cell adhesion and cell matrix interaction molecules can be regulated for engraftment of MSCs in diseased tissue or organ.

REFERENCES

1. Mason C and Dunnill PA. Brief definition of regenerative medicine. *Regen. Med.*, 2008; 3:1-5.
2. Cselenyak A, Pankotai E, Horvath EM, Kiss L and Lacza Z. Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections. *BMC Cell Biol*., 2010; 11:29.
3. Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E and Rachmilewitz J. Human mesenchymal stem cells alter antigenpresenting cell maturation and induce T-cell unresponsiveness. *Blood*, 2005; 105: 2214-2219.
4. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A and Drexler H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet*, 2004; 364:141-148
5. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y and Noda M. A ras-related gene with transformation suppressor activity. *Cell*, 1989; 56:77-84.
6. Bourne HR, Sanders DA and McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature*, 1990; 348:125-132.
7. Bos JL, de Rooij J and Reedquist KA. Rap1 signalling: adhering to new models. *Nat. Rev. Mol. Cell. Biol.*, 2001; 2:369-377.
8. Jeyaraj SC, Unger NT and Chotani MA. Rap1 GTPases: An emerging role in the cardiovasculature. *Life Sci.*, 2011; 88:645-652.
9. Li Y, Yan J, De P, Chang HC, Yamauchi A and Christopherson KW. Rap1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins. *J. Immunol*., 2007; 179:8322-8231.
10. Schmidt M, Evellin S, Weernink PA, von Dorp F, Rehmann H and Lomasney JW. A new phospholipase-C–calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat. Cell. Biol.*, 2001; 3:1020-1024.
11. Bos JL. Linking Rap to cell adhesion. *Curr. Opin. Cell. Biol.*, 2005; 17:123-128.
12. McLeod SJ, Shum AJ, Lee RL, Takei F and Gold MR. The Rap GTPases regulate integrinmediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J. Biol. Chem.*, 2004; 279:12009-12019.
13. Rangarajan S, Enserink JM and Kuiperij HB. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J. Cell. Biol.*, 2003; 160:487-493.
14. Bivona TG, Wiener HH, Ahearn IM, Silletti J, Chiu VK and Philips MR. Rap1 up-regulation and activation on plasma membrane regulates T cell adhesion. *J. Cell. Biol*., 2004; 164: 461-470.
15. York RD, Molliver DC, Grewal SS, Stenberg PE, McCleskey EW and Stork PJ. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. *Mol. Cell. Biol.*, 2000; 20:8069-8083.
16. Haneef K, Naeem N, Iqbal H, Jamall S, Kabir N and Salim A. Gene expression pattern in rat bone marrow mesenchymal stem cells in response to hypoxia. *Pak. J. Biochem. Mol. Biol.*, 2011; 43: 90-93.
17. Barnett P and van den Hoff MJ. Rap1 activation in Cardiac regeneration: different cells same goal. *Med. Biol. Eng. Comput.*, 2011; 49:723-732.
18. Hattori M and Minato N. Rap1 GTPase: functions, regulation, and malignancy. *J. Biochem.*, 2003; 134:479-484.

19. Orlic D, Kajstura J and Chimenti S. Bone marrow cells regenerate infarcted myocardium. *Nature*, 2001; 410:701-705.
20. Carmona G, Chavakis E, Koehl U, Zeiher AM and Dimmeler S. Activation of Epac stimulates integrin-dependent homing of progenitor cells. *Blood*, 2008; 111:2640-2646.
21. Duquesnes N, Derangeon M, Métrich M, Lucas A, Mateo P, Li L, Morel E, Lezoualc'h, F. Bertrand and Pflugers C. Epac stimulation induces rapid increases in connexin43 phosphorylation and function without preconditioning effect. *Eur. J. Physiol.*, 2010; 460:731-741.