Expression of miRNA-210 in human bone marrow-derived mesenchymal stromal cells under oxygen deprivation

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Received: November 17, 2018; **Revised:** January 6, 2019; **Accepted:** January 8, 2019; **Published online:** January 14, 2018

Abstract: A major limitation in the development of efficient clinical protocols for mesenchymal stromal cell (MStroC)-based tissue regeneration therapy is the low retention and survival of MStroC in injured tissue after therapeutic administration. Low oxygen concentration preconditioning (LOP) during *ex vivo* cultivation of MStroC, as a method for mimicking oxygenation in their physiological microenvironment, has been shown to be beneficial in clinical trials using MStroC. Introducing hypoxia-mimicking molecules into MStroC during cultivation could be an advantageous LOP strategy. MicroRNA (miRNA) drugs are good candidates for this approach. Analysis of the expression of miRNA-210 in human bone marrow-derived MStroC in conditions of acute and extended hypoxia (24 to 72 h) was performed using RT-qPCR methodology. *HIF-1α* and *HIF-2α* gene knockdown cell lines were generated using lentiviral transduction of short hairpin RNA (shRNA) in order to examine whether miRNA-210 expression is regulated by transcription factor HIF-1 and/or HIF-2. We detected a significant increase in miRNA-210 expression in hypoxic conditions at time points of 24, 48 and 72 h ($p<0.05$). Knocking down of *HIF-1α* and *HIF-2α* genes indicated involvement of both transcription factors in the elevation of miRNA-210 expression. These results point to miRNA-210 as a good candidate for a hypoxia-mimicking molecule in LOP strategy.

Key words: mesenchymal stromal cells; oxygen deprivation; miRNA-210; HIF-1α; HIF-2α

INTRODUCTION

Mesenchymal stromal cells (MStroC) are very attractive for cell-based therapies in regenerative medicine [1,2]. They exert strong antiapoptotic, prosurvival and proangiogenic activities, the ability to modulate the immune response and a capacity to home at site of injury after transplantation [2,5-10]. Inside the MStroC population mesenchymal stem cells (MSC) reside, which are able to self-renew and differentiate in several cell lineages originating from three different embryonic layers [2-4]. Bone marrow is one of the most widely used sources of MStroC for clinical application [2].

A major limitation of the efficient clinical application of MStroC is the poor survival of cells at site of injury after therapeutic administration [4,11-14]. Toma et al. [14] reported that less than 1% of human bone marrow-derived MStroC (hBM MStroC) survived four days after

transplantation into ischemic heart. *Ex vivo* expansion of MStroC at atmospheric oxygen concentrations is not appropriate, since the atmospheric oxygen concentration represents a hyperoxic state. Two to eight percent oxygen concentration is present in the physiological microenvironment of MStroC [15]. In addition, injured tissue exists in a proinflammatory, cytotoxic microenvironment, which is also characterized by poor vascularization, leading to very low or no oxygen and nutrient supply [13,18-20]. Experiments performed at low oxygen concentration preconditioning (LOP) of MStroC revealed an increase in their therapeutic potential when applied in cardiac ischemia treatment, critical limb ischemia, traumatic brain injuries and liver regeneration [13,16-22]. Increased retention and survival of LOP MStroC at site of lesion has been reported, suggesting that LOP could be a key factor in enhancing tissue repair [13,16-19,23]. It has also been demonstrated that LOP can improve *ex vivo* expansion of other types of stem cells (SC), such as hematopoietic SC [24].

Metabolic adaptation, operating through a shift to anaerobic metabolism, followed by attenuation of oxidative phosphorylation (OXPHOS), increased glycolysis, maintenance of homeostasis of mitochondrial reactive oxidative species (mtROS) production and induction of the expression of the hypoxia-inducible factor (HIF) family of proteins, is hypothesized to be crucial for increased viability, retention and proliferation of MStroC under conditions of restricted nutrients and oxygen availability. These acquired characteristics enhance the therapeutic efficiency of MStroC [23,25,26].

HIF-1 and HIF-2, members of the HIF transcription factor family, are master regulators of cellular homeostasis, metabolic adaptation, survival and proliferation in states of restricted oxygen availability [46-48]. HIF-1 and HIF-2 are heterodimers, consisting of oxygen-dependent α subunits, HIF-1α or HIF-2α and constitutively expressed HIF-β subunits [46, 47].

Although the application of LOP MStroC demonstrated promising results, there are still limitations in the development of clinical protocols based exclusively on this approach. Small fluctuations in the partial pressure of oxygen, time of exposure, MStroC density and MStroC tissue origin, can significantly impact different outcomes of LOP MStroC treatment [23,27-29].

Introducing some molecules into MStroC that are capable of inducing metabolic adaptation and inducing HIF protein expression independently of the partial pressure of oxygen (hypoxia-mimicking molecules), could be a beneficial and supplementary strategy for LOP of MStroC. A challenge to this strategy is to find a suitable hypoxia-mimicking candidate molecule with no toxic and side effects [25,26].

MicroRNAs (miRNAs) are small single-stranded, non-coding, regulatory RNA molecules, involved in posttranslational regulation of gene expression [30-32]. They are important regulators of many physiological processes, including energy metabolism, cell cycle propagation, apoptotic cell death, proliferation and differentiation [30,33,34]. miRNAs are very attractive candidates for hypoxia-mimicking molecules because of their high specificity for targets [30,34]. Multiple strategies have been developed for an efficient delivery of miRNAs to cell cultures *ex vivo* and tissues *in vivo* [35]. A significant challenge to this strategy is to validate suitable miRNA candidates with well-established roles in physiological and pathological processes [36,37].

miRNA-210 is most often reported to be highly expressed as a result of oxygen deprivation in different cell lines, from a variety of cancer cells to human umbilical vein endothelial cells [16,38,39]. This miRNA is a direct target of both transcription factors HIF-1 and HIF-2 [38-42]. miRNA-210 was shown to influence the attenuation of electron transport chain (ETC) activity. It is also involved in the regulation of cell cycle and apoptotic cell death, as well as in the HIF-1α protein stability, which is the first response of a cell exposed to low partial pressure of oxygen [34,41,43-46].

The aim of the present study was to provide additional data on miRNA-210 and to evaluate its potential as a hypoxia-mimicking molecule in hBM MStroC, one of most attractive

sources of MStroC for clinical application. Since there is growing evidence that molecular mechanisms underlying cellular adaptation, survival and proliferation during short-term and long-term exposure to a low oxygen concentration do not overlap completely [47], we examined the time-dependent effect of low oxygen concentration on miRNA-210 expression in hBM MStroC. We also aimed to elucidate the role of HIF-1α and HIF-2α proteins, which are essential for HIF-1 and HIF-2 heterodimer function, in the regulation of miRNA-210 expression in hBM MStroC in extended oxygen deprivation. Additionally, we studied interindividual differences of miRNA-210 expression in the same model system.

MATERIALS AND METHODS

Culture of hBM MStroC

Experiments were performed on mononucleated adherent cells derived from 6 human adult healthy donors. Before use for research purpose it was previously established that the cells satisfy the minimal criteria recommended by International Society for Cellular Therapy (ISCT), to be accepted as MStroC [48].

Cell culture conditions

Experiments were performed by thawing the MStroC (passage 0), and seeding them into 75-cm^2 flasks at a cell density of 5000 per cm^2 in MStroC cell culture medium. For the first 24 h, all flasks were cultured at the standard cell-culture gas mixture: 20% O_2, 5% CO_2, humidity 95% and temperature 37°C. Incubation of the control cells was continued for 24 h, 48 h and 72 h at standard culture conditions. The flasks, which were used to test the experimental conditions, were transferred at an atmosphere with 3% O_2, 5% CO_2, 92% N_2 (mimicking physioxia in bone marrow), humidity 95% and temperature 37°C for next 24 h, 48 h and 72 h. Cells obtained under these experimental conditions were used to analyze miRNA-210 expression by RT-qPCR.

RNA extraction and RT-qPCR analysis

Lysis of PBS-rinsed adherent MStroC was induced by adding 1 mL of cold TRIzol reagent (Thermo Fisher Scientific, USA). Lysates were collected by scraping at 1% O_2 atmosphere for samples incubated at physioxia, and at atmospheric oxygen concentration for samples incubated at 20% O_2. Total RNA was isolated by the TRIzol reagent extraction procedure following the manufacturer's protocol, quantified and analyzed for quality by nanodrop technology and stored at -80°C for further use.

Reaction of reverse transcription was performed using an miScript II RT kit (Qiagen, Germany) with 1 µg of purified total RNA in a 10-µL reaction volume, according to the manufacturer's recommendations. A reverse transcription negative reaction (containing total RNA and reaction buffer but no RT enzyme) was performed for each sample in addition to reactions containing nuclease-free water instead of the RNA template, which served as negative controls. cDNA was diluted 1:10 in nuclease-free water and stored at -80˚C.

Detection of miRNA-210 expression was done with an miScript SYBR qPCR kit (Qiagen, Germany) using 1 µL of diluted cDNA in 12 µL of multiplex PCR reactions. Each reaction was performed in triplicate. 10× miScript primer assay (Human miScript Primer Assay Set) for miRNA-210 qPCR analysis was used (MS00003801, Qiagen, Germany). PCR amplification was performed using a BioRad CFX96 Real-Time System light thermocycler machine (C 1000 Touch, Thermal Cycler, CT005772, Singapore). Samples were initially denatured at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 s, annealing at 55˚C for 30 s, extension at 70˚C for 30 s. Fluorescence data were collected after the extension step. The obtained results were normalized with the *RNU6-2* control gene (miScript Primer Assays MS00033740, Qiagen, Germany). The relative difference in expression of miRNA-210 was determined by the regular ΔΔCt method.

Transduction of hBM MStroC cells

Short hairpin RNA (shRNA) constructs with cloned sequences specific for one of the genes *HIF-1α* (sequence 5'gatgttagctccctatatcccTTCAAGAGAgggatatagggagctaacatc3'), *HIF-2α* (sequence 5'aggccgtactgtcaacctcaaTTCAAGAGAttgaggttgacagtacggcct3') and *GFP* gene coding sequence as a reporter, were produced and packed in lentiviral particles. Transduction was performed into hBM MStroC and the cells were grown in cell culture medium up to 80% of confluence. Viral capsids containing shRNA specific either to *HIF-1α* or *HIF-2α* genes or an empty shRNA construct, which served as a control, were successively added into the hBM MStroC culture for two days at a multiplicity of infection (MOI) 50. hBM MStroC were grown in α-MEM medium supplemented with 10% FBS with no added antibiotics. After the second day, the medium was replaced by a virus-free medium. Fluorescence-activated cell sorting (FACS) analyses for GFP protein were performed to verify the quality and percentage of transduction. Further experimental procedures were performed on transduced cell populations with a purity of transduction greater than 99%, obtained by cell sorting. Total RNA was isolated from transduced cells by the TRIzol reagent according to the manufacturer's recommendations.

Statistical analysis

The average, median, minimal and maximal values were calculated for each analyzed group. Differences in the miRNA-210 expression levels between control and treated samples, as well as between different time points, were tested by non-parametric Wilcoxon signed-rank test. All tests were two-tailed and p values less than 0.05 indicated significant differences. Statistical analyses were performed using GraphPad Prism software ver. 6.01 (La Jolla, California, USA).

RESULTS

Increase of miRNA-210 expression in hBM MStroC in extended low oxygen concentration (72h oxygen deprivation)

We quantified the expression level of miRNA-210 in hBM MStroC derived from six human adult healthy bone-marrow donors exposed to an atmosphere with 3% oxygen concentration for 24 h, 48 h and 72 h. Control cells were incubated at 20% oxygen concentration for the same duration. We detected a significant increase in miRNA-210 expression at 3% oxygen concentration in comparison to the control, 20% oxygen concentration, at 24 h, 48 h and 72h ($p=0.031$, $p=0.031$, $p=0.031$, respectively) (Fig. 1). On average, after 24 h and 48 h, the levels of miRNA-210 were 2.97- and 2.59-fold higher in cells treated with 3% than in cells treated with 20% oxygen, whereas after 72 h, the average increase in miRNA-210 expression was more prominent – at 3% oxygen concentration, treated cells had a 5.07-fold higher expression than the controls.

When differences between the studied time points were examined, we detected a significant increase in miRNA-210 expression at 72 h as compared to 24 h ($p=0.031$). There were no significant differences between 24 h and 48 h, nor between 48 h and 72 h of exposure to low oxygen concentrations (Fig. 2).

Interindividual differences in miRNA-210 expression in hBM MStroC in oxygen deprivation conditions

Besides the differences between low and control oxygen conditions, as well as between different time points, we wanted to examine the existence of donor-specific differences in miRNA-210 expression at all three measuring points. The detected miRNA-210 expression in conditions of exposure to 3% oxygen concentration as compared to 20% oxygen concentration (control treatment) varied in cells obtained from the six different donors, and their median, minimal and maximal levels at 24 h, 48 h and 72 h were as follows: 2.53 (range 0.94-7.84), 2.68 (range 1.66-4.1) and 5.14 (range 1.39-33.71), respectively. According to this, the expression of miRNA-210 differed the most between the samples maintained at 72 h of hypoxia

(Fig. 1). The donor-specific differences indicate the need to consider a personalized approach in studying and developing donor-adapted protocols for putative miRNA-210 application as an miRNA drug.

miRNA-210 expression in hBM MStroC is both HIF-1α- and HIF-2α -dependent in conditions of extended (72 h) oxygen deprivation

To evaluate whether miRNA-210 expression at 72 h is regulated by HIF-1 and/or HIF-2 transcription factors, we created *HIF-1α* and *HIF-2α* gene knockdown hBM MStroC cell populations, obtained from 2 adult healthy bone-marrow donors, using lentiviral vectors and shRNA specific for each of the genes. Cell sorting was performed to obtain more than 99% purity of a transduced population. At an atmosphere of 3% oxygen concentration, cells transduced by empty shRNA exhibited a 25% decrease in miRNA-210 expression when compared to control cells that were not transduced (NT) (Fig. 3). Knockdowns of either *HIF-1α* or *HIF-2α* displayed 70% and 90% lower expression of miRNA-210 at 3% oxygen concentration, as compared to cells that did not have any gene knockdown (NT control cells) (Fig. 3). hBM MStroC cells with only the *HIF-2α* functional gene (*HIF-1α* knockdown) were still able to maintain 30% of the level of miRNA-210 expression as compared to NT control cells. However, when the *HIF-2α* gene was silenced and only *HIF-1α* was functional, 10% of the level of miRNA-210 expression was maintained in the cells in comparison to NT control cells that were expanded at 3% oxygen concentration (Fig. 3).

Our results demonstrated the involvement of both HIF-1α and HIF-2α transcription factors in the regulation of miRNA-210 expression in hBM MStroC at conditions of extended oxygen deprivation, with a slight predominance of HIF-2α.

DISCUSSION

Research groups have reported on the beneficial effects of oxygen deprivation in *ex vivo* cultures of MStroC for maintaining their proliferative capacity and differentiation potential [27-29,49]. However, the effects of low oxygen concentration exposure on cell population differ in a time-dependent manner. Acute hypoxia that lasts less than 24 h is associated with metabolic adaptation, cell cycle arrest and increased apoptosis, whereas extended exposure to hypoxia (more than 48 h), similar to a chronic hypoxia, is associated with cell proliferation and population growth [47].

Heterodimer transcription factor HIF-1 has a pivotal role in the cellular response to acute low partial pressure of oxygen. It is a regulator of a shift to glycolytic metabolism. It is also involved in attenuation of ETC activity and OXPHOS in mtROS homeostasis and cell cycle arrest [50]. However, extended exposure to low partial pressure of oxygen is predominantly regulated by the HIF-2 heterodimer, which promotes cell proliferation and population growth with no involvement in energetic metabolism regulation [51-54]. The finely-tuned interplay between HIF-1α and HIF-2α subunits expression, critical for HIF-1 and HIF-2 transcription factor-mediated gene regulation, suggests their important, distinct and time-dependent roles in maintaining cellular homeostasis in conditions of low oxygenation [51,54-56].

A knock out for the HIF-1α subunit [41] showed that HIF-1 transcription factor-dependent induction of miRNA-210 was important for hBM MStroC survival during the first 24 h of exposure to low partial pressure of oxygen. In our study, we demonstrated a new increase in the miRNA-210 level in hBM MStroC cell cultures after 72 h exposure to low oxygenation. The increase in miRNA-210 was both HIF-1α- and HIF-2α-subunit-dependent, suggesting the involvement of both transcription factors in the regulation. Our results revealed the predominance of the HIF-2α subunit and consequently the HIF-2 heterodimer role in miRNA-210 expression under conditions of extended exposure to low oxygen concentration, which lasted up to 72h. Therefore, we confirmed the importance of miRNA-210 in the molecular mechanism of hBM MStroC response not only to acute, but also to extended

exposure to low oxygen concentration. Additionally, being associated with HIF-1- and HIF-2-dependent subsets of genes, miRNA-210 appears to be a good candidate for a hypoxia-mimicking molecule.

There are additional data that support the potential application of miRNA-210 as a hypoxia-mimicking molecule in MStroC-based therapies in regenerative medicine.

Overexpression of miRNA-210 at atmospheric oxygen concentration was demonstrated to be associated with a decrease in prolyl hydroxylase (PHD) activity through a glycerol-3-phosphate dehydrogenase 1-like (GDH1L)-dependent mechanism and consequent accumulation of HIF-1α protein in cells [34,38,50]. As a positive regulator of HIF-1α, miRNA-210 contributes to increased glycolytic activity, decreased Acetyl-CoA delivery to ETC and mtROS homeostasis maintenance [50]. Additionally, miRNA-210 directly contributes to metabolic adaptation by targeting mRNAs coding for iron sulfur clusters of proteins, which are crucial for electron flow through complex I, II and III of ETC [43,46].

Additionally, in *ex vivo* conditions of complete oxygen or combination oxygen/nutrient deprivation, miRNA-210 is demonstrated to have an important cytoprotective role in supporting cell proliferation and preventing apoptosis [46]. Several research groups reported that the mRNA coding for the *MNT* gene, a negative regulator of c Myc, is a direct target of miRNA-210, which points to its role in cell cycle- and proliferation-supportive mechanisms [45]. Cyclic ischemic preconditioning of SC demonstrates an inhibition of apoptotic cell death by a miRNA-210/casp8ap2-dependent mechanism [46]. Moreover, gene therapy based on the application of mini gene coding for miRNA-210 performed on a mice model of myocardial infarction, showed significant improvement of cardiac function [46].

According to reports discussed above and results obtained in this study, miRNA-210 is involved in hBM MStroC homeostasis in conditions of restricted oxygen availability. It is worth considering miRNA-210 as a possible hypoxia-mimicking molecule. Evaluation of miRNA-210 expression profiles in a larger number of samples is necessary to confirm our hypothesis that miRNA-210 could be a candidate for a hypoxia-mimicking molecule. Furthermore, various experimental conditions, such as oxygen deprivation, up to complete anoxia, as well as extension of LOP-preconditioning periods, could be useful in testing the hypothesis. Moreover, the observed donor-specific differences suggest a need for developing an individualized approach adapted to each donor, for miRNA-210 application as a hypoxia-mimicking molecule.

CONCLUSION

This is the first study showing the involvement of miRNA-210 in the hBM MStroC response to extended exposure to low oxygen concentration (at 3% oxygen concentration for 72 h). Our results support the idea of using miRNA-210 as a mimicking molecule to create LOP, which was shown to increase the efficiency of hBM MStroC in tissue regeneration. Moreover, we detected interindividual differences in miRNA-210 expression in hBM MStroC during extended oxygen deprivation, which point to the necessity of considering a personalized approach in studying and developing donor-adapted protocols for putative miRNA-210 application as an miRNA drug.

Acknowledgments: This research was supported by Grant III41004 from the Ministry of Education, Science and Technological Development of the Republic of Serbia and EFS French Blood Institute, annual budget for Nuvelle Aquitanie Branch. The authors acknowledge the Vectorology facility, Vect'UB, Lab INSERM U1035, UMS INSERM 005 – CNRS 3427 – TMBCore, University of Bordeaux, France, for providing lentiviral particles with shRNA coding for HIF-1α or HIF-2α, Platforme UB'Facsility labarotorie UMR 3427/ US 005, French Ministry of Foreign Affairs for PhD fellow support, for Ms. Darija Lončarić and la Ligue Contre le Cancer, Bordeaux University, and the Lebanese association for development for PhD fellow support for Ms. Amani Ghousein.

Author contributions: DL – molecular analysis, preparation of the manuscript; BS – statistical analysis, preparation of the manuscript; AG – data collection, data analysis, critical review of the manuscript; MV-

experimental work, statistical analysis; VS – data analysis, preparation of the manuscript; AV – sample collection, critical review of the manuscript; CD – sample collection, critical review of the manuscript; CG – design of the study, preparation of the manuscript; ZI – design of the study, preparation of the manuscript; SP – design of the study, interpretation of the results, preparation of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Expression level of miRNA-210 under *ex vivo* oxygen deprivation conditions in MStroC derived from 6 adult human healthy bone-marrow samples. Plots demonstrate the change in expression levels of miRNA-210 between cells cultivated at 3% and 20% oxygen concentrations (O_2) at three time points, 24 h (**A**), 48 h (**B**) and 72 h (**C**). Samples from the same donor are connected with a line. Relative miRNA-210 expression level was calculated by the ΔΔCt method, and the ΔCt median value of the control sample was used as a calibrator. Statistical analyses were performed by the Wilcoxon signed-rank test and $p<0.05$ was considered statistically significant.

Fig. 2. Time-related differences in the expression of miRNA-210 in MStroC derived from adult human healthy bone-marrow samples. Expression level of miRNA-210 cultivated at 3% oxygen concentration is presented as the fold-change compared to the control sample, cultivated at 20% oxygen concentration. The expression level was calculated by the ΔΔCt method, and for each sample the ΔCt of the corresponding control was used as a calibrator. Statistical analyses between time points were performed by Wilcoxon signed-rank test and $p<0.05$ was considered statistically significant.

Fig. 3. Expression level of miRNA-210 in HIF-1α and HIF-2α-knockdown hBM MStroC cells under conditions of extended (72 h) oxygen deprivation. The experiment was conducted using cells from 2 adult human healthy bone-marrow donors. The level of miRNA-210 expression in control and knockdown cell populations was analyzed by RT-qPCR. The level of miRNA-210 expression was calculated relative to 20% oxygen concentration (control) at standard cell culture conditions by the ΔΔCt method. The results are presented as average values in percentages of miRNA-210 expression levels in non-transduced cells, shRNA control, HIF-1α shRNA and HIF-2α shRNA hBM MStroC cells expanded *ex vivo* at 3% oxygen concentration. The shRNA control denotes the expression level of miRNA-210 in cells transduced by the shRNA control construct. HIF-1α shRNA and HIF-2α shRNA represent expression levels of miRNA-210 in HIF-1α or HIF-2α gene knockdowns generated by transduction of specific shRNAs in hBM MStroC cells.

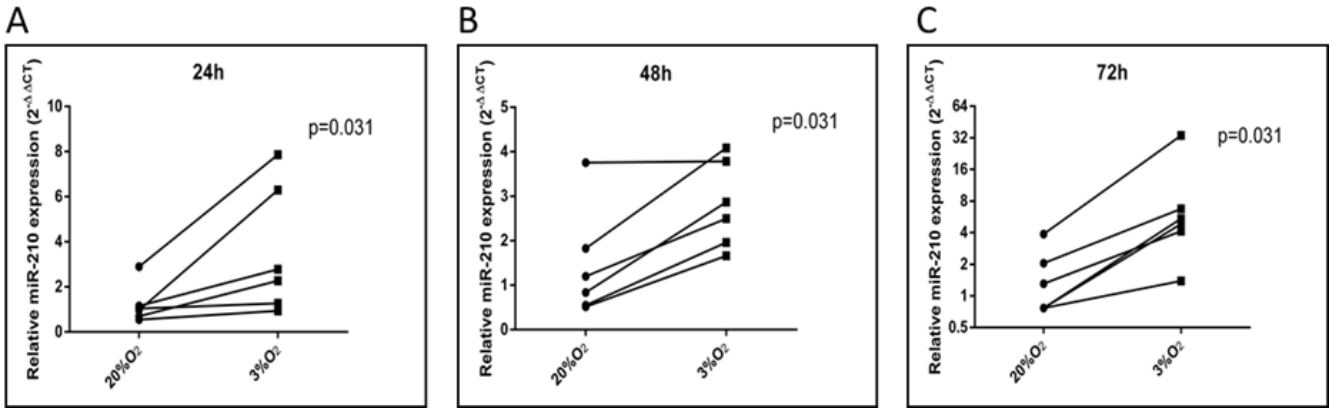
A
24h
Relative miR-210 expression (2^-ΔΔCT)
p=0.031
20%O2
3%O2
B
48h
Relative miR-210 expression (2^-ΔΔCT)
p=0.031
20%O2
3%O2
C
72h
Relative miR-210 expression (2^-ΔΔCT)
p=0.031
20%O2
3%O2

Fig. 1.

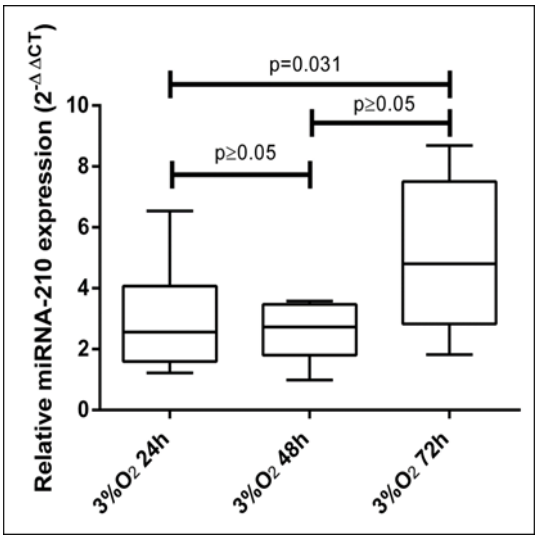
p=0.031
p≥0.05
p≥0.05
Relative miRNA-210 expression (2^-ΔΔCT)
10
8
6
4
2
0
3%O2 24h
3%O2 48h
3%O2 72h

Fig. 2.

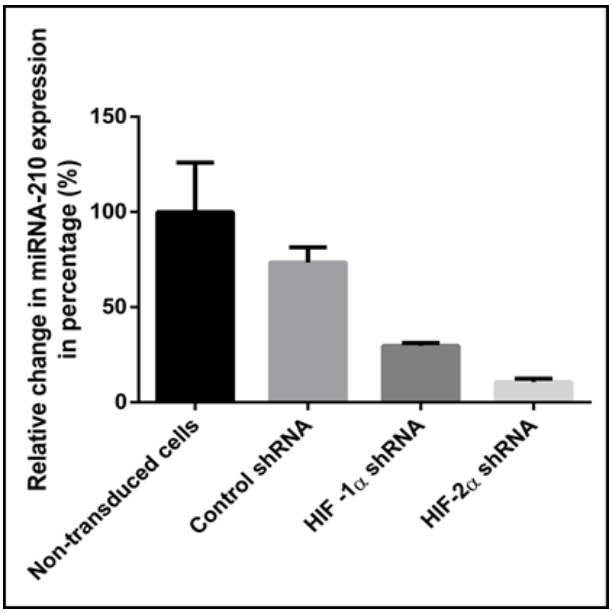
Relative change in miRNA-210 expression in percentage (%)
150
100
50
0
Non-transduced cells
Control shRNA
HIF -1α shRNA
HIF-2α shRNA

Fig. 3.