I. Research Project Objectives

The aim of this Project is to improve the umbilical cord Wharton's jelly mesenchymal stem cells (UC-MSCs) survival and their therapeutic properties prior to delivery by using preconditioning with defined hypoxia and hypoxia imitating agents. The hypothesis is that the usage of different level of hypoxia (1% O_2 vs 5% O_2) and hypoxia-mimetics with different mechanisms of action, such as desferoxamine mesylate (DFO), dimethyloxalylglycine (DMOG), and $CoCl_2$, vary in their preconditioning potential and the way they affect the cell response towards distinct types of injuries resembling the situation after delivery into the heart after myocardial infarction (MI). Other hypothesis of this Project states that the differences in therapeutic potential of different types of hypoxia-related preconditioning is caused by the divergences in the regulation of major hypoxia-inducible factors (HIF): HIF-1α and HIF-2α and in the expression of apoptosis-related, antioxidant, cytoprotective, antifibrotic and glycolytic factors. Final hypothesis declare that the best preconditioning strategy might differ depending on the reoxygenation of infracted heart what should be tested by different *in vitro* models: hypoxia and serum deprivation (HSD), as well as hypoxia and glucose- and serum- deprivation (HGD) representing hypoxic heart and H_2O_2 corresponding to heart with restored blood flow. Moreover, the aim of applied preconditioning is to strengthen the survival of cells after delivery giving them shield to protect themselves from harmful conditions of broken heart, mainly elevating their resistance to ROS and altering their metabolism. Hypoxia-related preconditioning will also boost MSCs therapeutic efficacy giving them better tools to reach their goal - elevated proangiogenic capacity as hypoxia is known to induce the expression of proangiogenic factors and proangiogenic action of MSC is crucial for their therapeutic properties.

Only *in vitro* studies are included in the Project as this is the first and crucial step to verify the therapeutic potential of different hypoxia-related preconditioning strategies in several models corresponding to situation in infracted heart: HSD, HGD, H_2O_2 and media from injured cardiomyocytes (ICM). Together with analysis of different paracrine action of UC-MSCs on cell types present in heart: cardiomyocytes, endothelial cells and fibroblasts and the verification of the role of HIF-1 and HIF-2, the Project will give the most complete view of the situation of UC-MSCs after delivery into infracted heart and give the best knowledge about their therapeutic properties.

II. Significance of the project

Prevention, treatment and regeneration represent three major elements of current medical therapeutic approaches used in tissue and organ repair. In the case of unsuccessful prevention or treatment, which does not restore the condition of damaged tissue, the regeneration approach is of great importance to be applied. It is an optimal approach especially for the treatment following MI since the surgery intervention and pharmacological methods are triggered to eliminate the cause of the disease (e.g. hypercholesterolemia) and to prevent the occurrence of subsequent events. However, until today there are no efficient ways to regenerate myocardial tissue. As the progressing damage of heart tissue affects functioning of the whole organism, it is necessary to address this issue in the field of life science and regenerative medicine.

Currently there are two ways to stimulate the tissue regeneration in patients suffering from organ injury including 1) the activation of endogenous progenitor cells e.g. by using erythropoietin (Epo), or 2) the delivery of exogenous progenitors (often allogenic) into damaged tissue. Since the therapies leading to the activation of resident progenitors in patients are often ineffective due to enlarged tissue damage (affecting also progenitors) or insufficient and late activation of bone marrow (BM)-derived progenitors (older patients), the scientific efforts are greatly focused on applications of allogenic progenitors.

Among stem cells investigated in the context of therapy the most widely tested are MSCs. There is no risk of tumorogenesis and no ethical concerns for the usage of MSCs. The therapy utilizing these cells may be based on cells of autologous origin including MSCs from bone marrow (BM-MSCs) or adipose tissue (AT-MSCs), or allogenic ones. In case of therapy utilizing allogenic MSCs, BM-MSCs or AT-MSCs from other patients can be used but for this approach there is another potent group of cells – UC-MSCs. Important aspects of MSCs-based therapy include proper quality and quantity of the cells as well as the time of delivery, as it would be crucial to use the cells almost immediately after MI. Unfortunately, MSCs properties decrease with age of the cell donor[1, 2]. Even more prominently, BM-MSCs from patients with chronic heart failure are characterized with reduced proliferation, downregulated cardiac natriuretic peptides signaling and enhanced transforming growth factor β (TGFβ) pathway. This would result in profibrotic phenotype of these cells after delivery making them not useful for the heart regeneration therapy[3].

Therefore is seems that autologous transplantations can be not a best choice for some patients. It is important to note that there are subtypes of MSCs differ in some of their properties, e.g. there are differences between UC-MSCs, AT-MSCs and BM-MSCs in their capability to suppress peripheral blood B cells, NK cells and T cells[4]. What is more, different subtypes of MSCs might diverge in their therapeutic potential, like elevated neuro-therapeutic properties of UC-MSCs over BM-MSCs[5]. Therefore, it seems that UC-MSCs represent the best MSCs type for the regeneration of infracted heart.

The usage of MSCs in the therapy after MI is currently under broad investigation and some of the research is in the clinical trials stage[6]. MSCs are shown to reduce infarcted area and improve cardiac function via immunomodulatory action, paracrine action and induction of the generation of capillaries and endogenous cardiomyocytes. However, the key factor diminishing the efficacy of such therapy is a very low survival rate of transferred cells. It has been shown that the survival of MSCs is lower than 0.44% at 4 days after delivery, what may be related to factors released by necrotic cells, local immune response, hypoxia, elevated level of ROS resulting from reoxygenation after reperfusion or lack of pro-survival signals or intercellular connections[7]. Therefore, in order to enhance therapeutic properties of MSCs it is of great importance to improve their survival after delivery into infarcted heart. For this purpose the strategy of MSCs preconditioning before their intramyocardial delivery has been applied. The preconditioning can be done using single factors, such as statins or Epo, but also more complex ones, such as hypoxia[7, 8].

The main goal of the Project is to compare the effect of preconditioning of UC-MSCs with different defined hypoxia conditions and various hypoxia imitating agents: DFO, DMOG and $CoCl_2$ to enhance their therapeutic potential prior to transplantation into injured myocardium.

The major response under hypoxic conditions is driven by HIFs. These transcription factors in normoxia are hydroxylated by enzymes called prolyl hydroxylases (PHDs) in the reaction that requires O_2 and α-ketoglutarate as substrates, iron as an activating metal and ascorbate as a cofactor, and degraded in the proteasome. In the conditions of reduced O_2 availability, HIFs are stabilized and translocated to the nucleus where they induce gene expression. What is more, HIF-1α and HIF-2α are regulated differentially with hypoxia with more rapid HIF-1α stabilization and the increase in the level of HIF-2α during later phase of the response, during prolonged hypoxia and upon higher O_2 concentrations compared to HIF-1α [9, 10]. Additionally, these two factors regulate their own expression. HIF-1α diminishes HIF-2α expression via activation of NADPH oxidase 2 (Nox2) expression and consequently elevation of ROS amount and then Ca^{2+} level and activity of calpain. The decrease of HIF-1α presence evoked by HIF-2α is mediated via induction of antioxidant enzyme superoxide dismutase 2 (SOD2) and subsequent diminishment of ROS level leading to mTOR kinase activation and inhibition of prolyl hydroxylation[11].

HIFs have been shown to be induced during MI in the peri-infarct area in rats and human[12]. While in the peri-infarctious tissue both HIF-1α and HIF-2α were stabilized, the elevation of HIF-2α was also detected in the areas remote from the infarction site and it progressed with time. Moreover, micro- and macrovascular endothelial cells expressed HIF-2α more frequently than HIF-1α. HIF-2α stabilization in non-infarcted area might be linked with imbalanced perfusion and oxygen consumption, being a result of elevated myocardium work and increased diffusion[13]. Interestingly, studies using HIF-2α KO mice showed that this transcription factor protects kidney from ischemia/reperfusion injury via the amelioration of oxidative stress in the endothelium[14]. Protective role of HIF-1α towards MI was demonstrated using HIF-$1\alpha^{+/-}$ mice as they were more prone to cardiac injuries evoked by ischemia/reperfusion, with the effect of HIF-1α mediated by induction of Epo[15]. What is more, rat cardiomyocytes overexpressing constitutively active HIF-1α are resistant to ischemia/reperfusion injury in *in vitro* model[16]. The role of HIFs is also proven with disruption of PHD-1, which also attenuates ischemia/reperfusion injury in heart[17].

HIF isoforms differ with their target genes and thus with the time when they are important in the response during MI. HIF-1α is crucial in the early phase inducing transition from aerobic to anaerobic metabolism, as it controls factors involved in glucose metabolism. HIF-1α also affects mitochondrial metabolism by inducing the expression of puryvate dehydrogenase kinase 1, an enzyme reducing mitochondrial respiration, by alterations in the cytochrome oxidase complex and by induction of COX4-2, LON and hexokinase II, a glycolytic enzyme that shifts glucose away from mitochondria. In contrast, HIF-2α seems to be crucial for long term response, including coming back to oxygenated state. HIF-2α regulates factors important in maintaining the mitochondrial homeostasis, including antioxidant SOD2 and frataxin, an oxidative stress-regulated chaperone for aconitase[12]. In contrast, in my previous studies I demonstrated that HIF-1α reduces anxioxidant response downregulating Nrf2, a major transcription factor responsible for antioxidant response, and expression of heme oxygenase-1 (HO-1), which also exhibits antioxidant properties in HMEC-1 cells[18]. I have also participated in the study which demonstrated that HIF-1 and HIF-2 regulate differently IL-8 expression [19]. Therefore it seem plausible that they can regulate differentially the immune response. Taken together, it seems that HIFs are important regulators of MI but

both isoforms might play different role during MI. Therefore important part of this Project will be to investigate the role of HIF-1α and HIF-2α in preconditioning of UC-MSCs using hypoxia and hypoxia-mimetics.

Compounds planned to be used in this Project: $CoCl_2$, DFO and DMOG resemble hypoxia conditions via inactivation of PHDs. DFO chelates iron, DMOG acts as an analoque of α-ketoglutarate[20], while cobalt works as an iron displacing metal and a factor depleting intracellular ascorbate[21]. Although all of these compounds inhibit PHDs, their mechanisms of action differ between each other. Importantly, DMOG, as an α-ketoglutarate analogue, was demonstrated to inhibit cell metabolism even before HIF stabilization and independently of HIF-1α and HIF-2α activation. DMOG downregulates glutamine/α-ketoglutarate metabolic axis, supresses oxidative phosphorylation and activates glycolytic ATP flux. Moreover, it decreases O_2 consumption and increases O_2 availability in tissues[22]. These non-HIF-related effects of DMOG seem to be perfect for the treatment of ischemic heart. Other compound planned to be used as hypoxia mimics, DFO works as a chelator of Fe^{2+}[23]. Fe^{2+} participates in Fenton reaction generating highly reactive and toxic hydroxyl radical and hydroxide ion, and therefore DFO exhibits antioxidant properties via scavenging Fe^{2+}[24]. Antioxidant properties of DFO I also demonstrated in my previous studies[24] included in my PhD thesis[25]. Other compound to be used in this Project, cobalt, apart stabilizing HIFs, can affect cellular oxidative stress level and induce HO-1 expression in HIF-independent but Nrf2- and MafG-dependent way[26]. These properties of DFO and $CoCl_2$ might also be crucial for the protection of MSCs from ROS generated during heart reperfusion, especially as antioxidant factors is considered for the treatment of MI[27], e.g. adenoviral overexpression of thioredoxin-1 in MSCs elevates proangiogenic factors production, diminishes fibrosis and improves heart function in rat model of MI[28]. Concluding, different strength of hypoxia (1% vs 5% O_2) and different types of hypoxia-mimetics might vary in their effect on HIFs stabilization and might affect cell response in HIF-independent way. Therefore, comparing the effect of different strength of hypoxia (1% vs 5% O_2) and ones exerted by hypoxia mimics, will allow to find the best preconditioning method to improve UC-MSCs properties and survival for heart regeneration after myocardial infarction (Fig. 1).

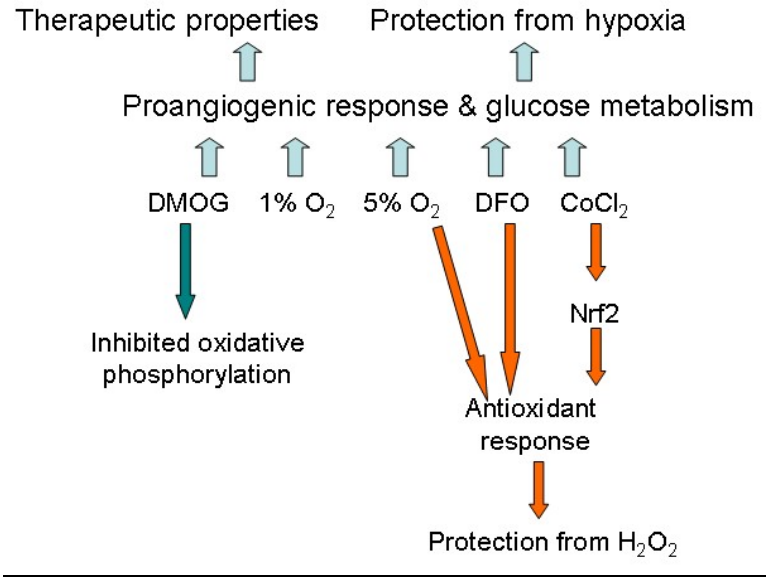

Fig. 1. The effects exerted by hypoxia and hypoxia mimetics on diiferent aspects of UC-MSC survival and therapeutic properties

There are some data showing that preconditioning of MSCs with hypoxia and hypoxia imitating factors improves cell therapeutic properties[29–33]. Transplantation of MSCs in rat model of MI demonstrated that hypoxic preconditioning (24h, 0.5% O_2) reduced death of delivered cells, elevated angiogenesis and improved morphological and functional parameters of heart compared to non-treated MSCs. In *in vitro* analysis hypoxia elevated expression of proangiogenic and prosurvival factors, including HIF-1, angiopoietin-1, vascular endothelial growth factor (VEGF), Flk-1 (VEGF receptor), Epo, Bcl2 and Bcl-xL and concomitantly decreased caspase-3 activation[29]. Preconditioning of BM-MSCs with 0.5% O_2 for 24h enhanced cell migration and homing both *in vitro* and *in vivo* after intravenous administration into rats subjected to permanent MI via enhancement of potassium channel Kv2.1 expression and focal adhesion kinase (FAK) activity[30]. In another study preconditioning of BM-MSCs with 3% O_2 for 24h improved cell

migration, adhesion and survival under oxidative stress conditions via PI3K/Akt-HIF-1α-CXCR4/CXCR7 pathway. CXCR4 were responsible for enhanced adhesion of preconditioned cells whereas only CXCR7 was necessary to resistance to oxidative stress. Moreover, treatment of BM-MSCs with DFO and $CoCl_2$ mimicked the effect of hypoxia on cell migration, adhesion and survival[31]. Another HIF stabilizer – DMOG attenuated BM-MSCs death and apoptosis induced by serum deprivation. It reduced mitochondrial cytochrome c release, nuclear translocation of apoptosis inducing factor (AIF), promoted Akt phosphorylation, stabilized HIF-1α and induced Glut1, a glucose transporter, via PI3K-Akt pathway[32]. DMOG also induced the expression of HIF-1α, Glut1 and VEGF after 24h of treatment of BM-MSCs, reduced H_2O_2-induced cell death in serum free conditions and enhanced viability and angiogenic activity of cells in ischemic heart model in rats[33]. Still, there are no studies investigating the effect of MSCs preconditioning with 5% hypoxia.

Nevertheless, precise studies unravelling the divergences in applied hypoxic or hypoxia mimetic conditions have never been conducted. As it was discussed above, it is of great importance to precisely investigate this very promising strategy to get the best possible method of improving therapeutic conditions of UC-MSCs. In this Project the effect of different strength of hypoxia (1% O_2 vs 5% O_2) for MSCs preconditioning will be compared for the first time. Additionally, this will be the first study evaluating hypoxia with hypoxia imitating agents with regards to HIF-independent action of hypoxia-mimetics. In this project the effect of preconditioning with hypoxia (1%, 5% O_2) and hypoxia-mimetics on antioxidant, cytoprotective, apoptosis-related, proangiogenic, metabolic-related and antifibrotic factors will be analysed. Moreover, although hypoxia and hypoxia imitating compounds were investigated before in the context of preconditioning of MSCs, the impact on antioxidant, cytoprotective, profibrotic and glucose metabolism-related factors was not analysed sofar. Moreover, the effects exerted by tested compounds were analyzed in single *in vitro* models of MI, what gives only partial information about the actual effect exerted by preconditioning in the context of heart regeneration. Additionally, HIF-independent properties of hypoxia mimetics have not been considered to be important. The effect of stimuli on cell metabolism or antioxidant response is of big importance as it might affect the surviving upon delivery or the reaction to reoxygenation. This will be also the first study to analyse the effect of preconditioning on expression of p53-upregulated modulator of apoptosis (PUMA), which reduction was shown to be important in cardioprotection[34]. Additionally, the specific role of HIF-1α and HIF-2α in observed effects will be verified, It will allow to investigate the mechanisms of observed changes and to determine HIF-independent effects, what was not addressed as of yet and is of great importance as discussed, above especially for pharmacological compounds planned to be used.

For the analysis of the survival of preconditioned UC-MSCs four *in vitro* models will be applied, which correspond to different types of injuries present in heart after MI: hypoxia and serum deprivation (HSD)[35] and hypoxia, glucose- and serum-deprivation (HGD)[36], corresponding to the lack of blood flow, H_2O_2 as a model of elevated ROS level during reoxygenation[37] and conditioned media from injured cardiomyocytes (ICM) corresponding to proapoptotic signals from dying cells (modified from:[38]). Although there are some studies in the field using hypoxia-related preconditioning as discussed above, they usually use only one model of tissue injury. The comparison of the effects exerted by preconditioning on MSCs survival and therapeutic properties using different *in vitro* models of injuries present in MI tissue has not been conducted as of yet. This will also allow to assess the effect exerted by conditions in injured heart on the therapeutic properties of MSCs. Moreover, comparing results obtained in different *in vitro* models will give the most complete view of therapeutic properties of preconditioned UC-MSCs,

Additionally, it is of importance to understand the paracrine effect exerted by MSCs on various cell types present in heart: cardiomyocytes[34], endothelial cells[36] and fibroblasts[39]. Paracrine therapeutic potential of MSCs is usually examined in the context of one cell type, usually cardiomyocytes[34] or endothelium[36]. This will be the first study to compare the paracrine effect of preconditioned MSCs on both cardiomyocytes and endothelium but also on cardiac fibroblasts, cells important in the scar formation. Additionally, in the Project the mechanism of observed alterations will be tested, mainly the role of HIF-1α and HIF-2α, what has not been investigated before. Additionally, the importance of HIF-2α in context of hypoxia-preconditioned MSC-based therapies has not been issued. These aspects are of great importance not only in the filed of regeneration but also in the context of hypoxia-related alterations within the cell.

The issues addressed in this Project will strongly trigger the progress both in the heart regeneration and in the regeneration field in general as it implies the need of analysing various aspects of preconditioning strategy of therapeutic cells, like impact on their survival in the tissue of delivery as well as intracellular properties (like ability to remove ROS and alterations in cell metabolism) and complex paracrine activity. Moreover, mechanisms underlying observed alterations with respect to the role of HIF-1α and HIF-2α will

be verified. This will let the Project to be used by a wide group of scientists. Cooperation within the Project among scientists with different expertise will increase the competence, widen the horizons and boost the knowledge about the topic. The presentation of this Project results on different conferences (stem cell-, hypoxia- or regeneration-related ones) and publishing them in prestigious, specialized journals will make them available for the scientists worldwide.

Finding the efficient method of heart regeneration is of great importance for current medicine, economy and society as cardiovascular diseases (CVDs) including MI are one of the biggest health problems worldwide. In "European Cardiovascular Disease Statistics" published in 2012 by European Heart Network and European Society of Cardiology it was reported that CVDs caused over 4 million of deaths in Europe, what accounts for 47% of all deaths, and over 1.9 million in the European Union, corresponding to 40% of deaths. Importantly, CVDs are the most common cause of death for men, except 6 countries, and for women in all European countries. The main form of CVDs is a coronary heart disease, which accounts for half of deaths[40]. In Poland CVDs are the main health problem and are responsible for almost half of deaths (178 000 in 2009). It is predicted that in 2020 they might cause as many as 200 000 deaths. Additionally, it is estimated that 87 500 patients suffered from MI in 2011. During that time 16 214 patients died because of MI making MI the most common cause of death among CVD in 2011 in Poland[41].

The MI occurrence is usually associated with the presence of accompanying diseases: coronary disease, high blood pressure, hypercholesterolemia or diabetes. Apart from physical problems, patients after MI very often suffer from depression, anxiety and insomnia. This is very often caused by the fact that MI episodes are sudden events. Additionally, after MI the physical abilities of patients are usually decreased, what is also a sudden change for them. This drop in physical abilities leads to the change of lifestyle. Moreover, patients after MI often need help from other people what affect their mood, relationship with family and cost of life. Very often MI leads to earlier retirement what greatly affects patients' psychological and financial situation.

According to the Central Statistical Office the high blood pressure and coronary disease were among five most frequent chronic diseases in 2009 in Poland with high blood pressure affecting every fifth person (the 2nd most common chronic disease) and coronary disease including one leading to MI present in almost 10% of people (the 5th most common chronic disease). Additionally, the increase in the occurrence of depression and anxieties was observed. In this analysis MI was shown to affect 1.7% of citizens. Among medications prescribed by physicians, the most common ones were for the reduction of blood pressure (44.7% of all prescriptions). The pills for reducing the cholesterol level account for 18.6% of all prescription, ones for other CVD for 22.8%, for depression -5.6%, anxiety – 5.9% and insomia – 8.1%[42]. This demonstrates that indeed MI and all linked diseases are important and frequent medical problems in Poland.

Apart for high mortality linked with CVDs and MI, there are also huge costs connected with them. There are several different types of costs associated with the treatment of patients with and after MI. Among them there are direct costs: hospitalization, surgery, interventions, visits to specialists (cardiologist, psychiatrist, diabetologist), medications with or without prescription, rehabilitation, diagnostic tests and in-direct ones, such as loss of productivity affecting efficiency of work, taking days off or earlier retirement of people. Among the costs there are ones paid by patients and their families, e.g. visiting doctors, rehabilitation, cost of medical equipment (glucometer, devices for blood pressure measurement), medications, diagnostic tests (holter, blood tests for cholesterol, glucose, CPK, ALT, AST, K^+, EKG, etc.). The costs paid by the government include visits in public hospitals (stays and visits in specialists), surgery, interventions, rehabilitation, diagnostic tests and refunded medications. Parts of the costs are paid by the social security system (pensions from ZUS and KRUS in Poland), social care system (nursing) and employers (illness benefits).

The analysis of the costs of acute coronary syndromes during first year after diagnosis showed, that the total cost was estimated around 3.3 bilion EUR in Germany, 3.1 bilion EUR in Italy, 1.9 bilion EUR in the UK, 1.3 bilion EUR in France and 1.0 bilion EUR in Spain. It accounted for 7 009 EUR in the UK and 12 086 EUR in the Italy per patient. The biggest cost was for hospital stay and revascularization procedures. Pharmaceuticals corresponded to 14-25% of total cost[43]. In Switzerland in 2008 acute coronary syndrome occurred 16 815 times for 14 955 patients out of whom 2 752 died in consequence. Total direct costs were amounted to 630 milion CHF for the society and 462 milion for health insurers. Production loss was assessed for 519 milion CHF. The loss of quality adjusted life year was calculated for 49 878[44]. In 2001 in the UK the average costs of cardiac rehabilitation to the health service per patients completing rehabilitation programme were 490 GBP[45]. In Poland the analysis done in 2011 by KPMG company indicated that the costs of diagnosis and therapy of CVDs was 15.3 bilion PLN, including hospitalization – 7.5 bilion PLN, ambulance care – 3.7 bilion PLN and medicines – 4.1 bilion PLN. The indirect costs connected with CVD were estimated for 26.6 bilion PLN including earlier retirement – 16.6 bilion PLN, the losses due to

premature mortality – 8 bilion PLN and the losses due to sick leaves – 2 bilion PLN[41]. Concluding, the costs for treatment of MI and related diseases are huge and are paid by several parts of society.

The efficient regenerative heart therapy will decrease the costs connected with MI, both direct and in-direct ones. There is a huge part of direct costs, which will be affected by such therapy, mostly hospitalization, rehabilitation, visiting the doctors and part of diagnostic tests (ones analysing heart parameters). As the medicines used by patients after MI (apart psychiatric-related ones) are focused on the prevention of the disease development and of the next acute episodes occurrence via the reduction of blood pressure, the diminishment of cholesterol level, the prevention of clothing, etc., they will have to be used regardless of heart regeneration. The renewal therapy will increase the quality of life, diminish the cost of care and nursing, reduce the number of people retiring from work, increase patients' independence thus lower the need for help, what will affect in-direct costs via reducing number of pensions paid and increasing the productivity. Reduction of the number of people who will retire after MI event will have an impact on patients themselves, the government, the social security system and employers (having qualified workers).

Apart being a serious economic problem, MI is also an important sociological issue. This is a result of decreased mobility and independence of patients. Additionally, due to additional costs connected with the disease and forced retirement for some people, the standards of their life decrease not only because of lower physical ability but also as a result of reduced monthly amount of money which they can spend, what affects both their psychical and physical status. The regeneration therapy itself and the possibility of this therapy will affect greatly the psychological state of patients not only by improving their conditions but also by giving them hope for improving their physical state. This will also reduce the need for the psychiatric help and improve contacts with friends and families.

Concluding, efficient heart regeneration therapy is of great importance for research as well as current medicine and will have great scientific as well as social and economic impact on the society. The results of this project might greatly improve the current knowledge in the field, conducted research as well as therapeutic strategies utilizing MSCs for heart repair.

III. Work Plan

1. Analysis of the impact of different types of hypoxia-related preconditioning on UC-MSC.

UC-MSCs will be treated with different strength (1% vs 5% O_2) and length (6h vs 24h) of hypoxia and hypoxia imitating agents with divergent mechanisms of action: $CoCl_2$, DFO and DMOG. Firstly, therapeutic properties of UC-MSCs after preconditioning will be verified: cell viability, proliferation, migration, apoptosis rate, ROS production, angiogenic potential as well as antioxidant response. Expression of proteins involved in glucose metabolism and cell protective, profibrotic and apoptosis-related factors as well HIF-1α and HIF-2α stabilization will also be analysed. In this step the morphology and UC-MSCs properties will be analyzed as well as selected parts of their transcriptome and level of HIF isoforms. This will allow the insight into UC-MSCs biology upon hypoxia-related preconditioning and give information about the different aspects of cell features.

2. Investigation of the survival and therapeutic properties of UC-MSCs in different *in vitro* MI models.

Next, four different models corresponding to various aspects of the situation in infracted heart will be utilized to analyze the survival of preconditioned UC-MSCs. In the Project models corresponding to three different situations will be used: 1) models of the lack of restoration of the proper blood flow (lack of oxygen and nutrition): 1% O_2 and serum deprivation (HSD) and 1% O_2, glucose and serum deprivation (HGD), 2) model of heart reoxygenation- H_2O_2 and 3) model of toxic effect of injured tissue - treatment with conditioned media from injured cardiomyocytes (ICM). In this point not only survival (measured by analysing LDH release) will be tested but also therapeutic properties of UC-MSCs listed in point III.1, as conditions in infracted heart might greatly affect them. The utilization of different *in vitro* models is crucial to completely understand the conditions to which preconditioned UC-MSCs will be exposed after intramyocardial delivery, thus to predict their survival and therapeutic properties when delivered to patients. It is also of big importance as preconditioning method might differ in the effect exerted on UC-MSCs compared to not-toxic environment.

3. Analysis of the paracrine action of preconditioned UC-MSCs.

In the next step the paracrine properties of UC-MSCs, which are known to contribute the most to therapeutic action of these cells, will be tested. The paracrine effect of preconditioned UC-MSCs will be

investigated using transfer assays on cardiomyocyte cell line, endothelial cell line and cardiac fibroblasts cell line. It is crucial to compare the effect exerted by preconditioned UC-MSCs on different cell types present in heart to obtain the most complete information. Even more importantly, the paracrine activity of UC-MSCs will not be tested on intact myocardial cells. In order to mirror in the best possible way the situation after the delivery, the cardiac cells will be treated by HSD, HGD and H_2O_2 before being exposed to factors secreted by preconditioned UC-MSC.

Based on steps 1-3 the best hypoxia-related preconditioning method will be chosen.

Application of different models of tissue injury as well as performing the transfer assays using different types of myocardial cell lines will provide the most complete information about therapeutic potential of preconditioned MSCs. Combining the information from 1-3 will allow to predict the possible mechanisms of observed effects with respect to changes in apoptosis index, cell metabolism, cytoprotective/antioxidant response, angiogenesis indication and scar formation.

4. **Investigation of the role of HIF-1α and HIF-2α in enhancement of therapeutic properties of UC-MSCs by hypoxia-related preconditioning.**

Finally, the role of HIF-1α and HIF-2α in observed effects will be analysed as these two transcription factors are known to be induced by hypoxia in different way and to share some of their target genes (proangiogenic ones) but exert different effect on others (antioxidant).

This will allow to add the new knowledge to our understanding about the mechanism of observed changes with respect to the specific roles of HIF-1α and HIF-2α and verify HIF-independent effects for preconditioning, what is especially important for usage of $CoCl_2$, DFO and DMOG.

The hypotheses of the Project are confirmed by preliminary data, which show that hypoxia and hypoxia imitating compounds differ with the effect their exert on HSD and H_2O_2-induced cell death and selected gene expression (Fig. 2). It is interesting as all tested pharmacological compounds inhibit PHD and one could estimate, that there will be no big differences in the effect they exerted during preconditioning on the mortality of UC-MSCs. However, obtained preliminary data demonstrate striking differences in the effect of different types of preconditioning on mortality of UC-MSCs depending on type of MI *in vitro* model used (Fig. 2A-C).

Preliminary results show that used preconditioning methods exert different effects in HSD and H_2O_2 toxicity models, which models corresponds to different situation in infracted heart. Pilot experiment measuring LDH release demonstrated that preconditioning of UC-MSCs with 100 and 200 μM $CoCl_2$ and 1% O_2 for 24h decreased cell mortality elevated by 24h HSD. Concomitantly, 5% O_2 had no effect whereas opposite effect was observed for DFO as 250 μM DFO elevated the HSD-induced cell mortality (Fig. 2A). The effect of 24h preconditioning of UC-MSCs was also investigated using injury model corresponding to reoxygenation, a treatment with 600 μM H_2O_2 for 24h. Interestingly, also in this model hypoxia mimetics differed in the effect they exerted. In this model the preconditioning with 200 μM $CoCl_2$ decreased (Fig. 2B) whereas 1000 μM DMOG very strongly increased (Fig. 2C) H_2O_2-triggered LDH release. For 100 μM $CoCl_2$ and 5% O_2 the tendency towards downregulation ($0.05<p<0.1$) was observed (Fig. 2B).

These preliminary data indicate that the effect exerted by different hypoxia strength and hypoxia-mimetics diverge between each other and the most complete protection (protection in both MI *in vitro* models) is achieved using 200 μM $CoCl_2$. What is more, the situation of infracted heart – hypoxic vs reoxygenated might require different hypoxic preconditioning. Even more importantly, it seems that some hypoxia mimetics might be beneficial or toxic depending on the situation after delivery. This situation took place for DMOG, which elevated the toxicity of H_2O_2 (reoxygenated heart), while it was not toxic delivered alone. Similar situation took place in case of DFO but for this compound it potentiated the mortality evoked by HSD (a model of hypoxic heart). Nevertheless, in order to completely understand the therapeutic capability of each preconditioning these data should be repeated and other *in vitro* models (HGD and ICM) should be tested as well as the analysis of the whole set of UC-MSCs features including their paracrine activity shall be conducted. There are also differences for preconditioning with different level of O_2, as 1% O_2 is protective in HSD *in vitro* model while 5% O_2 is more protective towards H_2O_2-evoked cell death, being a reoxygenation *in vitro* model what corresponds to heart with restored blood flow. These promising results fully confirm the need of this Project, however, these are only preliminary results and it needs a lot of research to be conduced, including the comparison of the effects of hypoxia-related preconditioning in other *in vitro* MI models (ICM, HGD) and very precise analysis of properties of UC-MSC important for their therapeutic action, including their paracine activity.

Further data indicate that observed discrepancies might be caused by different effect on gene expression evoked by each stimulus. Real-time PCR analysis of mRNA for Glut1, a transportr for glucose, showed that all of 6h-prestimulations increase its expression, although 5% O_2 have the weakest effect (Fig. 2D). Further analysis demonstrated that 6h treatment with 100 µM DFO and 5% O_2 decreased PUMA mRNA level, while the tendency towards downregulation was observed for 200 µM $CoCl_2$ and 500 µM DMOG, whereas 1% O_2 exerted no effect (Fig. 2E). These data demonstrate that different stimuli diverge in the effect they exerted on the regulation of selected gene expression(Fig. 2D-E). Nevertheless, these are only preliminary data and they need to be confirmed and compared with other properties of UC-MSCs. These results were obtained using UC-MSCs on passages 5-7. The cell phenotype was assessed using flow cytometry: CD29(+), CD90(+), CD105(+), CD34(-) and CD45(-).

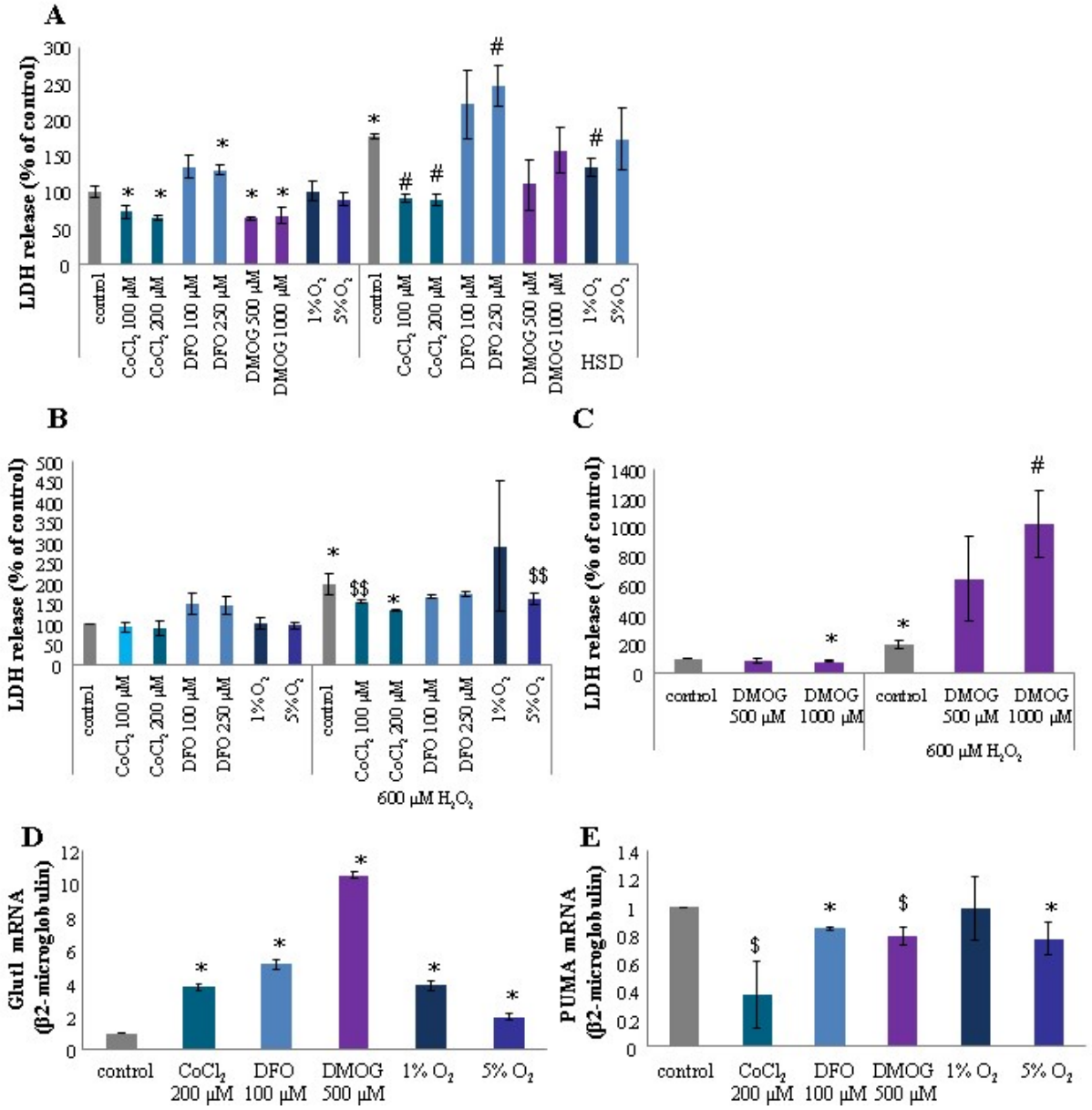

Fig. 2. Effects exerted by 1% and 5% O_2 and hypoxia imitating agents on UC-MSCs. UC-MSCs were preconditioned with hypoxia or hypoxia-mimetics for 24h followed with HSD for 24h (A) or treatment with 600 µM H_2O_2 for 24h (B-C), then cell death was assessed by measuring LDH release (A-C). UC-MSCs were cultured in the presence of hypoxia or hypoxia mimetics for 6h (D-E). Analysis of mRNA expression for Glut1 (D) and PUMA (E), was done using real-time PCR. A-pilot experiments, B, E – mean from 2-3 experiments, C-mean from 3 experiments, D – mean from 2 experiments; * $p<0.05$ vs control, \$ $0.05<p<0.01$ vs control; # $p<0.05$ vs control in HSD/H_2O_2 group, \$\$ $0.05<p<0.01$ vs control H_2O_2 group

I have the experience with UC-MSCs (isolation, culturing and conducting experiments) which I obtained working in the team of dr hab. Ewa Zuba-Surma, prof. JU in the Department of Cell Biology on the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University (FBB&B JU). I have used DFO and hypoxia in my previous studies including ones demonstrated in my PhD thesis[25]. I am a

first author of the original paper in which I am investigating the effect of hypoxia, HIF-1 and HIF-2 on ochratoxin A-diminished VEGF expression in LLC-PK1 cells[46]. Additionally, I am second author in other 2 original papers[18] [47] and third author in another one[19] focusing on the regulation of gene expression by hypoxia and HIFs in endothelial cells HMEC-1. Moreover, I am a first author of the original article in which I am utilizing antioxidant properties of DFO to investigate the mechanism of alterations in gene expression evoked by ochratoxin A in LLC-PK1 cells[24]. I am also a fist author of a review about hypoxia, HIF-1, HIF-2 and oxidative stress[48]. Finally, I am familiar with endothelial cell biology, as I am a co-author of three original papers about the regulation of gene expression in human microvascular endothelial cell line - HMEC-1[18] [19] [47]. Additionally, most of the methods included in this Project I have used conducting my previous research. Altogether it demonstrates that my expertise gives a solid theoretical and practical background for the feasibility of the Project.

IV. Research Methodology

Human UC-MSCs of 10 different individuals will be bought from ATCC. Human immortalized cardiac fibroblasts, human immortalized cardiomyocytes and human immortalized cardiac microvascular endothelial cells will be bought from abm. Cell phenotype will be confirmed by flow cytometry with the analysis of the presence of several human MSCs (hMSCs) markers such as CD29, CD90, CD105 as well as CD34 and CD45 (hematopoietic antigens). All experiments will be conducted on UC-MSCs between passages 5-7 according to scheme (Fig. 3). The preconditioning of UC-MSCs will be done with different levels of hypoxia: 1% O_2 and 5% O_2 using incubator chamber with regulated O_2 concentration as well as hypoxia imitating compounds: $CoCl_2$, DFO and DMOG for 6 and 24h. Only one concentration of each stimulus will be chosen based on my previous experience, pilot data and published data from studies on UC-MSCs. Thus 200 μM $CoCl_2$[49], 100 μM DFO[50] and 500 μM DMOG[32] will be used. The number of different assays planned to be done will be reduced as only selected tests will be applied to assess the cell properties of interest, such as LDH for toxicity.

Firstly, **UC-MSCs properties after preconditioning** will be verified. Apoptotic index will be assessed using Anexin V apoptosis kit, cell death will be tested analysing LDH release, cell proliferation will be investigated using BrdU incorporation, while cell migration will be examined using Transwell assay. ROS production will be measured using DCFH-DA, what allows detecting H_2O_2 generated during reoxygenation. Cell phenotype will be verified using standard markers for UC-MSCs characterization (CD29, CD90, CD105, CD34 and CD45) as well as endothelial markers (Gata-2, Tie-2, vWF, VE-cadherin) by flow cytometry and expression of selected antigens will be investigated also on mRNA level by real-time PCR. Expression of HIF transcription factors (HIF-1α and HIF-2α), cytoprotective factors (Nrf2, frataxin, HSPs), antioxidant enzymes (catalase, SOD2, GSTA1), factors involved in glucose metabolism (PDK1, Glut1), apoptosis-connected factors (p53, PUMA, Mdm2), proangiogenic factors (Epo, VEGF) and profibrotic factors (TGFβ1 and TGFβ2) will be analysed on mRNA level (Real-time PCR) and on protein level (western blot, immunofluorscent staining) (Fig. 3 part 1).

In the analysis of **UC-MSCs survival and therapeutic properties in different models of injury** in HSD and HGD protocols 1% O_2 will be used, as this situation reflects lack of proper blood flow, thus severe hypoxic conditions. In reoxygenation model H_2O_2 will be applied as it corresponds to reoxygenation of whole tissue. Important part of the analysis will be the usage of media from injured cardiomyocyte cell line, what allows assessing the response of UC-MSCs to proapoptotic signals coming from dying cells. Apart from analysis of UC-MSCs survival (by LDH release) also the therapeutic properties of cells cultured in these models will be assessed (Fig. 3 part 2).

During the experiments UC-MSCs will be cultured with hypoxia/hypoxia mimetics for 6h or 24h. Then the medium will be removed and cells will be cultured in medium without serum in 1% O_2 (HSD) or in medium without both serum and glucose in 1% O_2 (HGD) or 600 μM H_2O_2 in complete medium for 6h for gene expression analysis (Real-time PCR) and for 24h for analysis of cell death (LDH release), apoptosis (Annexin V staining), cell proliferation (BrdU incorporation), cell migration (transwell assay), cell phenotype (flow cytometry staining for hMSC and endothelial markers), gene expression (listed below, Real-time PCR), intracellular ROS level (DCFH-DA) and production of proangiogenic factors (luminex/ELISA). For the experiments using conditioned media, cardiomyocytes will be cultured in ischemic conditions – 1% O_2 and lack of nutrients (medium without serum and without glucose) for 24h. The media from control cardiomyocytes as well as injured ones (HGD) and cardiomyocytes after 1% O_2 alone and the lack of

nutrients alone will be transfer on UC-MSCs, which were preconditioned with hypoxia/hypoxia mimetics for 24h. The properties of UC-MSCs will be tested after 6h or 24h according to the scheme (Fig. 3).

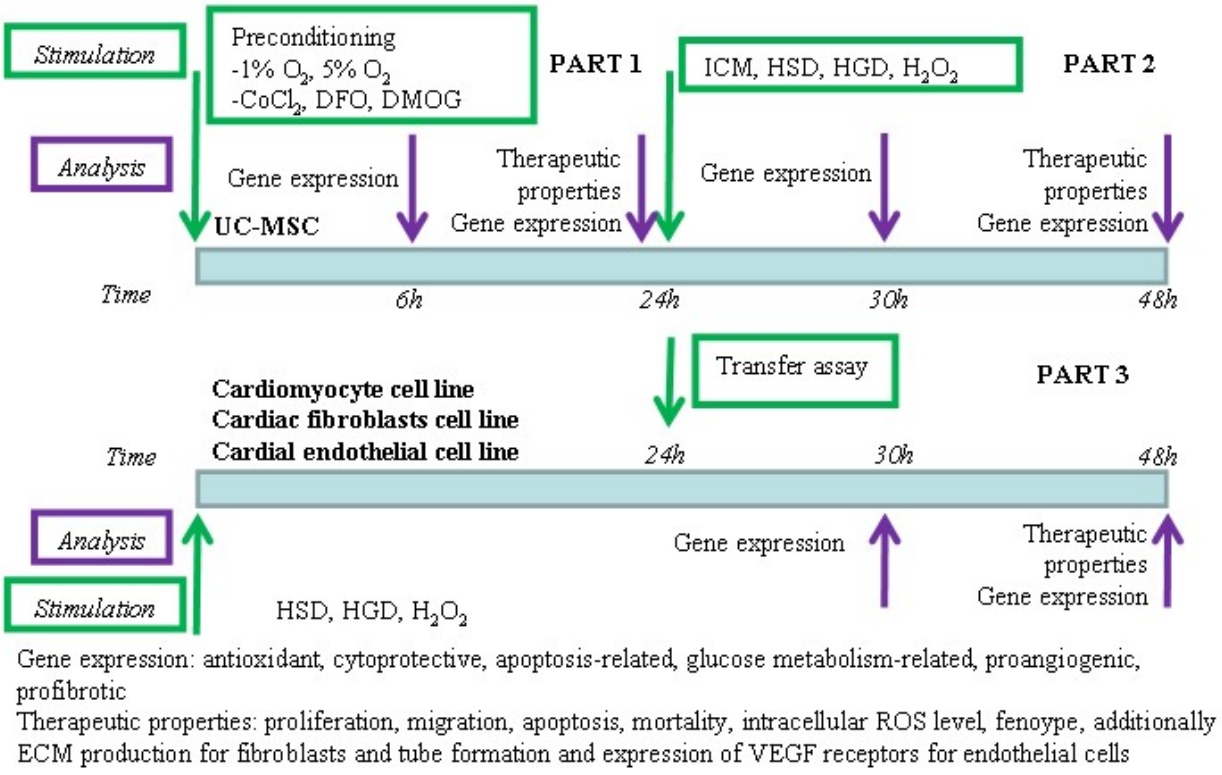

Fig. 3 Scheme of experiments. UC-MSCs will be cultured in different hypoxia conditions as well as in the presence of hypoxia imitating compounds. Cell properties will be analysed and culture media will be used for transfer assays using cardiomyocyte, cardiac fibroblast and cardiac microvascular endothelial cell lines as indicated.

During the analysis of **the paracrine effect of preconditioned UC-MSCs,** transfer assays using different cell types will be performed. Recipient cells will be cultured in HSD and HGD conditions as well as with H_2O_2. ELISA tests will be used on of transferred media for identification of factors, which might be responsible for the protective action of UC-MSCs. For all cardiac cell lines cell death will be assessed (LDH release), apoptosis (Annexin V staining), cell proliferation (BrdU incorporation), intracellular ROS level (DCHF-DA) as well as gene expression of apoptosis-related, cytoprotective, glucose metabolism-related, profibrotic and antioxidant factors (PUMA, p53, Mdm2, frataxin, HSPs, Nrf2, PDK1, Glut1, TGFβ1, TGFβ2, catalase,SOD2, GSTA1, real-time PCR). For endothelial cells, angiogenic potential will be verified using tube formation assay on Matrigel, and expression of angiogenenesis-related factors (VEGF receptors) will be assessed using real-time PCR. In case of fibroblasts, the expression of extracellular matrix (ECM) proteins (collagens, fibronectin) will be tested by real-time PCR (Fig. 3 part 3).

Verification of **the role of HIF-1α and HIF-2α in the improvement of therapeutic properties of UC-MSCs by preconditioning** will be done using 2 approaches: 1) pharmacological inhibitors of HIF-1 (Hypoxia inducible factor-1α Inhibitor - CAS 934593-90-5, or Chrysin) and HIF-2 (SML0883) and 2) siRNA targeting HIF-1α and HIF-2α, respectively. Pharmacological HIF inhibitors will be applied 1h before preconditioning. For choosing the siRNA transfection method with the highest efficacy, transfection with Lipofectamine® 2000[51], DharmaFECT® Transfection Reagent[52] and electroporation[53] will be tested. Transfection efficacy will be verified vs cells transfected with control siRNA on mRNA level by real-time PCR and on protein level by Western blot. The activity of HIFs will be measured using TransAM® HIF Transcription Factor ELISA Kit. Pharmacological HIF inhibitors inhibit HIF activity, whereas transduction using siRNA decreases mRNA level for these transcription factors.

UC-MSCs will be cultured on Primaria™ cell culture plates (Thermo Fisher Scientific). Cell death will be measured analysing LDH release to the culture medium (Pierce LDH Cytotoxicity Assay kit, Life Technologies). BrdU incorporation assay (Roche) will be utilized to analyze cell proliferation. Intracellular ROS level will be verified using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Paracrine activity of UC-MSCs will be tested measuring VEGF, bFGF, HGF, IGF-1, TGFβs and Epo in the culture medium by ELISA (R&D Systems). Cell apoptosis will be measured with Anexin V staining (Thermo Fisher

Scientific) using flow cytometry. HIF activation will be analyzed using TransAM® HIF Transcription Factor ELISA Kit (Active Motif). Cell phenotype will be confirmed using markers for UC-MSCs (CD29, CD90, CD105, CD34 and CD45) on mRNA level by real-time PCR and on the protein level by flow cytometry. Expression of apoptosis-related factors (p53, PUMA, Mdm2, Bcl2, Bax), proangiogenic factors (Epo, VEGF) and profibrotic factors (TGFβ1 and TGFβ2), cytoprotective factors (Nrf2, frataxin, HSPs), antioxidant enzymes (catalase, SOD2, GSTA1) and glycolysis-related genes (PDK1, Glut1) will be quantified on mRNA level using real-time PCR and on the protein level using western blot, immunofluorescent staining or ELISA. Cell migration will be examined using Transwell Migration Assay (Thermo Fisher Scientific). Transfection with siRNA against HIF-1α, HIF-2α and control siRNA (Thermo Fisher Scientific) will be done using Lipofectamine® 2000 (Thermo Fisher Scientific) and DharmaFECT® Transfection Reagent (Dhermacon). Endothelial cell tube formation will be assessed seeding these cells to Matrigel (Thermo Fischer Scientific). HIF-2 antaginist - SML0883 (Sigma-Aldrich) and HIF-1a inhibitors - CAS 934593-90-5 and Chrysin (Santa Cruz Biotechnology) will be utilized to inhibit HIFs. Statistical analysis will be done with one-way ANOVA and Bonferonni's test for multi-group comparison. Statistical analysis of comparing 2 independent groups will be done using t-test. Calculations will be done using Microsoft Excel, GraphPad Prism Demo and R Studio.

Equipment necessary for conducting this Project is available in the Department of Cell Biochemistry (DCB) at FBB&B JU or is planned to be bought in the budget of the Project (incubator with regulated oxygen level). DCB is equipped with instruments necessary for conducting this project, including centrifuges, Nanodrop (NanoDrop®ND-1000, NanoDrop Technologies), PCR systems (TProfessional Basic ThermoCycler, Biometra) and Real-Time PCR systems (Illumina EcoTM) for RNA expression analysis, flow cytometer (for the analysis of cell phenotype and Annexin V staining, BD FACSCalibur™), laminars, inverted light microscope (Nikon Eclipse TS100), fluorescent microscope (Leica DM IL LED), TC20™ automated cell counter (Bio-Rad) and incubator chambers for cell culture as well as electroporation system for cell transfection (Gene Pulser® II Electroporation System, Bio-Rad) with additional modules: the capacitance extender (CE module, Bio-Rad) and the pulse controller (PC module, Bio-Rad). DCB at the FBB&B JU also possess the Synergy H1 Hybrid Multi-Mode Reader (BioTek), electrophoresis system for DNA, RNA and protein analysis (Bio-Rad), system for gel visualization (Polygen), Trans-Blot Turbo Transfer System (Bio-Rad) for transfer during western blot and western blot and chemiluminescence imaging system (Fusion Fx, VILBER). Thus all equipment necessary for conducting this research is available for applicant at FBB&B JU.

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