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Influence of oxidative stress on bone marrow cells – characterisation of mesenchymal stromal cells lacking *Hmox1*

Wpływ stresu oksydacyjnego na komórki szpikowe – charakterystyka komórek zrębu tkankowego pozbawionych *Hmox1*

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3. Abstract

Diabetes can be associated with endothelial dysfunction and decrease in number and activity of various proangiogenic cells mobilized from the bone marrow. It has been shown in a mouse model, that heme oxygenase 1 (HO-1, *Hmox1*), the stress inducible enzyme degrading heme to biliverdin, carbon monoxide (II) and ferrous ion Fe^{2+}, is an important protein that influences viability and functions of both endothelial cells and bone marrow-derived proangiogenic cells. The aim of this work was therefore to evaluate how the stress factors, such as oxidative stress and hyperglycemia, as well as chronic complications in patients with type 2 diabetes and exercise in patients with intermittent claudication influence the mobilization and functions of subpopulations of proangiogenic bone marrow cells and what can be a role played there by HO-1.

We found that stress related to the exercise and muscle ischemia in intermittent claudication patients leads to increase in the number of circulating $CD45^{dim}CD34^+CD133^+KDR^+$ cells, decrease in *HMOX1* expression in leukocytes and decrease in tumour necrosis factor-α (TNFα) level in plasma. Three-month treadmill training programme resulted in these patients in decreased expression of tissue inhibitor of plasminogen activator-1 (tPAI-1), while single exercise, both before and after the training programme, led to the decreased plasma concentration of monocyte chemoattractant protein-1 (MCP-1) and increased superoxide dysmutase-1 (*SOD1*) expression in leukocytes.

Patients with type 2 diabetes were characterized with decreased numbers of circulating cells with proangiogenic, mesenchymal or hematopoietic phenotype, decreased expression of *HMOX1* and increased *HMOX2* level in leukocytes. Type 2 diabetes complications, especially diabetic foot syndrome, were associated with enhanced *HMOX1* expression in leukocytes and increased numbers of circulating $CD45^{dim}CD31^+CD133^+$ proangiogenic, $CD45^-CD29^+CD90^+$ mesenchymal, and $Lin^- CD45^+CD133^+$ hematopoietic cells. Regardless of the presence of complications, all type 2 diabetes patients were characterized with decreased catalase expression and increased plasma TNFα.

Murine bone marrow-derived proangiogenic cells either isolated from diabetic animals or lacking heme oxygenase 1 show decreased angiogenic potential and paracrine activity. Therefore, we checked whether pharmacological stimulation of

HMOX1 expression could enhance proangiogenic activity of human peripheral blood CD34$^+$ cells mobilized from the bone marrow with granulocyte colony stimulating factor (G-CSF) treatment. Sorted CD34$^+$ cells were cultured in serum-free defined medium and stimulated with atorvastatin, acetylsalicylic acid, sulforaphane, resveratrol or metformin. Only sulforaphane, known Nrf2 transcription factor activator, increased *HMOX1* expression. However, we observed increased paracrine angiogenic activity *in vitro* only in media from cells stimulated with atorvastatin, hence this was not related to HO-1 activation. Moreover, despite significant effect observed *in vitro*, we did not confirm effectiveness of such treatment *in vivo,* in a Matrigel plug assay.

Decreased numbers of circulating cells with mesenchymal phenotype and lower *HMOX1* expression in patients with type 2 diabetes encouraged us to characterize murine bone marrow mesenchymal stromal cells (MSC) lacking *Hmox1*. Cells were harvested from murine long bones with collagenase type II treatment. Isolated cells were purified from contaminating hematopoietic fraction with MACS. *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* MSCs showed similar ability to form colonies of fibroblastoid cells in CFU-F assay, what suggests similar number of such cells in bone marrows of wild type and knock-out mice. Isolated *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* cells were then characterized with similar phenotype. Namely, they expressed CD29, CD49e, CD73, CD90, CD105, CD106, CD140a and were devoid of hematopoietic markers CD45, CD11b, Gr-1, and endothelial markers CD31 and CD34. Both cell types showed similar proliferation in normal and increased glucose concentration. What is more, *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* MSCs produced similar amounts of growth factors and inflammatory mediators: G-CSF, chemokine (C-X-C motif) ligand 1 (CXCL1), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), vascular endothelial growth factor (VEGF), and MCP-1. Also, both MSC types similarly differentiated to adipocytes and osteoblasts. Interestingly, undifferentiated MSCs *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* formed similar numbers of tube-like structures on Matrigel in both low and high glucose media. Endothelial differentiation of mesenchymal stromal cells led to the increase in expression of some endothelial markers such as *Kdr* and von Willebrand factor or improved binding of acetylated LDLs. However, it did not enhance MSC angiogenic properties either *in vitro* or *in vivo*.

All known cells devoid of functional heme oxygenase 1 tested so far have shown decreased viability in oxidative stress, especially in the presence of heme. Surprisingly, mesenchymal stromal cells, both *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* are not sensitive

to hemin in 50 μmol/L concentration, even though such dose leads to increased levels of cellular hydrogen peroxide. Pronounced cell death in $Hmox1^{-/-}$ but also $Hmox1^{+/+}$ cells is observed in 200 μmol/L hemin concentration. Interestingly, low sensitivity to hemin does not result from the disturbed heme transport since both cell types similarly uptake hemin from the media.

In order to clarify the mechanism of MSC $Hmox1^{-/-}$ resistance to hemin we analysed expression of genes related to antioxidant response, heme transport and synthesis in mesenchymal stromal cells and primary fibroblasts isolated $Hmox1^{+/+}$ or $Hmox1^{-/-}$ mice. MSCs, regardless of the $Hmox1$ levels, were characterized with higher extracellular superoxide dismutase ($Sod3$) expression than fibroblasts. MSC $Hmox1^{-/-}$ cells stimulated with hemin upregulated catalytic and modifier subunits of glutamate-cysteine ligase ($Gclc$ and $Gclm$), key enzyme for the glutathione synthesis, glutathione synthetase (Gss) and reductase (Gsr), what altogether may lead to the increased level of reduced glutathione. What is more, $Hmox1^{-/-}$ cells in response to hemin treatment increased expression of peroxiredoxin 6 ($Prdx6$), the only member of peroxiredoxin family that uses glutathione instead of thioredoxin. $Hmox1^{-/-}$ MSCs, in response to hemin, upregulated expression of the receptor for feline leukemia virus subgroup C ($Flvcr1$), known heme exporter. Finally, both in MSCs and fibroblasts, hemin decreased expression of δ-aminolevulinate synthase 1 ($Alas1$) but not levels of mRNA of other heme synthesis pathway genes or heme importers.

To sum up, type 2 diabetes is associated with lower heme oxygenase-1 expression and decreased numbers of circulating progenitor cells. Complications of diabetes change the profile of circulating cells what undermines the feasibility to use number of such cells to assess progress of the disease. Lack of heme oxygenase 1 impairs paracrine angiogenic potential of bone marrow proangiogenic cells, but pharmacological enhancement of $HMOX1$ expression in human CD34$^+$ cells does not improve their paracrine activity. Contrary to the bone marrow proangiogenic cells, mesenchymal stromal cells devoid of $Hmox1$ gene do not show signs of impaired functions and differentiation. Their high resistance to stress may result from increased expression of glutathione pathway genes.

4. Streszczenie

Cukrzyca może wiązać się z dysfunkcją komórek śródbłonka oraz z obniżeniem liczby i aktywności krążących komórek proangiogennych mobilizowanych ze szpiku. Wcześniejsze badania wykonane u myszy wykazały, że ważnym białkiem ułatwiającym zachowanie żywotności i funkcji zarówno komórek śródbłonka jak i proangiogennych komórek szpikowych jest oksygenaza hemowa-1 (HO-1, *Hmox1*), enzym indukowany odpowiedzi na stres, katalizujący reakcję rozkładu hemu do biliwerdyny, tlenku węgla (II) i jonów Fe^{2+}. Celem prezentowanej pracy było sprawdzenie na ile czynniki stresowe takie jak stres oksydacyjny i hiperglikemia oraz chroniczne powikłania rozwijające się u pacjentów z cukrzycą typu 2 i wysiłek fizyczny u pacjentów z chromaniem przestankowym wpływają na mobilizację i funkcje subpopulacji proangiogennych komórek szpikowych oraz na ile wpływ ten jest modyfikowany przez HO-1.

Stres związany z wysiłkiem fizycznym i niedotlenieniem mięśni u pacjentów z chromaniem przestankowym, zespołem objawowym towarzyszącym miażdżycy naczyń obwodowych, prowadził do zwiększenia liczby krążących komórek proangiogennych $CD45^{dim}CD34^+CD133^+KDR^+$ oraz obniżenia ekspresji *HMOX1* w leukocytach i poziomu czynnika nekrozy nowotworów α (TNFα) w osoczu. U pacjentów po trzymiesięcznym treningu na bieżni zaobserwowaliśmy obniżenie poziomu tkankowego inhibitora aktywatora plazminogenu 1 (tPAI-1), zaś jednorazowy wysiłek fizyczny zarówno przed jak i po cyklu treningowym wiązał się ze spadkiem poziomu białka chemotaktycznego monocytów 1 (MCP-1) w osoczu i zwiększeniem ekspresji dysmutazy ponadtlenkowej 1 (SOD1) w leukocytach krwi obwodowej.

U pacjentów z niepowikłaną cukrzycą typu 2 stwierdziliśmy obniżoną liczbę krążących komórek o fenotypie proangiogennym, mezenchymalnym i hematopoetycznym oraz zmniejszoną ekspresję *HMOX1* i podniesiony poziom *HMOX2* w leukocytach. Powikłania cukrzycy, w szczególności zespół stopy cukrzycowej, wiązały się z nasileniem ekspresji *HMOX1* oraz zwiększeniem liczby krążących komórek proangiogennych $CD45^{dim}CD31^+CD133^+$, mezenchymalnych $CD45^-CD29^+CD90^+$ i hematopoetycznych $Lin^-CD45^+CD133^+$. Niezależnie od obecności lub braku powikłań, u pacjentów z cukrzycą typu 2 zaobserwowaliśmy obniżoną ekspresję katalazy w leukocytach i podniesiony poziom TNFα w osoczu.

Mysie szpikowe komórki proangiogenne izolowane od zwierząt z cukrzycą lub pozbawione funkcjonalnego genu *Hmox1* mają obniżony potencjał angiogenny

i zaburzoną aktywność parakrynną. Postanowiliśmy zatem sprawdzić czy zwiększenie ekspresji *HMOX1* za pomocą stymulacji farmakologicznej może nasilić potencjał angiogenny ludzkich komórek CD34$^+$ mobilizowanych do krwi obwodowej czynnikiem stymulującym tworzenie kolonii granulocytów (G-CSF). W tym celu, sortowane komórki CD34$^+$, hodowane w zdefiniowanych mediach bezsurowiczych, stymulowaliśmy atorwastatyną, kwasem acetylosalicylowym, sulforafanem, resweratrolem lub metforminą. Okazało się, że jedynie sulforafan, aktywator czynnika transkrypcyjnego Nrf2, podnosił w nich ekspresję *HMOX1*. Jednak nasilenie parakrynnej aktywności angiogennej zaobserwowaliśmy *in vitro* tylko w przypadku mediów znad komórek stymulowanych atorwastatyną, co wskazuje że efekt ten nie zależał od aktywacji HO-1. Ponadto, mimo istotnego wpływu zaobserwowanego w komórkach hodowanych *in vitro*, efekt ten nie był widoczny *in vivo* w mysim modelu podskórnych implantów matriżelowych.

Niższa liczba krążących komórek o fenotypie mezenchymalnym oraz obniżona ekspresja oksygenazy hemowej-1 w leukocytach pacjentów z cukrzycą typu 2 skłoniły nas do sprawdzenia właściwości mysich mezenchymalnych komórek zrębu (MSC) pozbawionych funkcjonalnego genu *Hmox1*. Komórki te uzyskiwaliśmy poprzez trawienie kolagenazą typu II fragmentów mysich kości udowych i piszczelowych. Komórki MSC były następnie oczyszczane z frakcji hematopoetycznej CD45$^+$ za pomocą metody MACS. Wykazaliśmy, że komórki szpikowe *Hmox1*$^{+/+}$ i *Hmox1*$^{-/-}$ miały podobną zdolność do tworzenia kolonii komórek fibroblastoidalnych (CFU-F), co świadczy o porównywalnej liczbie MSC. Komórki MSC *Hmox1*$^{+/+}$ i *Hmox1*$^{-/-}$ miały taki sam fenotyp tj. wykazywały ekspresję markerów powierzchniowych CD29, CD73, CD90, CD49e, CD105, CD106 i CD140a przy jednoczesnym braku markerów hematopoetycznych CD45 i CD11b oraz śródbłonkowych CD31 i CD34. Ponadto MSC *Hmox1*$^{+/+}$ oraz *Hmox1*$^{-/-}$ charakteryzowała podobna proliferacja, zarówno w warunkach kontrolnych jak i w zwiększonym stężeniu glukozy. Niezależnie od poziomu ekspresji *Hmox1*, mysie szpikowe komórki MSC produkowały *in vitro* zbliżoną ilość czynników wzrostu i czynników zapalnych: G-CSF, interleukiny 6 (IL-6), CXCL1 (KC), czynnika hamującego białaczkę (LIF), chemokiny z rodziny CXC indukowanej lipopolisacharydem (LIX), czynnika wzrostu śródbłonka naczyniowego (VEGF) oraz MCP-1. Komórki MSC *Hmox1*$^{+/+}$ i *Hmox1*$^{-/-}$ podobnie różnicowały do adipocytów i osteoblastów. Różnicowanie komórek MSC do komórek śródbłonka prowadziło natomiast do otrzymania komórek wykazujących ekspresję niektórych markerów

śródbłonkowych takich jak Kdr i czynnik von Willebranda oraz do zwiększenia wiązania acetylowanych LDL, ale nie poprawiało właściwości angiogennych *in vitro* i *in vivo*. Nieróżnicowane komórki MSC *Hmox1*$^{+/+}$ i *Hmox1*$^{-/-}$ tworzyły podobną ilość struktur naczyniopodobnych na matriżelu zarówno w warunkach niskiego jak i wysokiego stężenia glukozy.

Wszystkie opisywane w publikacjach komórki pozbawione genu *Hmox1* mają obniżoną żywotność w warunkach stresu oksydacyjnego, zwłaszcza wywołanego obecnością hemu. Nasze badania nieoczekiwanie wykazały, że komórki mezenchymalne zarówno *Hmox1*$^{+/+}$ jak i *Hmox1*$^{-/-}$ nie są wrażliwe na toksyczne działanie heminy, nawet podanej w stężeniu 50 μmol/L, mimo że wzrasta w nich poziom nadtlenku wodoru. Zwiększona śmiertelność komórek MSC *Hmox1*$^{-/-}$ oraz *Hmox1*$^{+/+}$ występuje dopiero przy stężeniu 200 μmol/L. Niska wrażliwość komórek *Hmox1*$^{-/-}$ na heminę nie wynika z zaburzonego transportu hemu, gdyż komórki MSC obu genotypów w równym stopniu pobierają hem z pożywki.

Aby wyjaśnić oporność MSC *Hmox1*$^{-/-}$ sprawdziliśmy ekspresję genów związanych ze stresem oksydacyjnym oraz transportem i syntezą hemu w MSC i fibroblastach izolowanych z myszy *Hmox1*$^{+/+}$ lub *Hmox1*$^{-/-}$. MSC, niezależnie od poziomu oksygenazy hemowej-1 i stymulacji heminą, wykazywały wyższy poziom zewnątrzkomórkowej dysmutazy ponadtlenkowej (Sod3) niż fibroblasty. Komórki MSC *Hmox1*$^{-/-}$ w obecności heminy zwiększały ekspresję podjednostki katalitycznej i modyfikującej ligazy glutamylo-cysteinowej (Gclc i Gclm) – enzymu kluczowego dla syntezy glutationu, syntetazy glutationu (Gss) oraz reduktazy glutationowej (Gsr), co może prowadzić do zwiększenia puli zredukowanego glutationu. Ponadto, w komórkach MSC *Hmox1*$^{-/-}$ pod wpływem heminy rosła ekspresja peroksyredoksyny 6 (Prdx6) – jedynej peroksyredoksyny wykorzystującej glutation jako czynnik redukujący, oraz ekspresja eksportera hemu – receptora wirusa kociej białaczki podgrupy C (FLVCR). Zarówno w MSC jak i w fibroblastach stymulacja heminą obniżała ekspresję syntazy δ-aminolewulinianu 1 (Alas1), nie wpływała zaś na geny enzymów syntezy hemu i importerów hemu.

Podsumowując, cukrzyca typu 2 wiąże się z obniżeniem ekspresji oksygenazy hemowej-1 oraz zmniejszeniem liczby krążących komórek progenitorowych. Powikłania cukrzycy zmieniają profil krążących komórek, co podważa możliwość stosowania ich liczby jako markera postępu choroby. Brak oksygenazy hemowej-1 zaburza parakrynną aktywność komórek szpikowych, ale farmakologiczne zwiększenie

jej poziomu w ludzkich komórkach CD34$^+$ nie poprawia ich potencjału proangiogennego. W przeciwieństwie do szpikowych komórek proangiogennych, komórki mezenchymalne pozbawione oksygenazy hemowej-1 nie wykazują zaburzeń funkcji i różnicowania, a ich duża oporność na stres oksydacyjny jest związana z podniesieniem ekspresji genów szlaku glutationu.

5. Introduction

Stem and progenitor cells are considered promising tools for both diagnostics and regenerative medicine. Transplantation of bone marrow or mobilized hematopoietic stem and progenitor cells become a routine procedure used to treat many diseases. What is more, there are currently many clinical trials in which efficiency of various types of stem cells is assessed in the regeneration of damaged organs. Especially, numerous studies focus on cells that were described as multipotent, thus able to differentiate to many cell types, *i.e.* on so called mesenchymal stem or stromal cells. However, still little is known on how different stress factors, especially diseases such as diabetes and its complications or atherosclerosis, affect numbers of various populations of circulating cells. Therefore, we tested numbers of circulating proangiogenic cells (PAC), mesenchymal stromal cells (MSC) and hematopoietic stem cells (HSC) in patients with different types of diabetic foot syndrome – common complication of type 2 diabetes and numbers of proangiogenic cells in atherosclerotic patients after the exercise that can lead to ischemia and reperfusion.

Many clinical trials are unsuccessful because transplanted cells are vulnerable and do not survive in the recipient. One of the strategies that is often exploited in order to increase survival of transplanted cells is overexpression of cytoprotective enzyme – heme oxygenase-1. However, one must take into the consideration that changed expression of HMOX1 can affect a plethora of cell features, especially stem cell differentiation [1], and even lead to tumorigenesis [2]. Noteworthy, expression of heme oxygenase-1 and its changes in response to stress factors differ in the human population because of *HMOX1* promoter polymorphism [3]. Moreover, some diseases, especially diabetes, can also lead to the decreased expression of heme oxygenase-1 [4]. Therefore, taking into consideration that aforementioned mesenchymal stromal cells are key elements of niches for other stem cells i.e. hematopoietic stem cells, we decided to investigate the role of HO-1 in MSCs. To this aim, we characterized mesenchymal stromal cells isolated from mice lacking functional *Hmox1* gene.

5.1. Diabetes and its complications.

Tight control of glucose levels is a prerequisite to maintain body homeostasis. Blood levels of glucose are regulated by pancreas that releases insulin and glucagon – hormones, which lower or increase blood glucose concentration, respectively.

Dysfunctions of insulin production or changed tissue response to insulin lead to the prolonged increase in blood glucose concentration, known as diabetes mellitus. Type 1 diabetes mellitus (T1DM), previously called insulin-dependent diabetes, is an autoimmune disease that results from destruction of β-cells, which produce insulin. Type 2 diabetes mellitus (T2DM), also known as noninsulin-dependent diabetes, is primarily caused by tissue unresponsiveness to the insulin. However, type 2 diabetes also leads to the decrease in β-cell function [5]. There are also rare types of inherited monogenic diabetes, so called MODY (maturity onset diabetes of the young) type, caused by mutations *i.e.* in *HNF4A, HNF1A* or *GCK* genes.

Prevalence of diabetes in Poland is 9.2% and is higher than average prevalence in OECD countries [6]. Poorly controlled glycemia in diabetic patients may lead to the development of various complications, of which many are of cardiovascular or neurological origin. Moreover, neuropathy, peripheral arterial disease and foot trauma can further cause various forms of diabetic foot syndrome (DFS). DFS develops in 12-25% of diabetic patients and is a frequent cause of amputations. It can manifest as Charcot osteoneuropathy or ulcerations, which are often infected. The pathogenesis of DFS, in addition to well established risk factors i.e. long diabetes duration and chronic hyperglycemia [7], may be associated with alterations in stem or progenitor cell mobilization [8], changes in growth factors [9,10], and impairment in cellular antioxidant capacity [11,12] (Figure 1). However, little is known about how these factors may be influenced by different forms of DFS, especially when the ulcer is further complicated with the infection or when it is healed.

5.2. Circulating proangiogenic cells

Development of new blood vessels is necessary for the effective tissue regeneration. Therefore, the mechanisms which allow for the regulation of angiogenesis, are of great interest. The process of neovascularization can be influenced not only by the residual endothelial cells but also by the circulating stem and progenitor cells. Such proangiogenic cells (PACs) of bone marrow origin were known as endothelial progenitor cells (EPCs). They are enriched in the population co-expressing CD34, CD133, KDR with low or absent expression of pan-hematopoietic marker CD45 [13]. In the cell culture, they can bind *Ulex europeaus* lectin and acetylated low-density lipoprotein (LDL) [14]. However, the exact phenotype, origin and precise definition of endothelial progenitor cells still remain the matter of debate. Furthermore, the features

of endothelial progenitor cells *i.e.* phenotype, expression of endothelial markers and ability to form tube-like structures on Matrigel, can be mimicked by blood monocytes [15]. Interestingly, peripheral blood monocytes can take up platelet microparticles released during standard monocyte isolation procedures. Such uptake can lead to the acquisition of endothelial markers *i.e.* von Willebrand factor, CD31, and ability to bind lectin by monocytes [16]. Therefore, cells named endothelial progenitor cells are considered now to be rather proangiogenic monocytes than endothelial precursors [17]. This change is not of only nomenclature significance but indicates for the different mechanisms of action, necessary to be investigated for better translational application.

Circulating proangiogenic cells can support neovascularization through different mechanisms: differentiating to the endothelial-like cells or through paracrine stimulation of endothelial cells *in situ*. The latter mechanism, however, seems to be the most important. Proangiogenic cells produce more vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF), than differentiated human umbilical cord endothelial cells (HUVEC). Moreover, conditioned media from PACs better induce endothelial cell migration than those from HUVECs [18]. Interestingly, HUVEC cells treated with the endothelial progenitor conditioned media upregulated antioxidant genes coding for catalase, copper/zinc superoxide dismutase, manganese superoxide dismutase and were more resistant to oxidative stress and apoptosis [19]. What is more, endothelial progenitors produce proangiogenic microvesicles, which improve vascularization of pancreatic islets [20] or ischaemic hind limbs in animal model [21] and protect kidneys from ischaemia-reperfusion injury [22] with the transfer of pro-angiogenic miR-126 and miR-296. Microvesicles derived from proangiogenic cells can also induce angiogenic program in endothelial cells with the transfer of mRNA encoding for transcripts involved in PI3K/Akt pathway [23].

5.3. Effects of diabetes on circulating proangiogenic cells [24]

The decreased numbers of circulating proangiogenic cells, assessed with flow cytometry or with colony forming unit (CFU) assay, were reported in patients with type 1 [25] or type 2 diabetes [8,26-28]. Similar effect was observed in animal models: streptozotocin-treated mice and rats [29] for type 1 diabetes or *db/db* mice [30,31] and Zucker Diabetic Fatty rats [32,33] for type 2 diabetes. Importantly, decreased number of circulating CD34$^+$ cells can be found already in people with impaired glucose tolerance

[28,34,35] or in rats with metabolic syndrome [32]. Moreover, other populations, such as MSCs or HSCs, which can contribute to revascularization and wound healing [36-39] can be influenced. MSCs, which are precursors of connective tissue cells i.e. fibroblasts, osteoblasts and chondrocytes [40], are of special interest in the Charcot foot that affects both bones and joints [41]. What is more, the role of very small embryonic-like stem cells (VSEL), claimed to be pluripotent and mobilized in stroke, severe burns and acute myocardial infarction (reviewed in [42]), has not been yet studied in the complications of type 2 diabetes. Low number of circulating progenitor cells can result from both cell extrinsic and intrinsic factors.

Cell extrinsic factors influencing circulating progenitor cells in diabetes

Numbers and function of circulating progenitors can be influenced by the metabolites found in diabetic patients as well as by the changed stem cell niche. First of all, oxidized small and dense LDLs from diabetic patients induced in healthy PACs activation of Akt, accumulation of p53, upregulation of p21 and dephosphorylation of retinoblastoma protein [43]. Moreover, such cells were characterized with increased reactive oxygen species (ROS) levels as well with decreased expression of manganese superoxide dismutase while catalase remained unchanged. Interestingly, proangiogenic cells from type 2 diabetes patients showed activation of Akt/p53/p21 pathway and accelerated senescence [43]. Furthermore, advanced glycation end products (AGE), formed in diabetic patients due to the high levels of blood glucose and increased oxidative stress [44], can also influence circulating proangiogenic cells. Accordingly, PACs isolated from healthy donors showed increased apoptosis, decreased Bcl2 and increased Bax, NFκB, COX2 and caspase 3 mRNA when stimulated with 200 mg/L AGE. What is more, stimulation with AGE decreased both endothelial nitric oxide synthase (NOS3) expression and nitric oxide (NO) release in healthy PACs [45].

However, the greater impact on the numbers of circulating proangiogenic cells seems to have altered bone marrow stem cell niche that can be affected by both neuropathy and vasculopathy. Signals from sympathetic innervation [46,47] as well as circadian oscillations [48] are crucial for correct egress of various bone marrow cells to the bloodstream. Bone marrow of BBZDR/Wor type 2 diabetic rats contained less nerve fibers and expressed lower levels of genes involved in the regulation of circadian rhythm, i.e. *Per2* and *Bmal1* [49]. Diabetic rats showed decreased amplitude in circadian release of Thy1^{+}CD133^{+} progenitor cells, accompanied with higher number of

such cells in bone marrow. Moreover, cells from diabetic bone marrow formed less colonies and were characterized with decreased proliferation and migration [49]. In another study, number of circulating $CD34^+$ cells was decreased in diabetic patients with diabetic autonomic neuropathy in comparison to diabetic patients without such complication [50]. Also in mouse models of both type 1 and type 2 diabetes numbers of nerve endings positive for tyrosinase hydroxylase were decreased. Mobilization of Lin^- $Sca1^+ckit^+$ hematopoietic stem and progenitor cells as well as $CD34^+Kdr^+$ proangiogenic cells in response to granulocyte colony stimulating factor (G-CSF) or hind limb ischemia was reduced in type 1 and type 2 diabetic mice and in sympanectomized animals. Decreased mobilization of $Lin^-Sca1^+ckit^+$ cells in response to G-CSF could be rescued with desipramine – inhibitor of norepinephrine reuptake. The effect of neuropathy on stem/progenitor mobilization was related to the increase in p66Shc and L-selectin expression and reduction of Sirt1 [50]. On the other hand, Ferraro and co-workers showed that diabetes alters bone marrow stem cell niche what is associated with increased innervation and sympathetic hyperactivity that leads to aberrant CXCL12 regulation [51].

Type 2 diabetes patients as well as streptozotocin-treated mice or *db/db* mice were characterized with decreased mobilization of hematopoietic stem and progenitor cells (HSPC) in response to G-CSF treatment. Moreover, there were more HSPC cells in the bone marrow of diabetic mice, but those cells showed impaired migration to CXCL12 gradient and increased adhesion to fibronectin, while having up-regulated L-selectin. Bone marrow of diabetic patients and mice contained decreased numbers of osteoblasts but numbers of $Nestin^+$ MSCs remained unchanged. Furthermore, diabetic osteoblasts in steady state expressed more Kit ligand, what may lead to decreased egress of progenitor cells to the bloodstream. Decrease in niche levels of CXCL12 is crucial for stem cell mobilization from the bone marrow [46]. Noteworthy, $Nestin^+$ mesenchymal stromal cells in diabetic mice exhibited unchanged levels of CXCL12 in response to G-CSF, whereas it decreased in healthy bone marrow [51].

While low level of CXCL12 in the niche is necessary for progenitor cell egress, high concentration of CXCL12 in the blood or in the site of injury ensures proper stem cell mobilization and homing. Dipeptidylpeptidase IV (DPPIV), also known as CD26, is an enzyme that has an ability to cleave dipeptides from CXCL12 and CCL22 chemokines [52]. Inhibition of DPPIV activity during G-CSF-mediated mobilization of hematopoietic stem and progenitor cells in mice results in decreased numbers of cells

released from the bone marrow [53]. Serum activity of DPPIV is elevated in diabetic patients and correlates with $Hb1_{Ac}$ levels [54]. Therefore, it may decrease activity of plasma CXCL12 and inhibit stem and progenitor mobilization to the blood. In fact, sitagliptin, inhibitor of DPPIV activity, increased numbers of circulating $CD34^+KDR^+$ proangiogenic cells in T2DM patients and plasma concentrations of CXCL12 [27]. Importantly, mice treated with plerixafor, partial agonist of CXCR4 (CXCL12 receptor), increased serum levels of CXCL12 and improved wound healing in diabetic mice [55].

Another factor crucial for mobilization of stem and progenitor cells form the bone marrow to the bloodstream is nitric oxide [56] and its synthesis can be influenced by sympathetic innervation [57]. Expression of endothelial nitric oxide synthase by diabetic bone marrow stroma of streptozotocin-treated mice is decreased [58]. On the other hand, Gallagher and co-workers showed, that total bone marrow endothelial NOS was unchanged but its phosphorylation was significantly decreased in the mouse model of type 1 diabetes [59].

Finally, numbers and function of circulating proangiogenic cells can be changed with pharmacological treatment administered to diabetic patients. First of all, insulin increases *in vitro* numbers of colonies formed by proangiogenic cells from diabetic patients [60]. However, neither NPH-insulin nor glargine, insuline analogues, changed numbers of circulating $CD34^+KDR^+$ proangiogenic cells while both increased their outgrowth *in vitro* [61]. Similarly, numbers of $CD34^+$ or $CD34^+CD133^+$ proangiogenic cells in culture could be increased when patients were treated with rosiglitazone – agonist of PPARγ nuclear receptor [62]. Numbers of $CD45^{-/low}CD34^+CD133^+KDR^+$ proangiogenic cells were increased in type 2 diabetes patients treated with perindopril – inhibitor of angiotensin-converting enzyme [63]. As mentioned before, sitagliptin, inhibitor of DPPIV used in incretin-based therapies in diabetes, increase numbers of circulating proangiogenic cells in diabetic patients [27].

Cell intrinsic factors influencing circulating progenitor cells in diabetes

Proangiogenic cells from type 2 diabetes patients show decreased proliferation in comparison to cells from healthy donors [64]. Importantly, proangiogenic cells isolated form healthy donors but cultured in high glucose concentration (20 mmol/L) showed impaired proliferation and increased apoptosis, what was linked to the suppressed Akt activation [65]. What is more, $CD34^+$ progenitor cells from T2DM

patients are characterized with decreased expression of miR-155 [66] and concomitant increase of proapoptotic FOXO3a – validated miR-155 target [67]. Upregulated and localized to the nucleus FOXO3a can then lead to the increase in p21 and p27^{Kip1}, inhibition of cell cycle progression and finally to the apoptosis [66]. Of note, miR-155 was shown in endothelial cells to target as well Bach1 - repressor of heme oxygenase-1 expression [68].
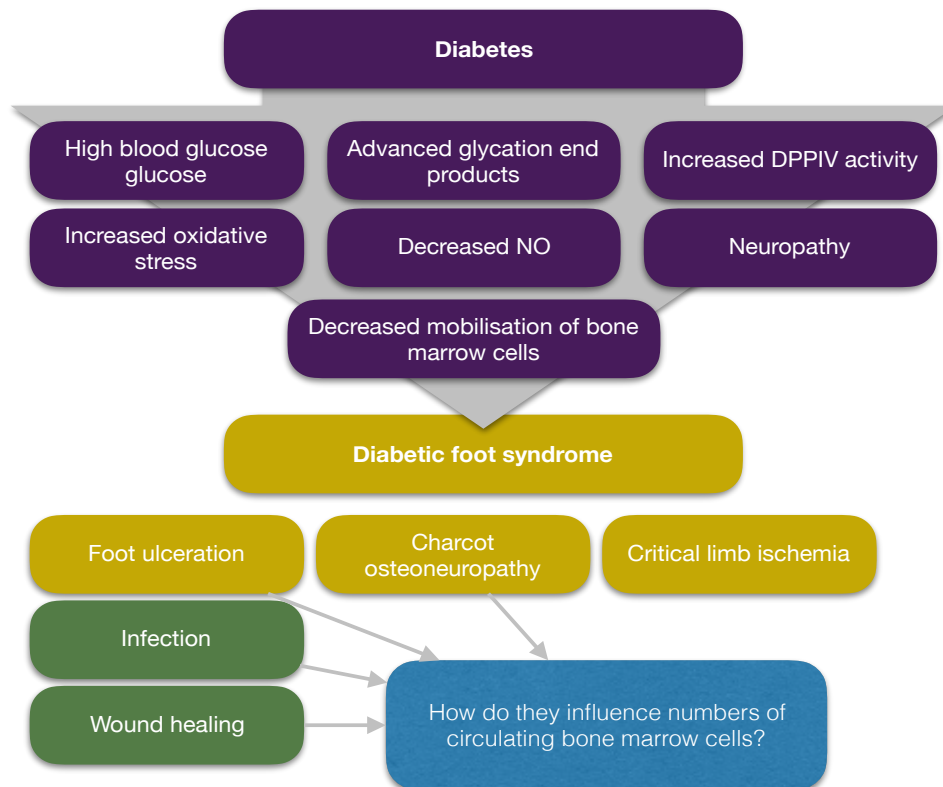
Figure 1. Factors contributing to the development of diabetic foot syndrome.

Proangiogenic cells isolated from peripheral blood of type 2 diabetes patients were characterized with decreased expression of miR-21, miR-27a, miR-27b, miR-126 and miR-130 [69]. Decrease in miR-126, which regulates Spred-1 expression, resulted in decreased proliferation, migration and increased apoptosis of PAC cells. Low miR-126 levels were associated also with inhibited PI3K/Akt/eNOS and Ras/Akt/VEGF pathways [69]. Furthermore, decreased expression of miR-130a in diabetic proangiogenic cells results in increased Runx3 gene expression, protein level and promoter activity. Inhibition of miR-130a decreased levels of VEGF, p-ERK and Akt1 in PACs, what can contribute to their dysfunction and decreased proliferation [70].

Proangiogenic cells isolated from *db/db* mice were less numerous than in control animals and characterized with decreased manganese superoxide dismutase expression and activity, what may lead to increased sensitivity to oxidative stress [30]. On the other hand, PAC cells from type 2 diabetes patients were shown to generate more superoxide anion radical while showing increased activity of NAD(P)H oxidase and superoxide dismutase and increased expression of both manganese and copper/zinc superoxide dismutases [71]. Such cells formed less colonies and produced smaller amounts of NO *in vitro* and were less abundant in peripheral blood of diabetic patients than in healthy controls [71]. Increased production of superoxide anion radical was also reported by Thum and co-workers in their publication, where PAC cells were characterized with protein kinase C activation and nitric oxide synthase uncoupling, related to the decreased tetrahydrobiopterin concentration [72]. Importantly, human proangiogenic cells from type 2 diabetes patients showed impaired adhesion to tumour necrosis factor-α (TNFα)-activated HUVEC cells as well as disturbed incorporation to tube-like structures *in vitro* [64].

5.4. Influence of exercise on circulating proangiogenic cells [73]

Intermittent claudication (IC), which is one of the most common manifestations of peripheral occlusive arterial disease (POAD), affects 12% of adult men between the ages of 55 and 74, and is associated with 30% of total mortality [74]. Risk of IC is increased by smoking, diabetes, hypertension or coronary heart disease [75]. Cycles of ischemia and reperfusion during the exercise and rest, respectively, may aggravate endothelial dysfunction, and contribute to the augmented activation of neutrophils [76,77], microalbuminuria [78] as well as increase in ROS [79], and subsequently worsen the atherosclerosis (Figure 2).

Since levels of plasma ROS scavengers are lower in IC patients [80], increase in ROS after the exercise, observed as well in healthy individuals [81], may be especially deleterious for the endothelium of claudicants. Furthermore, function and activity of immune cells, that are key players in development and progression of atherosclerosis, are regulated by microRNA, of which miR-146a and miR-155 seem to have high impact on lipid uptake, inflammatory cytokine and antioxidant response [82,83]. Finally, POAD patients have lower levels of circulating proangiogenic cells (CD34$^+$KDR$^+$ and CD34$^+$CD133$^+$) than healthy controls.
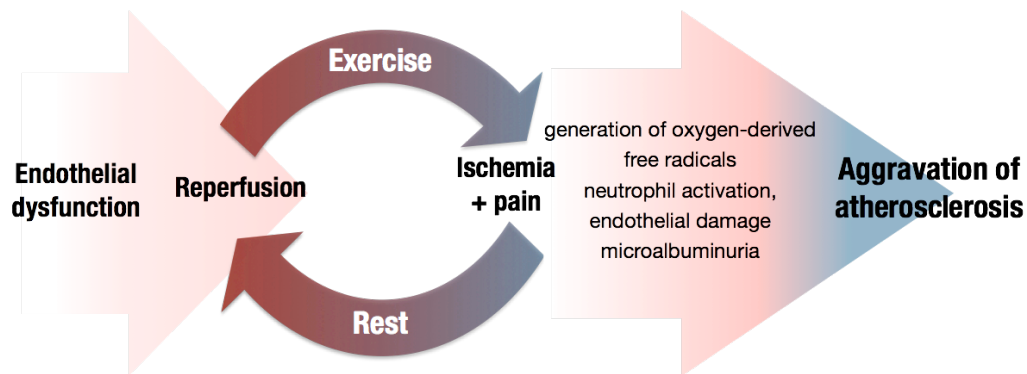
Figure 2. Mechanism for atherosclerosis aggravation induced by exercise in intermittent claudication patients.

Since 1960's supervised exercise training is considered as effective and low-cost therapy for IC [84]. However, still little is known about the influence of the training on the leukocyte antioxidant gene and microRNA response, while data on the mobilization of proangiogenic progenitor cells, and plasma levels of growth factors or inflammatory mediators are inconsistent.

In healthy volunteers increase in cells able to give rise of colonies able to bind *Ulex europeaus* lectin and acetylated LDL was observed already after 5-10 minutes after the exercise [85]. In other studies at least 30 minutes of exercise were necessary to increase numbers of circulating proangiogenic cells [86] and, although numbers of $CD34^+KDR^+$ cells were higher 10 minutes after the exercise, there was no difference in numbers of colony forming units [87]. Regular training could also increase numbers of proangiogenic cells in middle age and older men [88]. However, extreme exercise, such as marathon, could decrease PACs in runners in advanced age [89].

Supervised training was shown to increase numbers of circulating proangiogenic cells in various diseases. In patients with chronic heart failure (CHF) PAC numbers increased after 8 weeks of the training and returned to the baseline when training was discontinued [90]. Similarly, increase in PACs was observed in CHF patients even after shorter period [91]. In other study single exercise did not influence numbers but rather migration of circulating angiogenic cells in CHF patients [92]. Twelve week supervised running training increased number of circulating $CD34^+CD133^+KDR^+$ cells in patients with coronary artery disease [93]. Maximal treadmill exercise increased numbers of $CD34^+KDR^+$ cells in both healthy controls and peripheral occlusive artery disease patients, but increase was lower in the latter group [90].

5.5. Heme oxygenase-1

Heme oxygenase-1 (encoded by the *HMOX1* gene) is an enzyme degrading heme to carbon monoxide (CO), biliverdin and Fe^{2+} ions. Apart from its enzymatic activity heme oxygenase-1 influences cell survival, resistance to the oxidative stress and angiogenesis (Figure 3) [94]. We have recently shown that proangiogenic cells isolated from the bone marrow of *Hmox1* knock-out mice present impaired proliferation, migration and formation of capillaries [4]. What is more, overexpression of heme oxygenase-1 can lead to block of differentiation, i.e. in myoblasts [2].
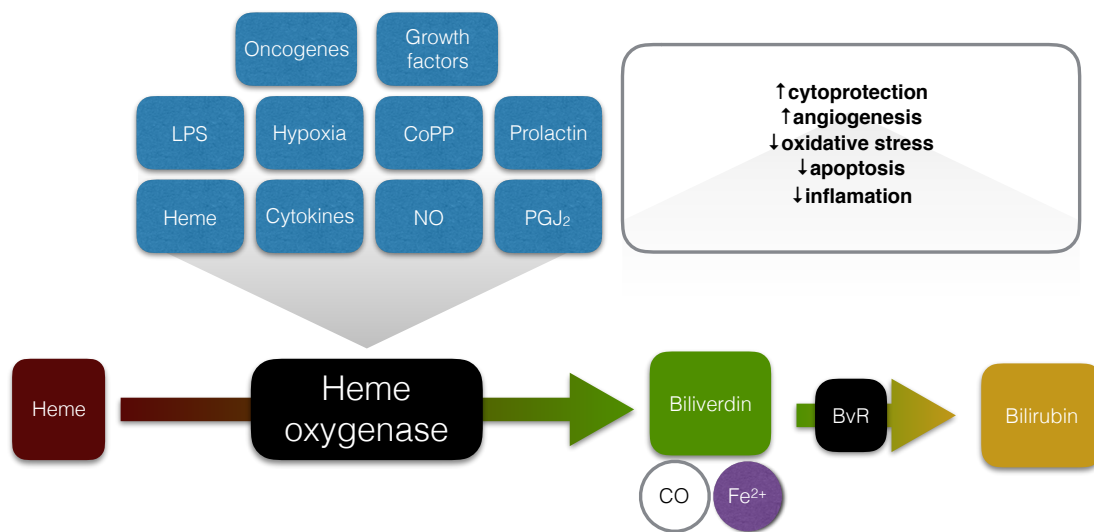
Figure 3. Induction of heme oxygenase-1 and its cytoprotective activity (based on the **[94]**).

5.6. Heme oxygenase-1 and Nrf2 in proangiogenic cells

Lack of heme oxygenase-1 or its low level can affect numbers and function of murine bone marrow proangiogenic cells. Numbers of Lin⁻CD45⁻ckit⁻Sca1⁺VEGFR2⁺ cells were lower in the bone marrow of *Hmox1⁻/⁻* mice [4]. *Hmox1⁻/⁻* PAC cells showed increased sensitivity to the hemin treatment in both low and high glucose conditions. Such cells were characterized as well with decreased proliferation, impaired migration in response to the complete medium and worse formation of capillaries in spheroid assay. All proangiogenic cells, regardless of the levels of *Hmox1*, were characterized with decreased paracrine angiogenic activity tested with Matrigel assay when cultured in hyperglycemic conditions. However, both paracrine proangiogenic potential *in vivo* and up-regulation of pro-angiogenic genes in hypoxia are reduced in the cells lacking heme oxygenase-1. The recovery of the blood flow was weaker in the ischemic limbs of

the diabetic mice, which received injections of the conditioned media from the *Hmox1*$^{-/-}$ bone marrow-derived proangiogenic cells in comparison to the media from wild type cells [4]. Furthermore, heme oxygenase-1 is crucial for the proangiogenic cell migration in response to the SDF-1 growth factor [95]. Proangiogenic cells isolated from *Hmox1*$^{-/-}$ mice showed impaired migration to the ischemic areas of injured retinas. Accordingly, in human and mouse mature endothelial cells heme oxygenase-1 was necessary for the SDF-1-induced migration [95].

Expression of heme oxygenase-1 can be induced by the nuclear factor E2-related factor 2 (Nrf2) transcription factor [96]. Bone marrow of *Nrf2*$^{-/-}$ mice contained less CD45$^-$Sca1$^+$ progenitor cells [97]. Such cells were furthermore less resistant to the treatment with H_2O_2, and showed decreased migration, proliferation and angiogenic properties *in vitro* than wild type control cells. What is more, *Nrf2*$^{-/-}$ proangiogenic cells did not upregulate VEGF in hypoxia. *In vivo,* proangiogenic cells *Nrf2*$^{-/-}$ attracted less host endothelial cells. However, *Nrf2*-deficient animals faster recovered blood flow in ischemic mice what was caused by increased inflammatory angiogenesis [97].

5.7. Statins as modulators of proangiogenic cell activity

Functions of proangiogenic cells can be changed with various drugs that are used currently in the clinic. Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase – an enzyme that controls production of cholesterol. Therefore, statins are a treatment of choice for lowering the cholesterol levels and normalization of high density lipoprotein (HDL)/LDL ratio.

Apart from their influence on the cholesterol synthesis, statins can also affect the biology of both endothelial cells and circulating PAC cells. First of all, statins enhance mobilization of proangiogenic cells from the bone marrow [98] *via* the PI3K/Akt pathway [99]. The positive effect of statins on the mobilization of proangiogenic cells was observed as well in the diabetes patients with atherosclerotic vascular disease [100], acute coronary syndromes, congestive heart failure, hypertension, hypercholesterolemia, vasoplastic anemia (reviewed in [101]). Surprisingly, longer administration of statins, i.e. longer than three months, could leads to the decrease in numbers of circulating proangiogenic cells [102]. Next, atorvastatin increases the proangiogenic cell proliferation and reduces their senescence [103] and levels of age-related reactive oxygen species [104]. Furthermore, proangiogenic cells pretreated with statins are more resistant to apoptosis caused by oxidized LDLs (oxLDLs) [105] and

homocysteine [106]. Stimulation of peripheral blood mononuclear cells with simvastatin enhanced the expression of endothelial markers and production of proangiogenic interleukin (IL) 8 [107], which seemed to be one of the most important PAC-derived paracrine mitogens for the mature endothelium [108]. Importantly, combined therapy with bone marrow-derived stromal cells and simvastatin was more effective in stroke patients than single therapies [109]. Finally, statins can induce expression of cytoprotective and proangiogenic heme oxygenase-1 via the ERK/p38 MAPK/PKG [110], C/EBPβ and AP-1 [111] in murine macrophages. The effect of statins on the heme oxygenase-1 expression in circulating CD34+ cells, enriched with proangiogenic cells, remains so far unknown.

Nobody so far has identified the factors responsible for the protective and proangiogenic effect of media from statin-stimulated CD34$^+$ cells. The proangiogenic paracrine activity of unstimulated PAC cells is well established and considered as the most important. However, the impact of statins on the production of released angiogenic cytokines and microRNAs needs to be elucidated. Moreover, heme oxygenase-1 plays an important role in the regulation of angiogenic properties of non-stimulated proangiogenic cells as well as endothelial cells. However, both the influence of statins on the heme oxygenase-1 in CD34$^+$ cells enriched with stem and progenitor cells (mainly of hematopoietic lineage), as well as its role in the regulation of proangiogenic paracrine effect of statins, have not been studied yet.

Therefore, there is a need for better understanding of molecular mechanisms, which may underlie impaired vascularization in patients with decreased *HMOX1* expression as well as angiogenic paracrine activity of peripheral blood proangiogenic cells. Importantly, the polymorphism of the *HMOX1* gene promoter affects expression of the heme oxygenase-1 in humans. Our team has shown that the long promoter alleles (with more GT repeats) result in the lower *HMOX1* expression [3]. Endothelial cells isolated from the people with the long promoter are more sensitive to oxidative stress and show lower proliferation response to VEGF than cells isolated from the people with short promoter alleles [3]. Hence, the influence of heme oxygenase-1 on the proangiogenic activity of cells, which may have potential clinical application, is an important subject of the studies.

Circulating proangiogenic cells, since their discovery by Asahara et al. in 1997 [14], have been an attractive subject of many research and clinical trials. However, biology of such cells, as well as their exact role, played in the site of vascular injury,

still remain unknown. On the other hand, statins are widely used drugs that may additionally influence biology of proangiogenic cells. Importantly, many people undergoing statin treatment are also those, who may be future recipients of prospective therapy with the proangiogenic cells or factors released from such cells. For that reason, enhancement of proangiogenic paracrine activity of autologous patient cells with known pharmaceuticals can have potential use for the therapy of diseases associated with impaired formation of blood vessels.

5.8. Mesenchymal stromal cells

Mesenchymal stromal cells (MSC), also known as mesenchymal stem cells, multipotent stromal cells or even medicinal stromal cells are heterogenous population of connective tissue cells that contain osteoblast and adipocyte progenitors, fibroblasts and smooth muscle cells [112]. Term "mesenchymal stem cells" was introduced in 1991 by A. Caplan [113]. However, cells with MSC characteristics were described by Friedestein already in 1968 [114]. In 2006 International Society for Stem Cell Therapy published the minimal criteria for human MSC that include adherence to the plastic in standard culture conditions, differentiation *in vitro* to adipocytes, osteoblasts and chondrocytes, and expression of CD73, CD90 and CD105 markers, while hematopoietic and endothelial markers CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR should be absent [115]. However, since that time many other markers for MSCs have been proposed: CD271 and CD106 [116], CD146 [117], nestin [118], Sca-1 and PDGFRα [119], leptin receptor LepR [120,121] or high expression of CXCL12 [122].

Importantly, the ability to differentiate to stromal lineages seems to vary in fractions of MSCs. Recently, lineage-tracing experiments allowed to recognize Gremlin+ [123] or $\alpha V^+Thy^-6C3^-CD105^-CD200^+$ [124] bone marrow cells as self-renewing bone stem cells able to regenerate bone tissue and form the environment for hematopoietic stem cells, while $CD45^-Tie2^+\alpha V^+$ population may generate adipocytes and endothelial cells [125]. Furthermore, formation of the stem cell niche and regulation of hematopoietic stem cells are the most important features of mesenchymal stromal cells (reviewed in [126]).

Cells characterized with MSC features *in vitro* were isolated from many tissues including adipose tissue [127], muscles [128] and tendons [129], dental pulp [130], skin [131], lungs [132], placenta, umbilical cord [133] (especially Wharton's jelly [134]) or even amniotic fluid [135] and breast milk [136]. However, cells obtained from different

tissues can express distinct panel of markers [137] and show dissimilar differentiation capabilities [138]. Furthermore, vast differentiation abilities are ascribed to mesenchymal stromal cells [139] including ability to give rise of neurons [140], endothelial cells [141], cardiomyocytes [142], smooth [143] and skeletal muscle cells [144] or even gastric epithelium [145]. Nevertheless, the importance of such phenomena, especially taking into consideration the heterogeneity of MSC in culture, is still a matter of debate (reviewed in [146]).

Another characteristic that is ascribed to mesenchymal stromal cells is the ability to modulate immune response. MSCs were shown to alleviate symptoms of many diseases that involve activation of immune response i.e. graft versus host disease [147]. The mechanism of the MSC immunomodulatory activity is complex and involves most of the types of immune cells (Figure 4). MSCs were shown to influence immune response with secreted factors such as IL-10, transforming growth factor-beta (TGFβ), prostaglandin E2, nitric oxide or indoleamine 2,3-dioxygenase 1 (IDO1) (reviewed in [148]).

Secreted factors can influence immune cell proliferation but also maturation. Especially, MSCs were shown to inhibit differentiation and maturation of dendritic cells derived from monocytes [149,150]. Furthermore, MSCs could induce differentiation of regulatory T cells [151]. Interestingly, cell to cell contact was considered to be redundant for MSC immunosuppressive activity when peripheral blood mononuclear cells were used, but necessary when purified T cell populations were used [152]. On the other hand, MSCs were shown to induce T cell apoptosis with direct cell to cell contact. T cell-derived apoptotic bodies stimulated then macrophages to induce T regulatory cells [153].
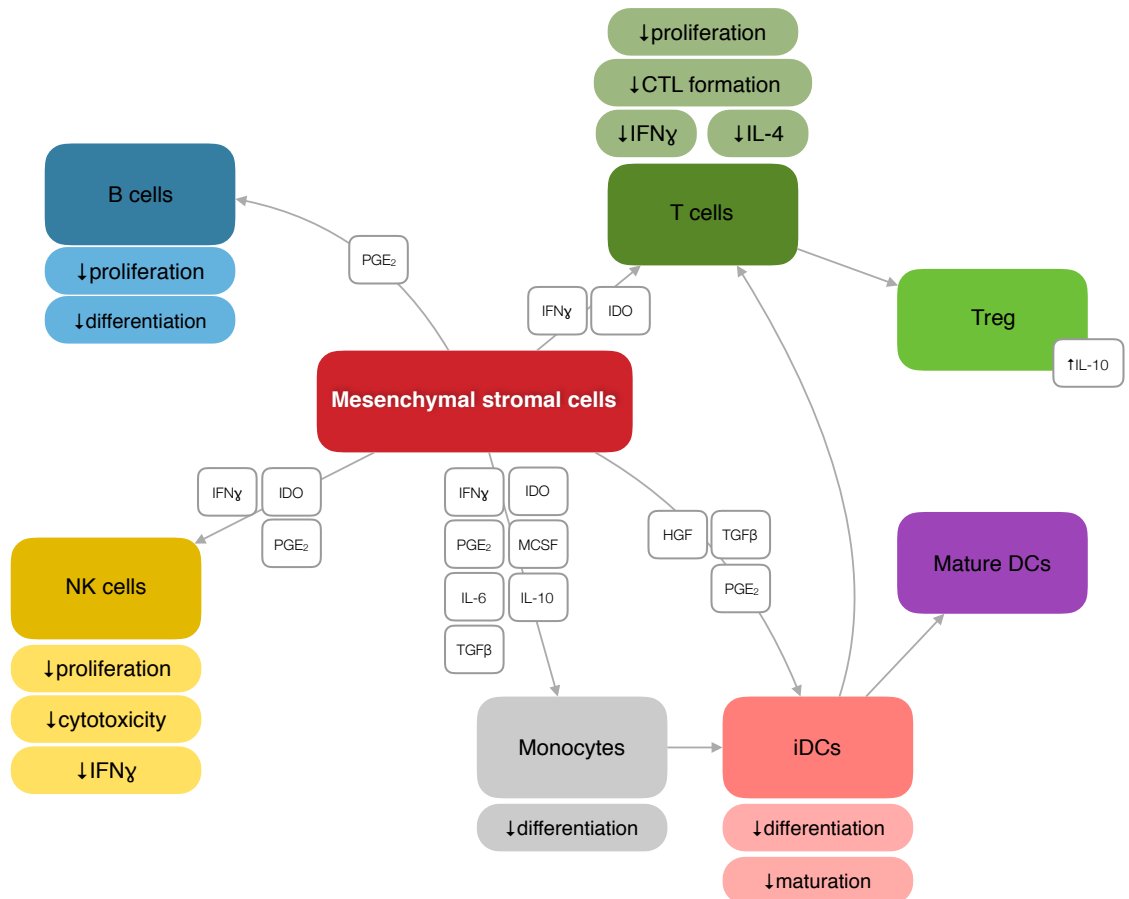
Figure 4. Influence of MSC on various types of immune cells (modified from [154]).

Recent evidence suggests that MSCs, especially allogeneic, are not immunomodulatory but rather immunoevasive and their influence on the immune system depends on the tissue context. Especially, MSCs can inhibit immune response in the immunosuppressive environment, i.e. in tumours (reviewed in [155]). Allogeneic MSCs turned out to induce immune response in many animal models including mouse [156], pigs [157], and rhesus macaques [158]. Furthermore, the immunosuppressive activity of MSCs could be compromised with the differentiation [159] or even long term culture [160]. Interestingly, adult human fibroblasts were shown to have similar immunosuppressive activity to that observed in MSCs [161,162].

Immunomodulation was suggested as one of the main MSC activities that may influence tumour growth [163]. However, mesenchymal stromal cells were shown to both inhibit or promote tumour progression. Bone marrow stromal cells inhibited growth and metastasis of Lewis lung carcinoma or B16 melanoma when inoculated together with malignant cells [164]. Immortalized rat mesenchymal progenitor cells inhibited growth of H1D2 colon carcinoma in rat model [165]. Tumours that contained mesenchymal cells were characterized with higher content of monocytes and

macrophages. Rats inoculated with 9L glioma mixed with syngeneic mesenchymal stromal cells showed better survival than control rats injected with 9L cells alone [166]. Effect of MSCs was further improved when they overexpressed IL-2. Human MSCs induced human ΔGli36 glioma cell death *in vitro*, decreased tumour growth and vessel density *in vivo* [167]. In another study human MSCs inhibited growth of Kaposi sarcoma with modulation of Akt pathway [168]. Furthermore, MSCs, when injected to pre-existing breast adenocarcinoma tumours in nude mice, inhibited tumour progression and induced remodelling of tumour vasculature [169]. Intraperitoneal delivery of rat MSCs prolonged survival of mice harbouring pancreatic cancer [170].

On the other hand, MSCs were shown to express markers of tumour-associated fibroblasts and promoted growth of Skov-3 ovarian carcinoma, probably with IL-6 secretion [171]. In another study human bone marrow-derived mesenchymal stromal cells promoted MDA-MB-231 breast cancer metastasis in immunodeficient mice with the secretion of CCL2, which increased cancer cell motility [172]. Conditioned media or co-culture with MSCs enhanced proliferation of B16 melanoma cells [173]. Tumours containing Lewis lung carcinoma or B16 melanoma cells mixed with MSCs were bigger and better vascularized. The effects of MSCs were probably mediated by secreted factors, such as leukemia inhibitory factor (LIF), macrophage-colony stimulating factor (M-CSF), macrophage inflammatory protein-2 (MIP-2) and VEGF [173]. Finally, MSCs could suppress the immune system and enabled growth of allogeneic B16 tumours in C3H mice [174].

5.9. Heme oxygenase-1 in mesenchymal stromal cells

Heme oxygenase-1 is well known for its cytoprotective activity. Therefore, most of the publications focus on the influence of its expression on the survival of transplanted mesenchymal stromal cells and its protective activity.

Rat MSCs transfected with the plasmid coding for human heme oxygenase-1 showed decreased apoptosis in hypoxia and higher resistance to H_2O_2 [175]. Furthermore, MSCs overexpressing *HMOX1* produced more VEGF than control cells when cultured in the presence of H_2O_2. The latter effect was reduced when PI3K or Akt kinases were inhibited. Moreover, transplantation of transfected MSCs decreased cardiomyocyte apoptosis and infarction size and enhanced vascularization in infarcted rat hearts [175]. Similar effect on infarcted hearts was observed when swine MSCs were used [176] or when rat MSCs were transduced with adenoviral vectors harbouring

Hmox1 gene [177,178]. Of note, swine MSC overexpressing *HMOX1* produced less proinflammatory TNFα and IL-6, and better survived ischemia/reperfusion treatment [176]. In our hands, swine bone marrow-derived cells transduced with adenoviral vectors encoding heme oxygenase-1 were characterized with better angiogenic activity *in vitro* [179]. Injection of cells overexpressing *Hmox1* improved left ventricular ejection fraction (LVEF) already 30 minutes after the infarction, what suggests rather paracrine effects of MSCs. Both control and *Hmox1* overexpressing cells had, however, similar and beneficial influence on LVEF on day 14 [179]. Treatment with cobalt protoporphyrin IX (CoPP), heme oxygenase-1 activator, enhanced proliferation of human mesenchymal stem cells and production of VEGF, while tin protoporphyrin IX (SnPP), heme oxygenase-1 inhibitor, had an opposite influence [180]. Furthermore, CoPP-treated MSCs accelerated wound healing in xenogeneic model of diabetic mice [180].

However, modulation of heme oxygenase-1 activity with SnPP in human MSCs affected their ability to inhibit T cell proliferation *in vitro*. Interestingly, effect of SnPP was not observed in rat MSCs, and T cell proliferation was restored only when concomitant treatment of nitric oxide synthase 2 was used [181]. Furthermore, heme oxygenase inhibition decreased ability of MSCs to induce Tr1 and Th3 regulatory cells and to elevate levels of their characteristic molecules, IL-10 and TGFβ, respectively. MSCs preconditioned with mixed lymphocyte reaction showed decreased HO-1 levels as well as immunomodulatory activity [182].

Finally, effect of heme oxygenase-1 on MSC differentiation to adipocytes and osteoblast was also studied. Nader Abraham and co-workers showed in the series of publications that enhanced expression of HO-1 in MSCs results in improved differentiation to osteoblasts, while its inhibition promotes adipogenesis [183-187]. On the other hand Zarjou and co-workers reported no differences in differentiation potential between MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ [188]. Also in other studies, overexpression of heme oxygenase-1 in MSCs had no effect on their differentiation [189,190]. What is more, rat primary osteoblasts treated with hemin or transduced with vectors with *Hmox1* showed inhibited osteogenesis [191]. Interestingly, although neuronal differentiation of mesenchymal stromal cells remain controversial, it was shown that expression of heme oxygenase-1 decreases in MSC-derived neuron-like cells. They were more sensitive for glutamate-mediated toxicity, what could be rescued with carbon monoxide donor [192].

Data on the influence of heme oxygenase-1 on mesenchymal stromal cells are often contradictory. Conjointly, copper or tin protoporphyrins were used in many studies to modulate HMOX1 activity, although they were shown to have many heme oxygenase-independent effects in various cell types [193,194]. Therefore, we decided to characterize murine bone marrow-derived mesenchymal stromal cells lacking *Hmox1* gene.

6. Aims of the work

The aim of the presented work was to evaluate how the stress factors, such as oxidative stress and hyperglycemia, as well as chronic complications in patients with type 2 diabetes and exercise in patients with intermittent claudication influence the mobilization and functions of subpopulations of bone marrow cells and what can be a role played here by HO-1.

Therefore, we planned:

1. To assess numbers of circulating proangiogenic cells, mesenchymal stromal cells and hematopoietic cells, expression of antioxidant genes and profile of plasma inflammatory mediators and growth factors in patients with type 2 diabetes and various forms of diabetic foot syndrome.

2. To evaluate the influence of exercise that induces ischemia and reperfusion in intermittent claudication patients on circulating proangiogenic cells, inflammatory mediators and growth factors and expression of antioxidant genes.

3. To assess the influence of pharmacological stimulation of heme oxygenase-1 in CD34^{+}-derived cells on their paracrine angiogenic activity.

4. To characterize bone marrow-derived mesenchymal stromal cells isolated from *Hmox1$^{+/+}$* or *Hmox1$^{-/-}$* mice.

7. Materials and methods

7.1. Type 2 diabetes patients [24]

We examined healthy volunteers (H, N=11) and five groups of type 2 diabetes patients: i) without DFS (T2DM, N=10), ii) with Charcot peripheral osteoneuropathy (ChPON, N=10), iii) with non-infected ulceration (DFU, N=17), iv) with infected ulceration (DFU-I, N=11), and v) with healed ulceration (DFU-H, N=6). The characteristics of the patients are shown in Table 1. The research complied with the Declaration of Helsinki and was approved by the local ethics committee. All patients provided written informed consent for the study.

Table 1. Characteristics of type 2 diabetes patients with or without diabetic foot syndrome and healthy controls. * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs H.

	H (*n*=13)	T2DM (*n*=11)	DFU (*n*=17)	DFU-I (*n*=11)	DFU-H (*n*=12)	CHPON (*n*=10)
Male / Female	5/ 8	2 / 8	14 / 3	9 / 1	8 / 4	10 / 0
Age	56.3 ± 8.3	59.9 ± 18.0	55.8 ± 4.3	59.3 ± 7.6	62.0 ± 6.1	57.2 ± 6.7
Duration of T2DM	NA	12.9 ± 9	14.8 ± 13	9.9 ± 9.1	13.9 ± 8.9	8.5 ± 8.9
HbA$_{1c}$ [%]	5.9 ± 0.2	9.0 ± 1.4 ****	8.6 ± 1.4 ****	8.4 ± 3.3*	7.3 ± 1.5**	8.2 ± 1.9**
Insulin [%]	0	90	92	100	100	100
Metformin [%]	0	70	55	0	92	36
Other Oral [%]	0	10	8	0	0	0
Body mass index	25.2 ± 1.5	35.1 ± 7.7	30.3 ± 6.4	26.9 ± 1.6	31.1 ± 5.6	28.7 ± 2.4
Erythrocytes [10^6/µL]	4.9 ± 0.3	4.8 ± 0.7	4.7 ± 0.4	3.9 ± 0.6 ***	4.6 ± 0.5	4.8 ± 0.6
Hemoglobin [g/dL]	14.2 ± 1.5	13.9 ± 2.0	13.4 ± 1.2	10.7 ± 1.4 ***	13.3 ± 2.0	14.0 ± 1.5
Hematocrit [%]	41.5 ± 3.0	41.2 ± 5.3	40.4 ± 2.3	32.5 ± 4.3	39.7 ± 4.5	41.3 ± 3.4
Leukocytes [10^3/µL]	6.6 ± 1.7	6.7 ± 1.3	7.5 ± 2.7	10.0 ± 3.7	5.8 ± 1.1	7.3 ± 1.2
Lymphocytes [10^3/µL]	2.2 ± 0.6	2.4 ± 0.6	2.2 ± 0.6	1.9 ± 0.3	1.6 ± 0.7*	2.2 ± 0.5
Neutrophiles [10^3/µL]	3.9 ± 1.3	3.5 ± 1.1	2.3 ± 4.5	7.6 ± 1.6*	3.8 ± 1.4	4.5 ± 0.9
Monocytes [10^3/µL]	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	1.0 ± 0.3 ****	0.5 ± 0.2	0.6 ± 0.1
Glucose [mg/mL]	92.4 ± 12.6	148.1 ± 31.0****	155.5 ± 70.9 *	101.5 ± 38.0	152.0 ± 53.8****	130.8 ± 38.0*
Total cholesterol [mg/dL]	203.2 ± 32.5	138.0 ± 54.6**	142.7 ± 39.8****	119.6 ± 34.8****	150.5 ± 41.3***	163.5 ± 30.4**
Blood urea nitrogen [mg/dL]	14.6 ± 2.4	17.8 ± 8.4	16.7 ± 8.9	16.0 ± 6.7	17.6 ± 4.7	15.5 ± 4.9
Total bilirubin [mg/dL]	0.60 ± 0.21	0.51 ± 0.12	0.39 ± 0.10 **	0.44 ± 0.15 *	0.52 ± 0.18	0.52 ± 0.13
GOT/AST [IU/mL]	16.3 ± 6.8	18.3 ± 10.2	20.2 ± 8.7	21.7 ± 9.9	21.3 ± 21.5	18.6 ± 10.2

7.2. Exercise training in POAD patients [73]

Twelve patients (8 females and 4 males) with POAD and Fontaine stage II IC have been recruited for this study. Table 2 identifies characteristics of patients at day 0 and day 90. The research complied with the Declaration of Helsinki and was approved by the local ethics committee. All patients provided written informed consent for the study.

Table 2. Characteristics of intermittent claudication patients subjected to the treadmill training.

	Stage of training	
	D0	**D90**
Age	65 ± 9	
Female/Male	8/4	
Body mass index	24.85 ± 4.35	24.97 ± 4.47
Ankle-brachial index	0.52 ± 0.15	0.55 ± 0.16
Total cholesterol [mmol/L]	4.63 ± 1.16	4.39 ± 0.92
High-density lipoprotein [mmol/L]	1.57 ± 0.33	1.66 ± 0.63
Low-density lipoprotein [mmol/L]	2.55 ± 0.96	2.34 ± 0.77
Triglycerides [mmol/L]	1.16 ± 0.45	1.27 ± 0.59
Hematocrit [%]	42.1 ± 3.9	41.3 ± 2.2
Erythrocytes [10^6/µL]	4.45 ± 0.34	4.47 ± 0.30
Leukocytes [10^3/µL]	6.19 ± 1.66	6.01 ± 1.01
Neutrophiles [10^3/µL]	4.03 ± 1.30	3.75 ± 1.01
Monocytes [10^3/µL]	0.56 ± 0.18	0.64 ± 0.28
Platelets[10^3/µL]	201 ± 57	184 ± 79
C-reactive protein [mg/L]	5.04 ± 7.78	2.04 ± 2.57
Fibrinogen [g/L]	4.07 ± 1.19	3.91 ± 0.95

Exercise programme consisted of 12 weeks of supervised, intermittent treadmill walking, 3 days per week. During each session patients walked on the treadmill (Gait Trainer, Biodex) till moderate claudication pain, then rested until claudication pain has abated and resumed walking. The intensity of claudication pain was determined on the 1 - 5 pain scale, where 1 = no pain, 2 = onset of claudication pain, 3 = mild pain, 4 = moderate pain, 5 = maximal claudication pain [195].

The walking exercises were performed at a speed of 3.2 km/h and grade that induce claudication within 3-5 minutes. In subsequent visits, the grade of the treadmill

was increased if the patients were able to walk for 8 minutes or longer without reaching moderate claudication pain [196]. Walking exercise time during the single session began at 30 minutes and progressively increased by 5 minutes per 2 weeks until a total of 55 minutes of exercise was accomplished.

The word 'exercise' is used throughout the dissertation to refer to the single treadmill exercise, whereas the term 'training' applies to the 3 month long training programme.

7.3. Walking ability [73]

Maximal walking time (MWT), defined as the point at which patient could no longer tolerate increase in the leg pain during walking, and time to the onset of claudication (pain-free walking time (PFWT)) were measured in 2nd Clinics of Internal Medicine, Jagiellonian University Medical College, Kraków, Poland before the programme and after 12 weeks. Patients performed graded treadmill test according to Gardner protocol [197], starting at 3.2 km/h at zero grade. Thereafter, with speed kept constant, the grade was increased 2% every 2 minutes until the maximal claudication pain.

7.4. Flow-mediated dilatation [73]

Brachial artery flow-mediated dilatation (FMD) was measured by dr Rafał Januszek, 2nd Clinics of Internal Medicine, Jagiellonian University Medical College, Kraków, Poland, at 7.00-8.00 a.m. after overnight fasting before the training and after 3 months using standard procedure [198]. The brachial artery was imaged 2 cm above the *antecubital fossa* using a high-resolution Echo-Doppler ultrasound (Sequoia 512, Acuson, linear probe 7 MHz). The mean of 3 maximal end-diastolic diameter measurements was assumed to calculate FMD value.

7.5. Blood sample collection [24,73]

Blood samples of type 2 diabetes patients were collected in three 5.0 mL EDTA tubes (Sarstedt) at Clinic of Metabolic Diseases, Jagiellonian University Medical College, Kraków, Poland at 08:00-09:00 a.m., after overnight fasting, and processed within 1 hour.

Blood samples of intermittent claudication patients were collected in EDTA tubes at vascular outpatient clinic before (D0) and after the 3 month training programme

(D90). At each time point, blood samples were collected before (T0) and at 1, 3 and 6 hours after the exercise (T+1h, T+3h and T+6h).

Plasma was transferred to the 1.5 mL tubes and frozen following the centrifugation for 10 minutes at 670 g. Total nucleated cells (TNCs) were obtained from the blood samples after the double ammonium chloride red blood cell lysis (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and resuspended in autoMACS Running Buffer (Miltenyi Biotec, Auburn, CA, USA) supplemented with 2% of foetal bovine serum (Lonza, Basel, Switzerland).

7.6. Analysis of cell subpopulations [24,73]

Cells were stained for 30 minutes in 4°C, then washed with PBS, collected on BD LSR II flow cytometer (Becton Dickinson) and analysed with BD FACS Diva software. Number of cells per 1 mL of peripheral blood (PB) was calculated based on the total leukocyte count (WBC, 10^3 cells per 1 mL of PB) and the percentage of each population within the collected events.

Diabetic foot syndrome [24]

The following subpopulations were analyzed based on their surface markers: i) EPC – $CD45^{dim}CD31^+CD133^+$ and $CD45^{dim}CD31^+CD34^+KDR^+$, ii) MSC – $CD45^-CD105^+STRO1^+$ and $CD45^-CD29^+CD90^+$, iii) HSC – $Lin^-CD45^+CD133^+$ and $Lin^-CD45^+CD34^+$. Additionally, we evaluated the $Lin^-CD45^-CD133^+$ and $Lin^-CD45^-CD34^+$ cells, described as very small embryonic-like (VSEL) cells. Peripheral blood TNC were stained using fluorescently conjugated antibodies: $CD45^{dim}CD31^+CD133^+$ cells were stained with anti-CD45-FITC (clone HI30, BD Biosciences, San Diego, CA, USA), anti-CD31-PE (clone WM59, biolegend, San Diego, Ca, USA), anti-CD133/AC133-APC (clone AC133, Miltenyi Biotec), $CD45^{dim}CD31^+CD34^+KDR^+$ with anti-CD45-FITC (clone HI30, BD Biosciences), anti-CD31-PE (clone WM59, biolegend), anti-CD34-PE-Cy5 (clone 581, BD Pharmingen), anti-KDR(CD309)-APC (clone Avas12, biolegend), $Lin^-CD45^+CD133^+$ and $Lin^-CD45^-CD133^+$ anti-CD45-PE (clone HI30, BD Pharmingen), anti-CD133/AC133-APC (clone AC133, Miltenyi Biotec), $Lin^-CD45^+CD34^+$ and $Lin^-CD45^-CD34^+$ with anti-CD45-PE (clone HI30, BD Pharmingen), anti-CD34-APC (clone 581, BD Pharmingen) and cocktail of antibodies for hematopoietic lineage markers (Lin) anti-CD2-FITC (clone RPA2.10), anti-CD3-FITC (clone UCHT1), anti-CD14-FITC (clone M5E2), anti-CD16-FITC (clone 3G8), anti-CD19-FITC (clone HIB19), anti-CD24-FITC (clone ML5), anti-CD56-FITC (clone

NCAM16.2), anti-CD66b-FITC (clone G10F5), anti-CD235a-FITC (clone GA-R2(HIR2)) all from BD Pharmingen; CD45$^-$CD105$^+$STRO1$^+$ cells were stained with anti-CD45-FITC (clone HI30, BD Biosciences), anti-CD105-PE (clone 43A3, biolegend), anti-STRO1-APC (clone STRO-1, biolegend), CD45$^-$CD29$^+$CD90$^+$ with anti-CD45-FITC (clone HI30, BD Biosciences), anti-CD29-PE-Cy5 (clone MAR4, BD Pharmingen), anti-CD90-PE (clone 5E10, biolegend).

Intermittent claudication [73]

Circulating CD45^{dim}CD31$^+$CD133$^+$ EPCs were stained with anti-CD45-FITC (BD Biosciences), anti-CD31-PE (Biolegend) and anti-CD133/1(AC133)-APC (Miltenyi Biotec) antibodies. CD45^{dim}CD34$^+$CD133$^+$KDR$^+$ and CD45^{dim}CD34$^+$CD133$^-$KDR$^+$ were assessed following the staining with anti-CD45-APC-Cy7 (Biolegend), anti-CD34-PE-Cy5 (BD Bioscience), anti-CD133/1(AC133)-PE (Miltenyi Biotec) and anti-CD309(KDR)-APC (Biolegend) antibodies. Additionally, all the nucleated events were stained with Hoechst 33342 to exclude debris, platelets and non-lysed erythrocytes from the analysis

7.7. Analysis of gene expression.

Total RNA was isolated with phenol-chloroform extraction, and reverse transcribed with the oligo(dT) primers and RevertAid reverse transcriptase (Fermentas) or with the NCode™ VILO™ miRNA cDNA synthesis kit (Invitrogen). The expression of genes was assessed with quantitative real-time PCR (qRT-PCR), which was performed in the StepOnePlus system (Applied Biosystems, Foster City, CA, USA) with the specific primers (Tables 3-5), cDNA and SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich, St. Louis, MO, USA), under conditions summarized in the Table 6. Expression of lipid metabolism genes in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ MSCs differentiated to adipocytes was assessed with TaqMan® Array Mouse Lipid Regulated Genes (Applied Biosystems) and TaqMan® Universal PCR Master Mix (Applied Biosystems) with PCR program described in the Table 6.

Table 3. Sequences of primers used for mouse genes. *recognize also other transcript variants

Gene	Primer	Sequence 5' - 3'	Product [bp]	mRNA ID
Mmu_Acta2	For	CAGGTGATCACCATTGGAAACGAACG	164	NM_007392.3
	Rev	GACAGGACGTTGTTAGCATAGAGATCC		
Mmu_Actb	For	TCCTTCGTTGCCGGTCCACA	93	NM_007393.5
	Rev	GCTTTGCACATGCCGGAGCC		
Mmu_Alad	For	GTGGGCTTGCGCTTTTAGAG	199	NM_008525.4
	Rev	CGAAGCAGTGGGTGAAAGTA		
Mmu_Alas1	For	TCTATATTTAGGGCCGCCAG	176	NM_020559.2
	Rev	TCGGGATAAGAATGGGCATC		
Mmu_Cat	For	TGACATGGTCTGGGACTTCTGG	192	NM_009804.2
	Rev	TTGATGCCCTGGTCGGTCTT		
Mmu_Cpox	For	CACTGCGATGACTCCTACAC	93	NM_007757.2
	Rev	GCCCCGATCATACAACAGA		
Mmu_Eef2(Ef2)	For	GACATCACCAAGGGTGTGCA	214	NM_007907.2
	Rev	TCAGCACACTGGCATAGAGG		
Mmu_Fabp4	For	TGTCTCCAGTGAAAACTTCG	119	NM_024406.2
	Rev	TGACCAAATCCCCATTTACG		
Mmu_Fech	For	ACCACATTGAGACGCTCTAC	88	NM_007998.6
	Rev	TCCGCTCTTCTGATGTTCTC		
Mmu_Fth1	For	CTTCGAGCCTGAGCCCTTTG	157	NM_010239.2
	Rev	CAGGTTGATCTGGCGGTTGA		
Mmu_Gclc	For	GGGCTGCTGTCCCAAG	223	NM_010295.2
	Rev	CAAGAACATCGCCTCCATTC		
Mmu_Gclm	For	GTTGCTATAGGCACCTCTGA	125	NM_008129.4
	Rev	GTCAAATCTGGTGGCATCAC		
Mmu_Gpx1	For	ACGATCCGGGACTACACC	190	NM_008160.6
	Rev	ACTTCTCAAACAATGTAAAATTGG		
Mmu_Gpx3	For	TTTAAGCAGTATGCAGGCAA	106	NM_008161.3
	Rev	GCCCAAGTTCTTCTTGTAGTG		
Mmu_Gpx4	For	CCGATATGCTGAGTGTGGTT	264	NM_008162.2 NM_001037741.2
	Rev	ACGCAGCCGTTCTTATCAAT		
Mmu_Gpx8	For	AAGATTTTAGGGCCGGAAGC	116	NM_027127.2
	Rev	TTCACGACTTGTCCCTCAG		
Mmu_Gsr	For	GGCACTTGCGTGAATGTTGG	150	NM_010344.4
	Rev	GGCATCCCTTTTCTGCTTGATG		
Mmu_Gss	For	TTGATATCTACAAGCAAGTCC	141	NM_008180.2
	Rev	TGGCAGAGATAGTGTTGATC		
Mmu_Gstk1	For	TATCACGGAGTATCAGAGCA	110	NM_029555.2
	Rev	GCTTGTTCTTCACCTGTTG		
Mmu_Gstp1	For	CTTGTCTGTATGGGCAGCTC	227	NM_013541.1
	Rev	GGCCTTCACGTAGTCATTCT		
Mmu_Hcp1	For	GATTGCTCTTCCTGTCATTGG	158	NM_026740.2
	Rev	CGGGTAGATGGAGTTGAAGAT		
Mmu_Hmbs	For	CTTTTCCTCTGGTCTTGCTTC	63	NM_001110251.1
	Rev	TTTGAGCCGTTTTCTTCCTT		
Mmu_Hmox1	For	CCTCACTGGCAGGAAATCATC	67	NM_010442.2
	Rev	CCTCGTGGAGACGCTTTACATA		
Mmu_Hmox2	For	TGACCGAGCAGAAAATACCC	94	NM_001136066.2*
	Rev	GAAGTAAAGTGCAGTGGTGGC		
Mmu_Hrg1	For	CTCCATCTTCTTCGTCTGGAC	106	NM_026353.4
	Rev	CATCACGTGAGTCACCAAGA		
Mmu_Kdr	For	CGGCCAAGTGATTGAGGCAG	114	NM_010612.2
	Rev	ATGAGGGCTCGATGCTCGCT		
Mmu_Mfsd7b	For	ATTGAATAAAATGCTCCAGTCATGA	80	NM_001081259.1*

Gene	Primer	Sequence 5' - 3'	Product	mRNA ID
(Flvcr1)	Rev	ATCTGGAACCTGTGCAGAAACA		
Mmu_Nox4	For	ATTTGCCTGGAAGAACCCAAG	101	NM_015760.5
	Rev	CATCGGTAAAGTCTCTCCGCA		
Mmu_Ppox	For	CCTAGCAAGTAAAGGGGTCA	198	NM_008911.2
	Rev	ATGGTACTCAGGATTCGAGC		
Mmu_Prdx3	For	GTCATCTTGCCTGGATCAAC	103	NM_007452.2
	Rev	CCGTAGTCTCGGGATATCTG		
Mmu_Prdx4	For	TCTGAATGACCTTCCTGTCG	139	NM_016764.4
	Rev	AGCTGGATCTGGGATTATTGT		
Mmu_Prdx5	For	AAAGAAGCAGGTTGGGAGTGT	114	NM_012021.2
	Rev	TTCCCCTTCAAATACCTCCACT		
Mmu_Prdx6	For	CTAACAACATGCCTGTGACG	80	NM_007453.3
	Rev	AGGGTAGAGGATAGACAGC		
Mmu_Ptgs2	For	AGGTCATTGGTGGAGAGGTG	79	NM_011198.4
(Cox2)	Rev	TCAGGGATGTGAGGAGGGTA		
Mmu_Runx2	For	ACAGTCTTCACAAATCCTCC	180	NM_001146038.2
	Rev	GACACCTACTCTCATACTGG		
Mmu_S100a4	For	CTTCCACAAATACTCAGGCAAAGAGG	141	NM_011311.2
(Fsp1)	Rev	AAGTTGCTCATCACCTTCTGGAATGC		
Mmu_Slc40a1	For	GTTTGCAGGAGTCATTGCTGCTA	80	NM_016917.2
(Fpn)	Rev	TTACATTTTCTTGCAGCAACTGTGT		
Mmu_Sod2	For	AGACCTGCCTTACGACTATG	163	NM_013671.3
	Rev	AGCGACCTGAGTTGTAACAT		
Mmu_Sod3	For	ACTTCACCAGAGGGAAAGAG	87	NM_011435.3
	Rev	AGCAAGCCGTAGAACAAGAA		
Mmu_Txn1	For	TCCCTCTGTGACAAGTATTCC	96	NM_011660.3
	Rev	CGGCATGCATTTGACTTCAC		
Mmu_Txnrd2	For	GGCTGACTATGTGGAACCTT	230	NM_013711.3
	Rev	GATGACCCCAGTTCAAGGATTT		
Mmu_Txnrd3	For	ACCTATGTCAACTCCTTCGG	97	NM_153162.3
	Rev	CAAACTTCGAAGCCGTGTAA		
Mmu_Txrdn1	For	TGCGGTGGACACTCTACTAAG	165	NM_001042523.1
	Rev	GAAAACCTGGAAACCAGCAA		
Mmu_Ucp3	For	ATCCTGCTGCTACCTAATGG	144	NM_009464.3
	Rev	GTTCCTTTGCTGCCTATGGA		
Mmu_Uros	For	GAAGCAGTGAAGCTGTGTTT	91	NM_009479.2
	Rev	CGTACACAGACTTGGCATTC		
Mmu_Vim	For	AGGCCGAGGAATGGTACAAGTCC	194	NM_011701.4
	Rev	ATTCTCTTCCATCTCACGCATCTGG		

Table 4. Sequences of primers used for human genes. *recognize also other transcript variants of the gene, **designed to recognize only Oct-4A form and not pseudogenes

Gene	Primer	Sequence 5' - 3'	Product	mRNA ID
Hsa_HMOX1	For	TCTATATTTAGGGCCGCCAG	165	NM_002133.2
	Rev	CAGCTCCTGCAACTCCTCAAA		
Hsa_HMOX2	For	GATCGTGGAGGAGGCCAACAAGG	150	NM_002134.3*
	Rev	TAGAAAGGGCATTTACGCATGTCTCC		
Hsa_SOD1	For	TGACATGGTCTGGGACTTCTGG	192	NM_001752.3
	Rev	TTGATGCCCTGGTCGGTCTT		
Hsa_SOD2	For	GGTTGGCTTGGTTTCAATAAGGAACG	108	NM_000636.2*
	Rev	ATCAATCCCCAGCAGTGGAATAAGG		
Hsa_SOD3	For	CCAATGACTGGCTCCCTCACG	198	NM_003102.2
	Rev	AGCAGGCAGGAACACAGTAGC		
Hsa_NQO1	For	CAAATCCTGGAAGGATGGAA	199	NM_000903.2*
	Rev	GGTTGTCAGTTGGGATGGAC		
Hsa_B2M	For	AATGCGGCATCTTCAAACCT	79	NM_004048.2
	Rev	TGACTTTGTCACAGCCCAAGATA		

Hsa_NOS2	For	CTCCAGATGAGCTTCTACCTCAAGC	116	NM_000625.4
	Rev	TATCTCCTTTGTTACCGCTTCCACC		
Hsa_POU5F1-1	For	GATGTGGTCCGAGTGTGGTTCT	68	NM_002701.5**
	Rev	TGTGCATAGTCGCTGCTTGAT		
Hsa_POU5F1-2	For	CGGAGCCCTGCACCGTCA	221	NM_002701.5
	Rev	GCAGATGGTCGTTTGGCTGAAT		

Table 5. Sequences of primers used for the analyses of miRNA expression.

Gene	Sequence 5' - 3'
miR-21-5p	TAGCTTATCAGACTGATGTTGA
miR-31-5p	AGGCAAGATGCTGGCATAGCT
miR-132-3p	TAACAGTCTACAGCCATGGTCG
miR-145-5p	GTCCAGTTTTCCCAGGAATCCCT
miR-146a-5p	TGAGAACTGAATTCCATGGGTT
miR-150-5p	TCTCCCAACCCTTGTACCAGTG
miR-155-5p	TTAATGCTAATCGTGATAGGGGT
miR-200c-3p	TAATACTGCCGGGTAATGATGGA
miR-301a-3p	CAGTGCAATAGTATTGTCAAAGC
miR-378a-3p	CTGGACTTGGAGTCAGAAGG
miR-378a-5p	CCTGACTCCAGGTCCTGTGT
U6 snRNA	CGCAAGGATGACACGCAAATTC

Table 6. PCR settings for the analysis of mRNA and miRNA levels.

mRNA / SYBR Green				
Hold	**40 cycles**			**Melt curve**
10 minutes	30 s	60 s	45 s	-
95 °C	95 °C	60 °C	72 °C	60-95 °C
mRNA (lipid regulated genes) / TaqMan				
Hold	**Hold**	**40 cycles**		
2 minutes	10 minutes	15 s		60 s
50 °C	95 °C	95 °C		60 °C
miRNA / SYBR Green				
Hold	**Hold**	**40 cycles**		**Melt curve**
2 minutes	2 minutes	15 s	60 s	-
50 °C	95 °C	95 °C	60 °C	60-95 °C

7.8. Multiplex immunoassays [24,73]

Concentrations of plasma sE-selectin, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), adiponectin, tissue inhibitor of plasminogen activator-1 (tPAI-1), matrix metalloproteinase-9 (MMP-9), myeloperoxidase were assessed with the Milliplex MAP Cardiovascular Panel I (Millipore); interleukin IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, monocyte chemotactic protein-1 (MCP-1), TNF-α, interferon-γ (IFN-γ), VEGF, epidermal growth factor

(EGF), fibroblast growth factor-2 (FGF-2), Flt-3 ligand, G-CSF with the Milliplex MAP Cytokine/Chemokine Panel I, and SDF-1α+β, LIF, thrombopoietin (TPO), stem cell factor (SCF) with the Milliplex MAP Cytokine/Chemokine Panel II reagents (both Millipore) on the Luminex *FlexMAP 3D* platform, according to the vendor's instructions. Concentration of factors produced by CD34^{+}-derived cells were measured with Cytokine Human 30-Plex Panel (Invitrogen) on the Luminex *FlexMAP 3D* platform. Levels of factors produced by MSC *Hmox1*^{+/+} or *Hmox1*^{-/-} were measured with Milliplex ® MAP Mouse Cytokine/Chemokine Bead Panel - 32 Plex (Millipore).

7.9. In cell ELISA [73]

Phosphorylation of NOS3 Ser1177 in human aortic endothelial cells (HAoEC) in response to the IC patient plasma was evaluated with the in cell ELISA. Briefly, 5.0 × 10^{3}/well of HAoECs were seeded on 96 well plate, 48 h prior to the stimulation with plasma, and starved with the EBM-2 (Lonza) supplemented with 0.5% fetal bovine serum (FBS) for 12 h before the stimulation. HAoECs were treated with 10% IC plasma in EBM-2 for 30 minutes, then washed with TBS, fixed with 4% formaldehyde in TBS and permeabilized. Activity of internal peroxidases was quenched with 1% H_2O_2. Then, cells were blocked with 3% BSA + 10% goat serum in TBS and incubated overnight in 4 °C with the rabbit antibody against phosphorylated NOS3 Ser1177 (Abcam). Next, cells were incubated with biotinylated goat anti-rabbit antibody (Vector Lab) and streptavidin conjugated with horseradish peroxidase (HRP) (R&D Systems). HRP activity was measured with the colorimetric assay and normalized to the Janus Green B (Sigma-Aldrich) whole cell stain.

HAoEC cells were purchasd from ATCC and culured in EGM2-MV medium (Lonza) supplemented with 10 % FBS (Lonza) and 1% penicillin with streptomycin (Sigma).

7.10.　　Isolation and culture of human peripheral blood CD34^{+}

Human peripheral blood CD34^{+} cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from a healthy volunteer, who was treated for 5 days with G-CSF. The research complied with the Declaration of Helsinki and was approved by the local ethics committee. Donor provided written informed consent for the study. PBMC were obtained with apheresis using COBE® Spectra Apheresis System in the Clinics of Hematology, Jagiellonian University Medical College, Kraków, Poland. Remaining erythrocytes were removed with centrifugation on Ficoll

Plus gradient (GE Healthcare). PBMC were then aliqoted and frozen in either Cryostore freezing medium (Stem Cell Technologies) or in PBS with 50% FBS and 10% DMSO. Fraction of CD34$^+$ and their phenotype was assessed with flow cytometry.

When necessary, aliquots of PBMC were thawed, cells were washed with PBS and treated for 30 minutes with 25 μg/mL DNase I (Roche) in PBS with 5.0 μmol/L Mg^{2+} in order to prevent cell clumping. Then cells were stained for 25 minutes with anti-human CD34-FITC antibody (BD Pharmingen) and for 10 minutes with 7-AAD (BD Pharmingen) in 4 °C. Stained cells were washed with 3 mL of PBS, resuspended in AutoMACS Running Buffer and filtered with 40 μm cell strainer (BD Biosciences). CD34$^+$7AAD$^-$ cells were sorted on Beckman Coulter MoFlo XDP FACS sorter (Figure 5).

Cells were cultured in U-shaped, non-adherent 96 well plates (150 μL, 5.0×10^3 per well) in StemSpan ACF medium supplemented with 20% BIT9500 serum replacement, CC100 mix of cytokines (all from Stem Cell Technologies), 50 ng/mL VEGF (Peprotech) and penicillin with streptomycin (Sigma). After 48 hours 50 μL of fresh medium was added, then 50 μL of medium was changed every other day. On the day 6 of culture cells were stimulated with 7.5, 15 or 30 μmol/L atorvastatin (Tocris), 7.5, 15 or 30 μmol/L resveratrol (Sigma), 125, 250 or 500 μmol/L acetylsalicylic acid (Sigma), 2.5, 5.0 or 10 μmol/L sulforaphane or 1.25, 2.5 or 5.0 mmol/L metformin (Sigma). Conditioned media were collected after 48 hours. Control, non-conditioned media, contained the same concentrations of stimulants and were kept for 48 hours in cell culture incubator in the same conditions as stimulated cells. Wash-out conditioned media were collected from stimulated cells after washing with PBS and reseeding on 96 well plates in fresh complete medium. DMSO, which was used to dissolve all stimulants, was added to the control wells.
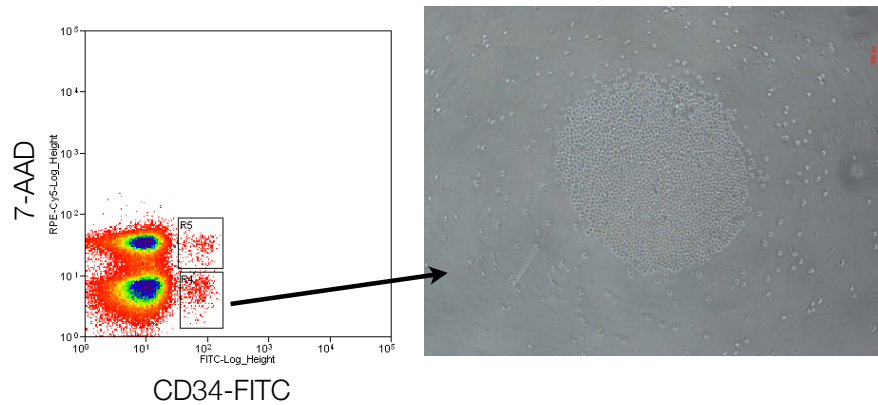
Figure 5. Strategy for sorting alive CD34$^+$7AAD$^-$ cells and sorted cells in culture.

7.11. Phenotyping of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells from G-CSF treated healthy volunteer were stained in AutoMACS Running Buffer in 4 °C with following antibodies: cocktail of antibodies for hematopoietic lineage markers (Lin) (anti-CD2-FITC (clone RPA2.10), anti-CD3-FITC (clone UCHT1), anti-CD14-FITC (clone M5E2), anti-CD16-FITC (clone 3G8), anti-CD19-FITC (clone HIB19), anti-CD24-FITC (clone ML5), anti-CD56-FITC (clone NCAM16.2), anti-CD66b-FITC (clone G10F5), anti-CD235a-FITC (clone GA-R2(HIR2)) all from BD Pharmingen, anti-CD45-APC-Cy7 (clone 2D1, BD Biosciences), anti-CD34-APC or anti-CD34-FITC (clone 581, BD Pharmingen), anti-CD133-PE (clone AC133, Miltenyi Biotec), anti-CD105-PE (clone 43A3, biolegend), anti-CD90-APC (clone 5E10, biolegend), anti-CD181-APC (clone 5A12, BD Pharmingen), anti-CD184(CXCR4)-APC (clone 12G5, biolegend), anti-CD11b-APC (clone M1/70, biolegend). Stained cells were then analysed on BD LSR Fortessa flow cytometer using BD FACS Diva Sofware (Becton Dickinson).

7.12. Animals

All procedures involving the use of animals were performed according to approved guidelines. Mice were maintained under the specific pathogen free conditions, in individually ventilated cages, with full access to food and water. All animal experiments were approved by the Local Ethical Committee for Animal Research at the Jagiellonian University.

7.13.   Isolation of murine fibroblasts

Murine adult tail fibroblasts were isolated from C57Bl6×FVB *Hmox1$^{+/+}$* or C57Bl6×FVB *Hmox1$^{-/-}$* according to the previously published protocol [199] and cultured in DMEM (Lonza) medium supplemented with 10% FBS (Lonza) and penicillin with streptomycin (Sigma Aldrich).

7.14.   Isolation of mesenchymal stromal cells

MSCs were isolated from femurs and tibia of C57Bl6×FVB *Hmox1$^{+/+}$* or C57Bl6×FVB *Hmox1$^{-/-}$*. Mice were sacrificed with ketamine/xylazine. Bones were resected under the sterile laminar flow hood and cut into small pieces (ca. 1 mm^2) with a bone cutter. Then bone chips were digested with 1 mg/mL type II collagenase (Gibco) for 90-120 minutes in 37 ^{o}C in rotary shaker (250 rpm). Released cells were washed once with PBS and resuspended in the growth medium (αMEM supplemented with 10% FBS (Lonza) and penicillin with streptomycin (Sigma-Aldrich). Cells were seeded in 6 well plates – bone marrow from one mouse per well. Medium was changed every 24 hours in the first 3 days and every 2-3 days thereafter, and cells were passaged when confluent. Importantly, prior to the MACS sorting cells were detached with short treatment with trypsin (2 minutes) in room temperature in order to decrease number of highly adherent macrophages in culture. After 3 passages mesenchymal stromal cells were further purified from CD45$^+$ fraction with MACS sorting (Figure 6).
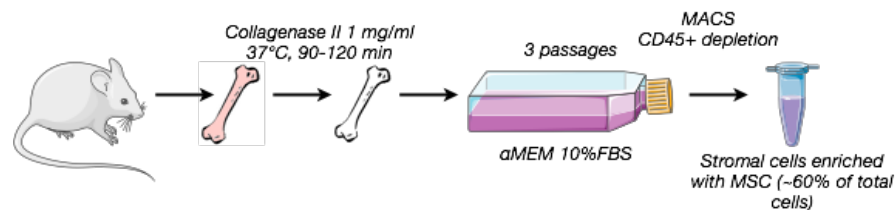
Figure 6. Isolation of murine bone marrow mesenchymal stromal cells (pictures of mouse, cell culture flask and a tube are from *Servier Medical Art*).

7.15.   MACS sorting of CD45$^-$ murine bone marrow stromal cells

Bone marrow-derived cells were detached with trypsin, washed with PBS, resuspended in AutoMACS running buffer and stained for 25 minutes with anti-mouse CD45 MicroBeads (Miltenyi) in 4 °C. Then cells were washed with 1 mL of PBS, resuspended in AutoMACS Running Buffer and separated on MACS MS columns or with AutoMACS (Miltenyi). Flow-through with CD45$^-$ cells was collected and columns with CD45$^+$ cells were discarded. Purified CD45$^-$ murine bone marrow mesenchymal

stromal cells were next counted and either used directly for the experiments or seeded for further culture (1.5-2.0×10^4 per 1 cm^2) in αMEM CM.

7.16. Analysis of Fibroblast- or Osteoblast-Colony Forming Units

Cells isolated from the bone marrow were counted using Türck solution to lyse red blood cells. 1×10^6 of bone marrow cells were seeded per well of 6 well plates. Cells were cultured for until colonies of fibroblastoid cells were formed. At that stage part of the wells was fixed and stained with crystal violet and the colonies of fibroblastoid cells were counted. Another part of the cells was treated with osteogenic differentiation medium for the next 3 weeks and then cells were stained with Alizarin Red S and positive colonies were counted.

7.17. Mesenchymal stromal cell phenotyping

Mesenchymal stromal cells cultured for 3 passages after the isolation were detached with trypsin, washed with PBS and stained for 25 minutes in AutoMACS Running Buffer in 4 °C with following antibodies: anti-mouse CD45 (clone 30F-11, BD Biosciences), anti-mouse CD29 (clone HMβ1-1, biolegend), anti-mouse CD31 (clone MEC13.3, BD Biosciences), anti-mouse CD34 (clone RAM34, BD Biosciences), anti-mouse CD90.2 (clone 30-H12, biolegend), anti-mouse CD105 (clone MJ7/18, biolegend), anti-mouse CD117 (c-kit) (clone 2B8, eBioscience), anti-mouse CD140a (clone APA5, eBioscience), anti-mouse Ly-6A/E (Sca1) (clone D7, eBioscience). Phenotype of the cells was assessed with BD LSR II or BD LSR Fortessa (Becton Dickinson).

7.18. Mesenchymal stromal cell: differentiation to osteoblasts

Mesenchymal stromal cells $Hmox1^{+/+}$ or $Hmox1^{-/-}$ were differentiated to osteoblasts with the protocol by [200]. Briefly, 2.5×10^4 of sorted CD45$^-$ bone marrow stromal cells were seeded per 1 well of 24 well plates. Osteoblast differentiation was induced for 3 weeks with αMEM CM supplemented with 0.1 μmol/L dexamethasone, 10 mmol/L β-glycerol phosphate and 50 μmol/L ascorbate-2-phosphate (all from Sigma Aldrich) and verified with AlizarinRed S staining and gene expression analysis. Control cells were cultured in αMEM CM.

7.19. Mesenchymal stromal cell: differentiation to adipocytes

Mesenchymal stromal cells $Hmox1^{+/+}$ or $Hmox1^{-/-}$ were differentiated to adipocytes with the protocol by [200]. Briefly, 2.5×10^4 of sorted CD45$^-$ bone marrow stromal cells were seeded per 1 well of 24 well plates. Adipocyte differentiation was induced for 3 weeks with αMEM CM supplemented with 1.0 μmol/L dexamethasone, 50 μmol/L 3-isobutyl-1-methylxanthine (IBMX) and 10 ng/mL insulin (all from Sigma-Aldrich) and verified with OilRedO staining and gene expression analysis. Control cells were cultured in αMEM CM.

7.20. Mesenchymal stromal cell: differentiation to myofibroblasts

Sorted CD45$^-$ bone marrow stromal cells were seeded in 24 well plates (2.5×10^4 per well). Myofibroblast differentiation was induced for 6 days with αMEM CM supplemented with 2 ng/mL recombinant human TGFβ1 (Peprotech) and confirmed with staining for αSMA and gene expression analysis. Control cells were cultured in αMEM CM.

7.21. Mesenchymal stromal cell: differentiation to endothelial cells

Sorted CD45$^-$ bone marrow stromal cells were seeded in 24 well plates (2.5×10^4 per well), coated with 0.25% gelatine (Sigma) and 20 μg/mL human plasma fibronectin (Sigma). Endothelial differentiation was induced for 7 days with αMEM CM. Medium was changed every other day. Control cells were cultured in non-coated cell culture plates in αMEM CM. Endothelial differentiation was assessed with the staining with acetylated LDLs and *Bandeiraea simplicifolia* lectin, gene expression analysis, phenotyping, and functional assays (*in vitro* Matrigel tube forming assay, spheroid assay).

7.22. Matrigel assays

Analysis of MSC endothelial character

Control MSCs or MSC-derived endothelial-like cells (MSC-ECs) stained with CellViolet were detached with trypsin, washed with PBS, and resuspended in respective culture medium. Cells were then counted and seeded in 50 μL of solidified Matrigel (5.0×10^3 cells per well). Part of the cells were seeded in co-culture with HAoEC or iPS-EC cells stained with PKH26 (1:1, altogether 1.0×10^4 cells per well).

iPS cells were generated by mgr Jacek Stępiewski as described in Stepniewski *et al.* [199]. Pluripotency of the resulting cells was confirmed by staining for SSEA-1 and NANOG and by teratoma formation after subcutaneous transplantation to the nude SCID mice as shown earlier [199]. Pluripotent iPS cells were subsequently differentiated toward endothelium by dr Neli Kachamakova-Trojanowska (iPS-EC) as described previously[199].

Analysis of paracrine angiogenic activity of conditioned media from CD34$^+$-derived cells

Assay was performed in 96 well plates with 50 μL of Matrigel per well. 1.0×10^4 HAoEC cells were seeded in each well in 50 μL of EBM2 medium supplemented with 2% FBS. After 30 minutes 50 μL of conditioned medium was added to the cells. Non-conditioned media with the same stimulants were used as controls. EBM-2 with 2% FBS was used as a negative control, whereas EGM-2 CM was a positive one. Pictures of formed structures were taken after 16 hours and then analysed with ImageJ software, with Angiogenesis Analyzer (source: *http://image.bio.methods.free.fr /ImageJ/?Angiogenesis-Analyzer-for-ImageJ&artpage=4-7#outil_sommaire_4*).

7.23. Spheroid assay

Angiogenic properties of mesenchymal stromal cells and MSC-ECs were assessed with spheroid assay. Spheroids were formed by 750 cells seeded per well in growth medium with 10% methylcellulose in non-adherent U-shaped 96 well plates (Greiner). Spheroids were formed in 24 hours. Then, they were collected with centrifugation (3 minutes, 50 *g*), resuspended in methylcellulose supplemented with 30% FBS, and embedded in collagen type I. Briefly, collagen type I was mixed with 10 × HBSS and neutralized with 0.2 mol/L NaOH. Then, 500 μL of collagen solution was mixed with 500 μL of spheroids in methylcellulose and seeded on 24 well plates. After 15 minutes of incubation in 37 °C spheroids were stimulated with growth medium and cultured for 24-48 hours.

7.24. Analysis of MSC and MSC-EC angiogenic activity *in vivo*

Experiments *in vivo* were done with cells obtained form C57BL6-*GFP* mice. MSCs or MSC-ECs were mixed with Matrigel and injected subcutaneously in two sites on mouse abdomen (3.0×10^5 cells in each plug, 250 μL). After two weeks mice were sacrificed and plugs were excised, then cut into small pieces and digested with the

enzyme mix: 3 U/mL liberase™ (Roche), 25 μg/mL hyaluronidase (Sigma-Aldrich), 25 μg/mL DNAse (Roche), and dispase 3 U/mL (Gibco) for 1 hour in 37 °C. Reaction was stopped with FBS. Cells were then filtered through 70 μm cell strainer (BD Biosciences), centrifuged (600 g, 10 minutes) and stained in 2% FBS in PBS in 4 °C for 20 minutes with following antibodies: anti-mouse CD31 PE (clone MEC13.3, BD Bioscences), anti-mouse CD45 APC (clone 30F-11, Bd Biosciences), anti-mouse Ly-6A/E (Sca-1) PE-Cy7 (clone D7, eBioscence), anti-mouse CD117 (c-kit) APC-Alexa Fluor® 780 (clone 2B8, eBioscience).

7.25.   Staining with CellViolet or PKH26

Cells were stained with CellViolet (life technologies) or with PKH26 (Sigma) according to manufacturer's protocol.

7.26.   Proliferation assay

Proliferation of mesenchymal stromal cells was assessed with BrdU method using Cell Proliferation ELISA (Roche), according to manufacturer's protocol. Low glucose medium contained 5 mmol/L glucose and high glucose medium contained 33 mmol/L glucose.

7.27.   LDH assay

Cytotoxicity of hemin or H_2O_2 in mesenchymal stromal cells was evaluated with CytoTox 96® NonRadioactive Cytotoxicity Assay (Promega), according to manufacturer's protocol. Low glucose medium contained 5 mmol/L glucose and high glucose medium contained 33 mmol/L glucose.

7.28.   7-AAD cell viability assay

Viability of mesenchymal stromal cells stimulated with high doses of hemin was assessed with 7-AAD staining. Stimulated and control cells were detached with trypsin, washed with PBS and resuspended in AutoMACS Running Buffer. Then cells were stained for 10 minutes with 7-AAD (BD Pharmingen) according to manufacturer's protocol and analysed on BD LSR Fortessa cytometer.

7.29.   Mesenchymal stromal cells: analysis of immunosuppressive activity in SCC VII-*luc* tumours *in vivo*

Murine squamous cell carcinoma SCC VII-*luc* cell line and MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$ cells were mixed (1.0×10^6 SCC VII-*luc* cells and 2.5×10^4 MSC per plug)

with Matrigel and injected subcutaneously to the C3H mice (syngeneic to the SCC VII-*luc* cells) in 2 sites on the murine abdomen in 250 µL per plug. Matrigel was let to form spheroid plugs. Growth of the tumours was monitored for 2 weeks and assessed on day 3, 7 and 14 with luminescence assay, and on day 14 with measurement of tumour size with the calliper. Tumours were excised on day 14 and digested with 3 U/mL liberase™ (Roche), 25 µg/mL hyaluronidase (Sigma Aldrich), 25 µg/mL DNAse (Roche), and dispase 3 U/mL (Gibco) for 1 hour in 37 °C. Reaction was stopped with FBS. Cells were then filtered through 70 µm cell strainer (BD Biosciences), centrifuged (600 *g*, 10 minutes) and stained in 2% FBS in PBS in 4 °C for 20 minutes with following antibodies: anti-mouse-CD45 APC-eFluor780 (clone 30-F11, ebioscience), anti-mouse CD11b PE-CF594 (clone M1/70, BD Pharmingen), anti-mouse Ly-6C PerCP-Cy™5.5 (clone AL-21, BD Pharmingen), anti-mouse Ly-6G BV605 (clone 1A8, BD Horizon™), anti-mouse F4/80 APC (clone BM8, biolegend), anti-mouse MHC II PE-Cy7 (clone M5/114.15.2, eBioscience) anti-mouse CD43 FITC (clone S7, BD Pharmingen), anti-mouse CD202b (Tie2) PE (clone TEK4, biolegend), anti-mouse CD11c Alexa Fluor® 700 (clone N418, eBioscience) and analysed on BD LSR Fortessa cytometer. Gating strategy is shown in Figure 7.
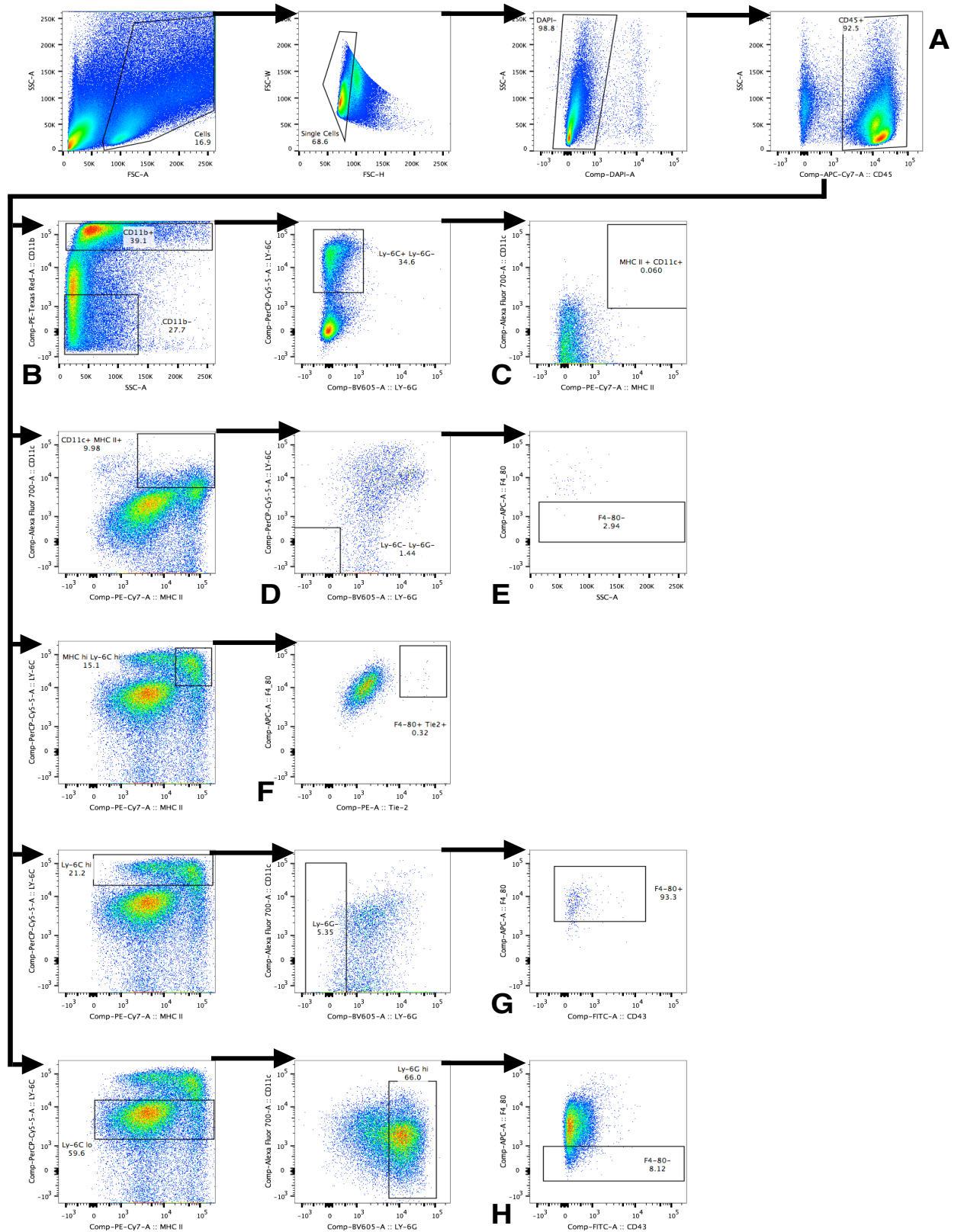
Figure 7. Gating for tumour infiltrating cells: total infiltrating cells CD45$^+$ **(A),** total CD11b$^+$ myeloid cells **(B)**, plasmocytoid dendritic cells **(C)**, CD11b$^+$Ly-6C$^-$Ly-6G$^-$MHCII$^+$CD11c$^+$ dendritic cells **(D)**, CD11b$^+$Ly-6C$^-$Ly-6G$^-$ MHCII$^+$CD11c$^+$F4/80$^-$ classical dendritic cells **(E)**, CD11b$^+$Ly-6C^{hi}MHCII^{hi}Tie2$^+$F4/80$^+$ Tie2$^+$ macrophages **(F)**, CD11b$^+$Ly-6C^{hi}Ly-6G$^-$F4/80$^+$ monocytoid MDSC **(G),** CD11b$^+$Ly-6C^{lo}Ly-6G$^+$F4/80$^-$ granulocytoid MDSC **(H).**

7.30. Measurement of cellular H_2O_2 levels

Levels of cellular H_2O_2 were measured with H2DCFDA assay. *Hmox1$^{+/+}$* or *Hmox1$^{-/-}$* MSCs and fibroblasts were stimulated for 6, 16, 24 or 48 hours with 50 μmol/L hemin (Frontiers Scientific). After each time-point the stimulated cells and non-stimulated controls were harvested with trypsin, washed with PBS and stained for 30 minutes with 0.1 μmol/L H2DCFDA (Sigma Aldrich) in PBS. Then cells were washed twice with PBS and DCFDA fluorescence was assessed with BD LSR Fortessa cytometer.

7.31. Heme cellular content assay

Hmox1$^{+/+}$ or *Hmox1$^{-/-}$* mesenchymal stromal cells were cultured in 24 well plates. Heme content was assessed with method by Foresti et al. ([201]) in non-stimulated cells (T0), after 2 hours of stimulation with 50 μmol/L hemin (T2) and after 2 additional hours of culture in fresh αMEM CM (T4) (Figure 44A). Cells were lysed with 80% formic acid (POCH S.A.) and the lysate was transferred to clear plastic 96 well plates. Absorbance was measured at λ=398 nm with GENios microplate reader (Tecan).

7.32. SnPP binding assay

Cell stimulated with tin protoporphyrin IX (SnPP) show fluorescence in APC channel. Therefore, we stimulated *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* MSCs with 10, 25 or 50 μmol/L SnPP for 6 hours and analysed their fluorescence with flow cytometry. Cells were detached with trypsin, washed and resuspended in AutoMACS Running Buffer. Flow cytometry analysis was performed on BD LSR Fortessa cytometer.

7.33. GSH/GSSG Assay

Levels of total GSH, total GSSG and GSH/GSSG ratio in MSCs and fibroblasts were assessed with GSH/GSSG-Glo™ Assay (Promega) according to manufacturer's protocol. 1.0×10^4 cells were seeded per well in 96 well plates. Cells were stimulated for 4 hours with 50 μmol/L hemin in αMEM CM and cultured for 2 hours in αMEM CM in order to let them recover GSH levels. Total GSH and total GSSG data are shown as a ratio to the control non-stimulated cells.

7.34. Statistical analysis

Statistical anlysis of the data was performed with GraphPad Prism software. Results are expressed as mean ± SD unless otherwise stated. Normality of data was checked with the D'Agostino & Pearson omnibus test when applicable. Statistical significance of diabetic foot syndrome data was assessed with the Mann-Whitney U-test and accepted at $p<0.05$ level. All data obtained in diabetic foot syndrome project has been correlated using Spearman's rank correlation. Differences in response to the exercise or trainings were assessed with the paired t-test. Statistical significance was accepted at $p<0.05$.

Data obtained in *in vitro* experiments were analysed with Student t-test when two groups of samples were used. In other case we used One-way or Two-Way Anova with Bonferroni post-test. Kind of statistical test applied to analyse given sets of data is provided in description of figures.

8. Results

8.1. Numbers of circulating stem and progenitor cells is affected by the complications of type 2 diabetes [24]

The number of EPCs defined as $CD45^{dim}CD31^+CD133^+$ (Figure 8A) or $CD45^{dim}CD31^+CD34^+KDR^+$ (Figure 8B) was decreased in type 2 diabetes patients in comparison to healthy controls, and in general was not further modified by diabetic foot syndrome forms. The exemption was infected diabetic foot ulcer, which led to an increase in the $CD45^{dim}CD31^+CD34^+KDR^+$ subpopulation (p=0.01 for DFU-I *versus* DFU). The number of these cells correlated negatively with $Hb1_{Ac}$ (Spearman r=-0.40, p=0.0025), although there were no statistical differences in $Hb1_{Ac}$ between the analyzed groups.

$CD45^-CD29^+CD90^+$ MSCs were less numerous in type 2 diabetes patients than in healthy volunteers (Figure 8C), while in groups with infected or healed ulcers their numbers increased to the control values. A similar pattern, however not significant, was observed for MSCs defined as $CD45^-CD105^+STRO1^+$ (Figure 8D). Patients with type 2 diabetes either with or without diabetic foot syndrome had also less $Lin^-CD45^+CD133^+$ HSCs (Figure 8E). Differences for $Lin^-CD45^+CD34^+$ HSCs were similar, but less pronounced (Figure 8F). The number of $Lin^-CD45^-CD34^+$ VSEL cells was increased in type 2 diabetes patients (p<0.01, T2DM *versus* H) (Figure 8G) and correlated positively with $Hb1_{Ac}$ (Spearman r=0.31, p=0.02). This rise was weaker in diabetic patients with foot ulceration, especially in DFU-I and DFU-H groups. The $Lin^-CD45^-CD133^+$ VSEL population was less numerous in comparisons to non-complicated type 2 diabetes group in all groups with diabetic foot syndrome (Figure 8H).
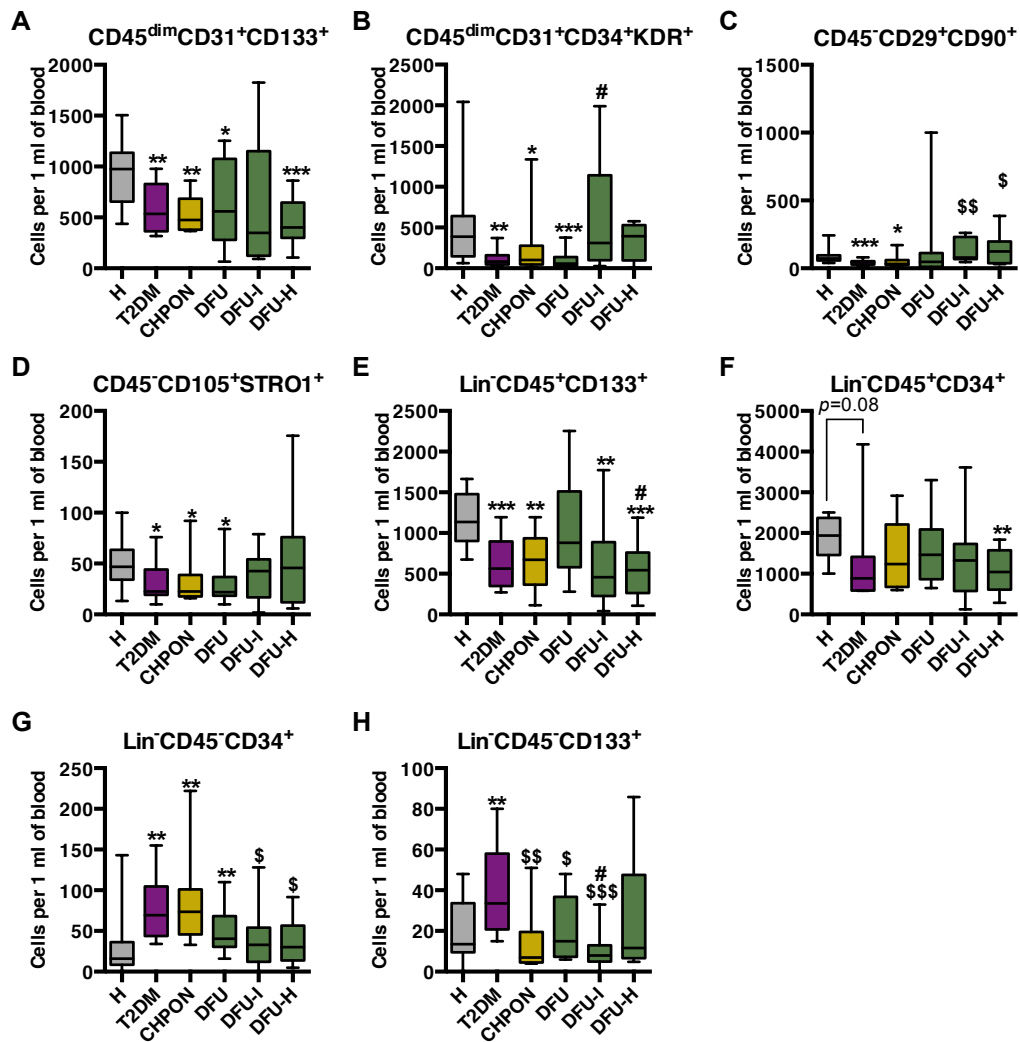
Figure 8. Numbers of circulating stem and progenitor cells in the peripheral blood of patients. **(A)** CD45^{dim}CD31$^+$CD133$^+$ EPC; **(B)** CD45^{dim}CD31$^+$CD34$^+$KDR$^+$ EPC; **(C)** CD45$^-$CD29$^+$CD90$^+$ **D)** CD45$^-$CD105$^+$STRO-1 MSC; **(E)** Lin$^-$CD45$^+$CD133$^+$ HSC; **(F)** Lin$^-$CD45$^+$CD34$^+$ HSC; **(G)** Lin$^-$CD45$^-$CD34$^+$ VSEL cells; **(H)** Lin$^-$CD45$^-$CD133$^+$ VSEL cells. Flow cytometry phenotyping. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *versus* H; $^\$p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ *versus* T2DM; $^\#p<0.05$ *versus* DFU. Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles [24].

Of note, populations of endothelial progenitor cells, mesenchymal stromal cells and hematopoietic stem cells/progenitors in general correlated positively with each other, whereas number of VSEL cells did not correlate with any other population (Table 7).

Table 7. Spearman correlations between numbers of circulating stem and progenitor cells [24].

	CD45-CD31+CD133+	CD45-CD105+STRO1+	Lin-CD45+CD133+	Lin-CD45-CD133+	Lin-CD45+CD34+	Lin-CD45-CD34+	CD45-CD31+CD34+KDR+	CD45-CD29+CD90+	
CD45-CD31+CD133+		0.34	0.72		0.66		0.35		
CD45-CD105+STRO1+	0.002						0.40		
Lin-CD45+CD133+	0.0000	NS			0.80				
Lin-CD45-CD133+	NS	NS	NS			0.23			RR
Lin-CD45+CD34+	0.0000	NS	0.0000	NS			0.37	0.27	
Lin-CD45-CD34+	NS	NS	NS	0.038	NS		-0.24	-0.23	
CD45-CD31+CD34+KDR+	0.001	0.0003	NS	NS	0.0048	0.038		0.26	
CD45-CD29+CD90+	NS	NS	NS	NS	0.0238	0.043	0.0022		
	P value								

Next, we checked whether the presence of stem cell subpopulations allowed for detection of the *POU5F1* transcript, which encodes OCT4A - a key regulator of pluripotency. The results indicate that primers designed to avoid detection of known pseudogenes (Primers Nowak et al. in Figure 9 are *Hsa_POU5F1-2* in Table 4) did not show any *POU5F1* expression in PB TNCs or bone marrow (Figure 9A,B), although amplified in the positive control (Figure 9C). On the other hand, primers Kucia et al. (*Hsa_POU5F1-1* in Table 4) bind in the exons 4 and 5 of the *POU5F1* gene and therefore do not distinguish OCT4A from B and B1 isoforms that do not function as regulators of pluripotency [202]. Furthermore, the latter pair of primers also recognizes many *POU5F1* pseudogenes what can further lead to the potential misinterpretation of data obtained.
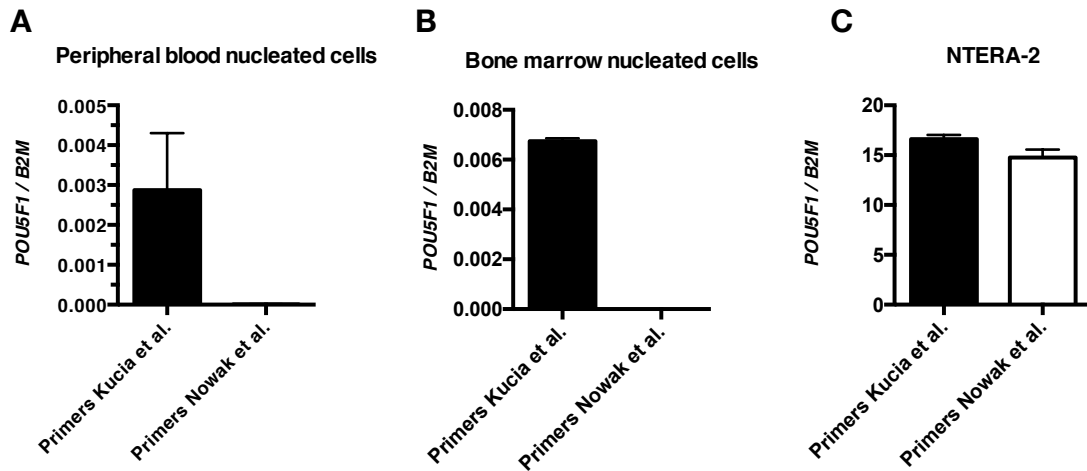
Figure 9. Expression of *POU5F1i (OCT4A)* assessed with two different primer pairs in **(A)** peripheral blood nucleated cells, **(B)** bone marrow nucleated cells, **(C)** pluripotent NTERA-2 cell line (human testicular teratoma)[24].

8.2. Total nucleated cells in diabetic patients are characterized with changed expressions of *HMOX1* and *HMOX2* [24]

Peripheral blood nucleated cells in all groups of diabetic patients displayed a downregulation of catalase (Figure 10A). A similar decrease in this group of patients was found for *HMOX1* (Figure 10B), regulated in response to oxidative stress by Nrf2 transcription factor. Here the inhibition of *HMOX1* was possibly not associated with Nrf2, as *NQO1* – another Nrf2 target – showed an opposite tendency (Figure 10C). The expression of *HMOX1* was augmented in all groups with foot disorders (Figure 10B) and correlated negatively with the both VSEL populations ($Lin^-CD45^-CD133^+$ $p=0.026$, Spearman r=-0.31; $Lin^-CD45^-CD34^+$ $p=0.008$, Spearman r=-0.37).
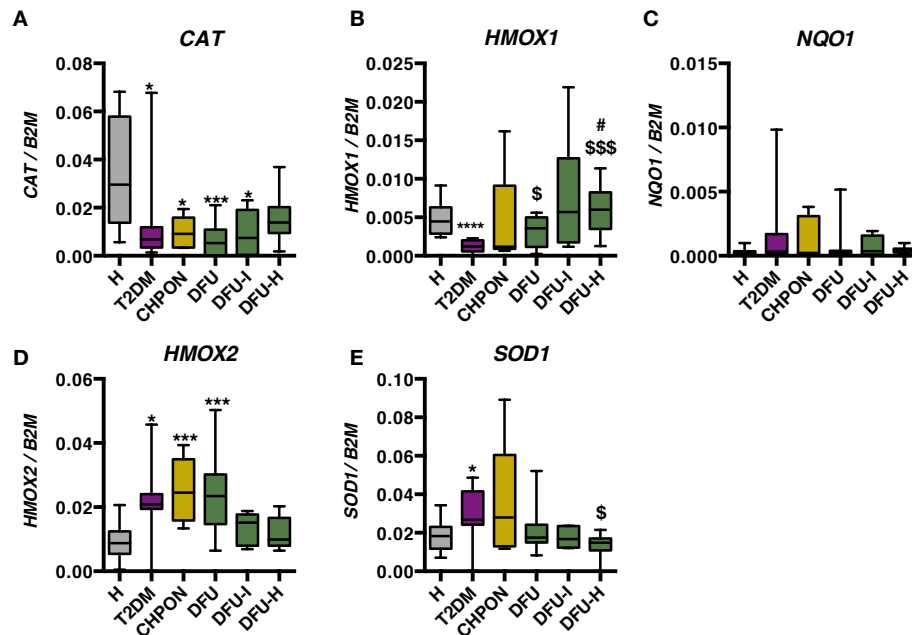
Figure 10. Expression of antioxidant genes in circulating total nucleated cells and concentration of plasma cytokines in peripheral blood of patients. **(A)** *CAT*, **(B)** *HMOX1*, **(C)** *NQO1*, **(D)** *HMOX2*, **(E)** *SOD1*. Expression of mRNA was determined by qRT-PCR. *B2M* served as a constitutive control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *versus* H; $^{\$}p<0.05$ *versus* T2DM; $^{\#}p<0.05$ *versus* DFU. Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles [24].

Interestingly, the expression of *HMOX2*, which is considered a constitutive gene, was higher in type 2 diabetes, Charcot osteoneuropathy (CHPON) and diabetic foot ulcer (DFU) groups than in healthy subjects (Figure 10D), what may be regarded as a kind of compensatory mechanism. Although no significant changes were found for *SOD1* (Figure 10E), its expression correlated positively with both VSEL populations (Lin^{-}CD45^{-}CD133^{+} $p=0.026$, Spearman r=0.31; Lin^{-}CD45^{-}CD34^{+} $p=0.008$, Spearman r=0.32) and *NQO1* ($p=0.0005$, Spearman r=0.46) but negatively with CD45$^{\text{dim}}$CD31^{+}CD34^{+}KDR^{+} EPCs ($p=0.024$, Spearman r=-0.26).

8.3. Diabetic patients have increased TNFα [24]

TNFα was elevated in all diabetic patients, regardless of the diabetic foot syndrome presence (Figure 11A). Type 2 diabetes patients also had a lowered concentration of EGF (Figure 11B), the effect less pronounced in groups with diabetic foot syndrome. Of note, level of plasma EGF correlated positively with the number of circulating CD45^{-}CD29^{+}CD90^{+} MSCs (Spearman r=0.32, $p=0.009$), negatively with the Lin^{-}CD45^{-}CD34^{+} VSEL population (Spearman r=-0.37, $p=0.002$) and Hb1$_{\text{Ac}}$ (Spearman r=-0.31, $p=0.02$). Interestingly, patients with Charcot osteoneuropathy were the only subjects with a reduced level of FGF-2 (Figure 11C). Concentrations of G-CSF, Flt-3-L,

SDF1α+β, LIF, TPO and SCF (Figure 11D-I) were similar in all groups and did not correlate with the numbers of stem or progenitor cells.
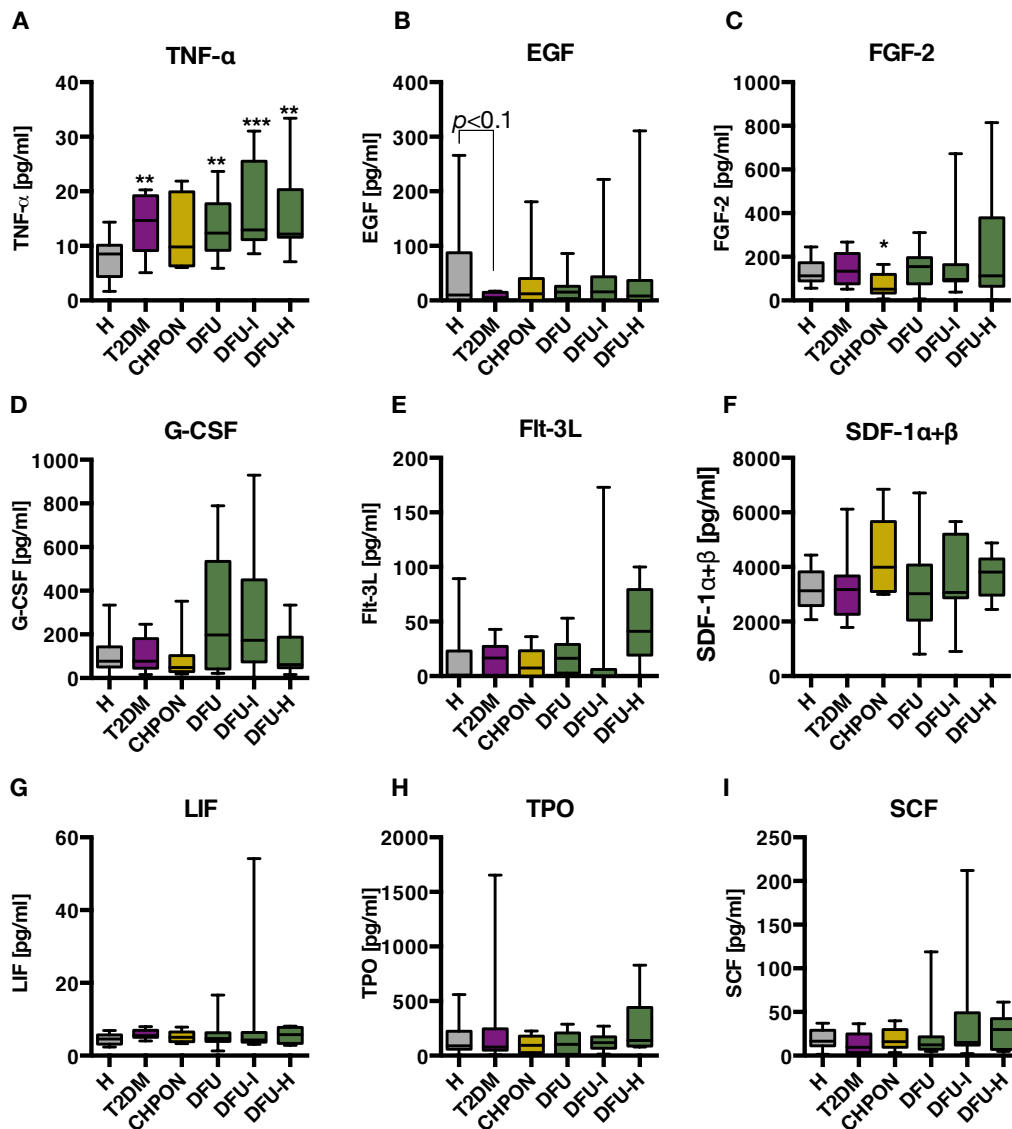
Figure 11. Plasma levels of **(A)** TNF-α **(B)** FGF-2 **(C)** EGF, **(D)** G-CSF, **(E)** Flt3-L, **(F)** SDF1α+β, **(G)** LIF, **(H)** TPO, **(I)** SCF. Concentration of cytokines in plasma was measured using Milliplex FlexMap 3D method. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *versus* H; $^\$p<0.05$ *versus* T2DM; $^\#p<0.05$ *versus* DFU. Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles [24].

To sum up, complications of type 2 diabetes, namely diabetic foot syndrome, especially when complex can affect numbers of various circulating stem and progenitor cells. This variability hampers the potential use of such characteristics as robust and specific marker of disease progression. Furthermore, profile of antioxidant gene expression, especially of heme oxygenases is changed in T2DM patients, what can suggest possible mechanism contributing to the development of complications of diabetes.

8.4. Treadmill training increases PFWT and MWT in intermittent claudication patients [73]

Treadmill training increased pain-free walking time from 155±46 s at baseline to 385±164 s at day 90 (*p*=0.01) (Figure 12A), and maximal walking time from 561±246 to 1045±480 s (*p*=0.003) (Figure 12B). Moreover, we have observed evident trend towards increase in the flow-mediated dilatation (Figure 12C) after the training.

Subsequently, we have checked if the improvement in the endothelial function could have resulted from the release of molecules that affect activity of NOS3. There was no change, however, in the level of NOS3 phospho-Ser1177 in HAoEC cell line in response to plasma collected from patients either after exercise or after the training (Figure 12D).
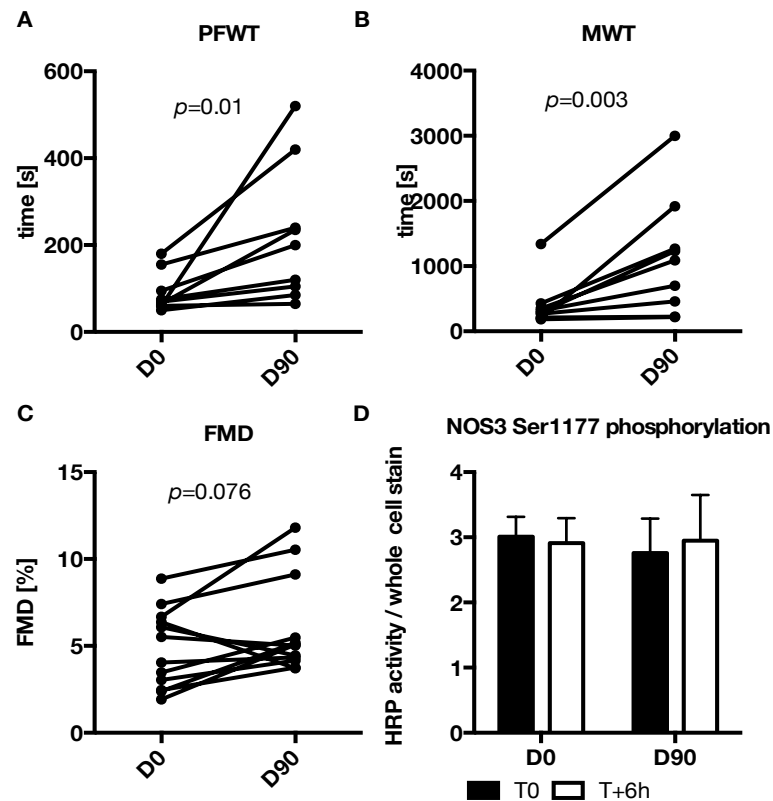
Figure 12. Pain-free walking time **(A),** maximal waling time **(B)** and flow mediated dilatation **(C)** in intermittent claudication patients before (D0) and after the 3 months training programme (D90). Each pair of points represent single patient, paired Student *t*-test. Potential of plasma collected from intermittent claudication patients before (T0) and 6 hours after single exercise (T+6h) before (D0) and after the 3 month training programme (D90) **(D)**. Data shown as mean + SD, Two-way Anova with Bonferroni post-test, N=12 [73].

8.5. Exercise increases number of circulating $CD45^{dim}CD34^+CD133^-$ KDR^+ proangiogenic progenitor cells [73]

Various populations of bone marrow-derived stem and progenitor cells enriched for the proangiogenic potential have been shown to contribute to the vascular homeostasis. Therefore, we have evaluated number of three populations of PACs after the exercise and treadmill training. Number of circulating $CD45^{dim}CD31^+CD133^+$ and $CD45^{dim}CD34^+CD133^+KDR^+$ cells did not change in response to the single exercise either on day 0 or on day 90 or in response to the training (Figure 13A,B). Number of more differentiated population of $CD45^{dim}CD34^+CD133^-KDR^+$ increased in the whole trained group 3 hours after the exercise on day 0 (Figure 13C), however remained stable on day 90 and over the training (Figure 13D).
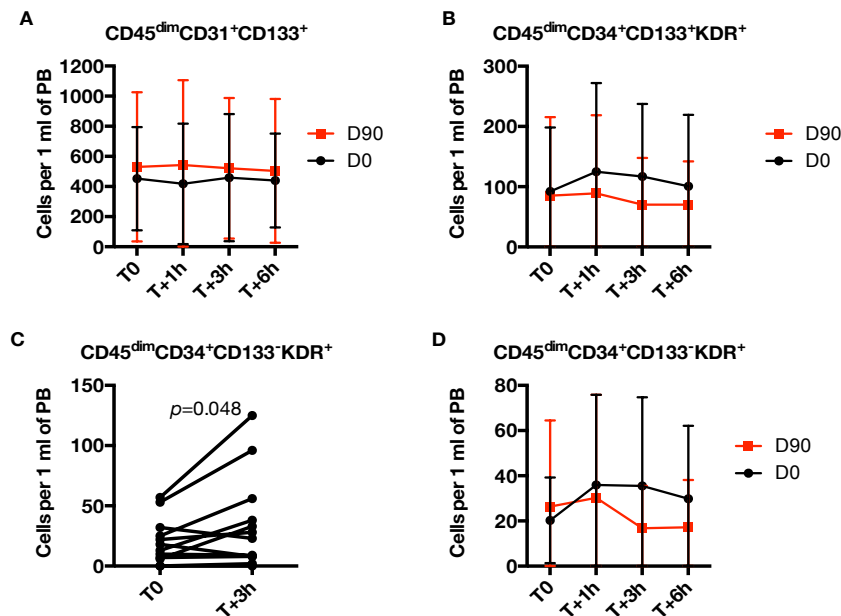
Figure 13. Number of circulating $CD45^{dim}CD31^+CD133^+$ **(A)**, $CD45^{dim}CD34^+CD133^+KDR^+$ **(B)** proangiogenic progenitor cells in response to the single exercise (T+1h, T+3h, T+6h vs T0) and training (D90 vs D0). Mobilization of $CD45^{dim}CD34^+CD133^-KDR^+$ PACs 3 hours after the exercise at the beginning of training series **(C).** Each pair of points represents one patient, paired Student t-test. Number of circulating $CD45^{dim}CD34^+CD133^-KDR^+$ **(D)** proangiogenic progenitor cells in response to the single exercise (T+1h, T+3h, T+6h vs T0) and training (D90 vs D0). Numbers of cirvulating cells were assessed with flow cytometry. Data are shown as mean ± SD (A,B,D) [73].

Interestingly, number of $CD45^{dim}CD34^+CD133^-KDR^+$ cells correlated positively with the ankle-brachial index ($p=0.035$, Spearman r=0.68) whereas $CD45^{dim}CD34^+CD133^+KDR^+$ with the maximal walking time ($p=0.017$, Spearman r=0.74) on day 90 (Table 8).

Table 8. Correlations of MWT, ABI, PFWT with the measured parameters before and after the training series [73].

D0			
Parameter A	**Parameter B**	**_p_**	**Spearman r**
MWT	PFWT	0.0047	0.82
	TC / HDL	0.0390	-0.61
	LDL	0.0234	-0.72
	LDL / HDL	0.0234	-0.72
ABI	RBC	0.0306	-0.69
	MONO	0.0390	0.67
PFWT	TC / HDL	0.0105	-0.78
	LDL	0.0037	-0.84
	LDL / HDL	0.0058	-0.82
D90			
Parameter A	**Parameter B**	**_p_**	**Spearman R**
MWT	IL-6	0.0268	0.71
	IL-12	0.0490	0.65
	miR-146a	0.0138	-0.80
	PFWT	0.0016	0.88
	TC / HDL	0.0174	-0.74
	HDL	0.0058	0.82
	LDL / HDL	0.0149	-0.76
	$CD45^{dim}CD34^{+}CD133^{+}KDR^{+}$	0.0174	0.74
ABI	PLT	0.0347	0.68
	$CD45^{dim}CD34^{+}CD133^{-}KDR^{+}$	0.0347	0.68
PFWT	IL-6	0.0037	0.84
	miR-146a	0.0214	-0.76
	TC / HDL	0.0390	-0.67
	LDL / HDL	0.0268	-0.70

8.6. Exercise changes the expression of antioxidant enzymes in IC patients [73]

Walking as well as treadmill exercise in intermittent claudication results in oxygen imbalance and ischemia-reperfusion cycles that may lead to oxidative damage of the endothelium. Therefore, we have evaluated expression of antioxidant genes *HMOX1*, *HMOX2* and *SOD1* as well as miRNAs associated with regulation of endothelial function in TNCs of IC patients subjected to the training.
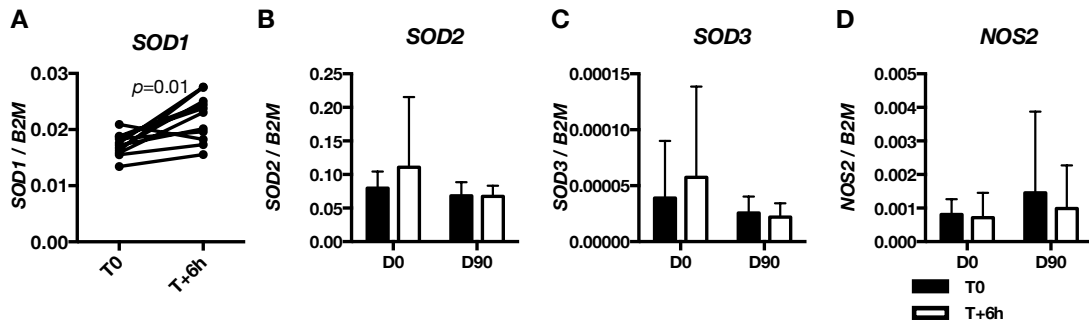
Figure 14. Influence of the single exercise on the expression of *SOD1* on day 0 before (T0) and 6 hours after the single exercise (T+6h) **(A).** Each pair of points represents one patients, paired Student *t*-test. Expression of *SOD2* **(B)**, *SOD3* **(C)** and *NOS2* on day 0 and day 90 of the training before (T0) and after the single exercise (T+6h). Expression of genes was assessed with qRT-PCR. Data shown as mean + SD, Two-way Anova with Bonferroni post-test, N=12 [73].

Expression of *SOD1* was enhanced 6 hours after the single exercise on day 0 and on day 90 (Figure 14A) while *SOD2*, *SOD3,* and *NOS2* did not change significantly both before and after the training programme (Figure 14B-D).
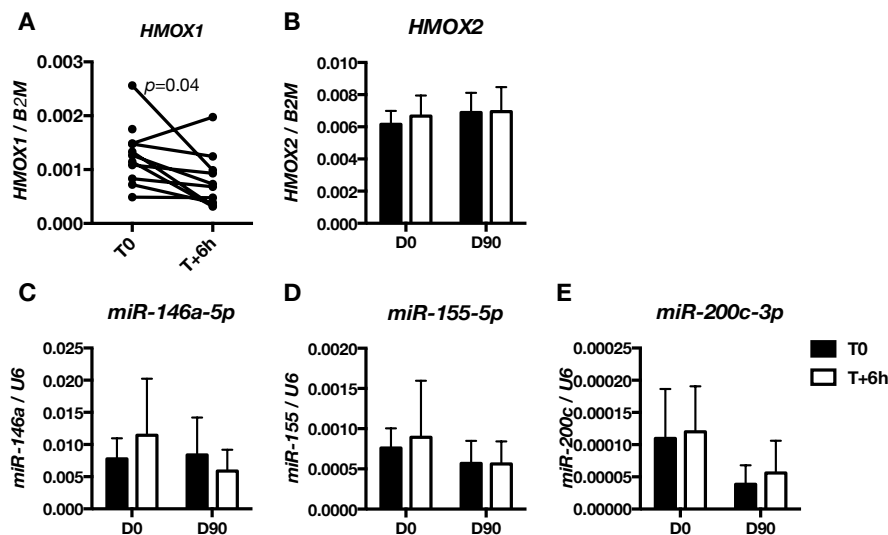
Figure 15. Expression of *HMOX1* in peripheral blood total nucleated cells before (T0) and 6 h after single exercise (T+6h) **(A)** Each pair of points represents one patient, paired Student *t*-test. Expresssion of *HMOX2* **(B)**, *miR-146a-5p* **(C)**, *miR-155-5p* **(D)**, *miR-200c-3p* **(E)** in peripheral blood total nucleated cells in response to the single exercise (T0 and T+6h) and training (D0 or D90). Expression of genes was assessed with qRT-PCR. Data are shown as mean + SD, Two-way Anova with Bonferroni post-test, N=12 [73].

Noteworthy, expression of *HMOX1* decreased in response to the single exercise at day 0 (Figure 15A). Moreover, there was a weak trend towards higher *HMOX2* expression ($p=0.06$) at D90 than at D0 (Figure 15B). We did not observe changes in the expression of miR-146a-5p, miR-155-5p and miR-200c-3p either in response to the exercise or to training (Figure 15C-E). There was, however, a significant negative correlation between the level of anti-inflammatory miR-146a and MWT or PFWT at

D90 (Spearman r=-0.80, *p*=0.014 and Spearman r=-0.76, *p*=0.021, respectively), (Table 8).

8.7. Treadmill training decreases plasma IL-6 but increases MCP-1 [73]

Ischemia-reperfusion cycles in IC during walking and rest may lead to the induction of inflammatory response and so aggravate the atherosclerosis. Therefore, we have evaluated impact of single exercise and training on the plasma levels of the growth factors and mediators of inflammation. Plasma IL-6 level in IC patients was lower on day 90 than on day 0 (Figure 16A). Concomitantly, we have observed increase in plasma MCP-1 level (Figure 16B).
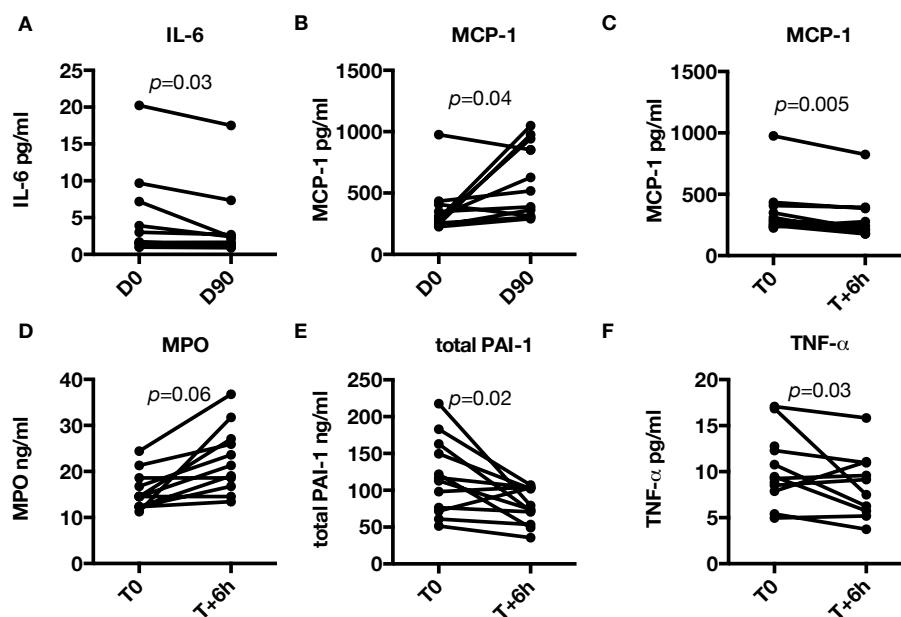
Figure 16. Plasma concentrations of IL-6 **(A)** and MCP-1 **(B)** before (D0) and after (D90) 3 months training programme. Plasma concentrations of MCP-1 before (T0) and after (T+6h) single exercise on day 0 **(C)**, MPO before (T0) and after (T+6h) single exercise on day 0 **(D)**, total PAI-1 before (T0) and after (T+6h) single exercise on day 90 **(E)** and TNF-α before (T0) and after (T+6h) single exercise on day 0 **(F).** Plasma concentrations of above factors were measured with multiplex assays on luminex platform. Each pair of points represents one patient, paired Student *t*-test [73].

Interestingly, concentration of MCP-1 decreased in response to the single exercise at D0 (Figure 16C) and D90. Moreover, there was an evident trend towards increased plasma myeloperoxidase level at day 0 (Figure 16D) Single exercise decreased total PAI-1 at D90 (Figure 16E) and TNF-α at D0 (Figure 16F).
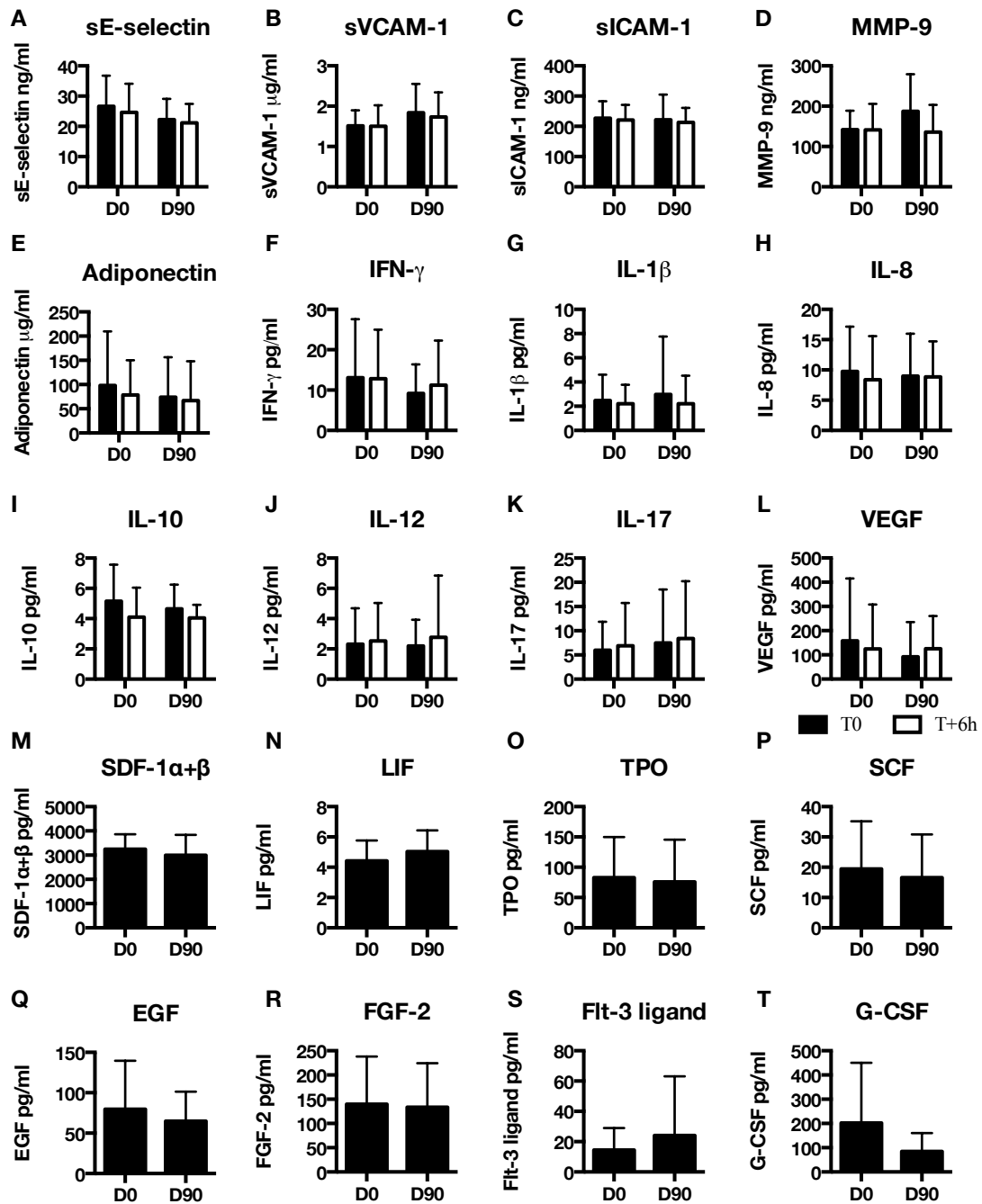
Figure 17. Plasma level of sE-selectin **(A)**, sVCAM-1 **(B)**, sICAM-1 **(C)**, MMP-9 **(D)**, adiponectin **(E)**, IFN-γ **(F)**, IL-1β **(G)**, IL-8 **(H)**, IL-10 **(I)**, IL-12 **(J)**, IL-17 **(K)**, VEGF **(L)** in response to the exercise and training. Plasma level of SDF-1α+β **(M)**, LIF **(N)**, TPO **(O)**, SCF **(P)**, EGF **(Q)**, FGF-2 **(R)**, Flt-3L **(S)**, G-CSF **(T)** in response to training. Concentrations of above factors were measured with multiplex assays on luminex platform. Data are shown as mean ± SD [73].

Furthermore, there was a trend towards lower level of soluble E-selectin at D90 in comparison to the D0 (Figure 17A). Neither training nor exercise changed plasma levels of sVCAM-1, sICAM-1, adiponectin, IL-1β, IL-8, IL-10, IL-12 IL-17, VEGF, IFN-γ, SDF-1α+β, G-CSF, Flt3 ligand, FGF-2, TPO, SCF and LIF (Figure 17B-T). Interestingly, concentrations of proinflammatory IL-6 and IL-12 in plasma of patients at day 90 were positively correlated with MWT (Spearman R=0.71, p=0.027, and Spearman R=0.65, p=0.049). IL-6 correlated also with PFWT (Spearman R=0.84, p=0.004) (Table 8).

Concluding, single exercise in non-trained intermittent claudication patients results in increase of numbers of circulating proangiogenic cells but also some inflammatory mediators. We may speculate that observed change in $CD45^{dim}CD34^+CD133^-KDR^+$ can be a marker of increased stress resulting from ischaemia and reperfusion, which is reduced in trained subjects. Similar trend was observed for inflammatory mediators eg. MPO and heme oxygenase-1, which is potent cytoprotective enzyme but its upregulation can also be regarded as a marker of cellular stress.

8.8. Effects of statins on proangiogenic paracrine activity of human peripheral blood CD34$^+$ cells

Paracrine angiogenic activity is considered as a key role played by circulating proangiogenic cells in the regulation of blood vessel formation. On the other hand, prospective beneficients of therapy with proangiogenic cells often are administered with a vast panel of medicines. Therefore, we decided to evaluate the influence of atorvastatin, acetylsalicylic acid, metformin but also sulforpahane and resveratrol on the proangiogenic activity of human peripheral blood CD34$^+$-derived cells.
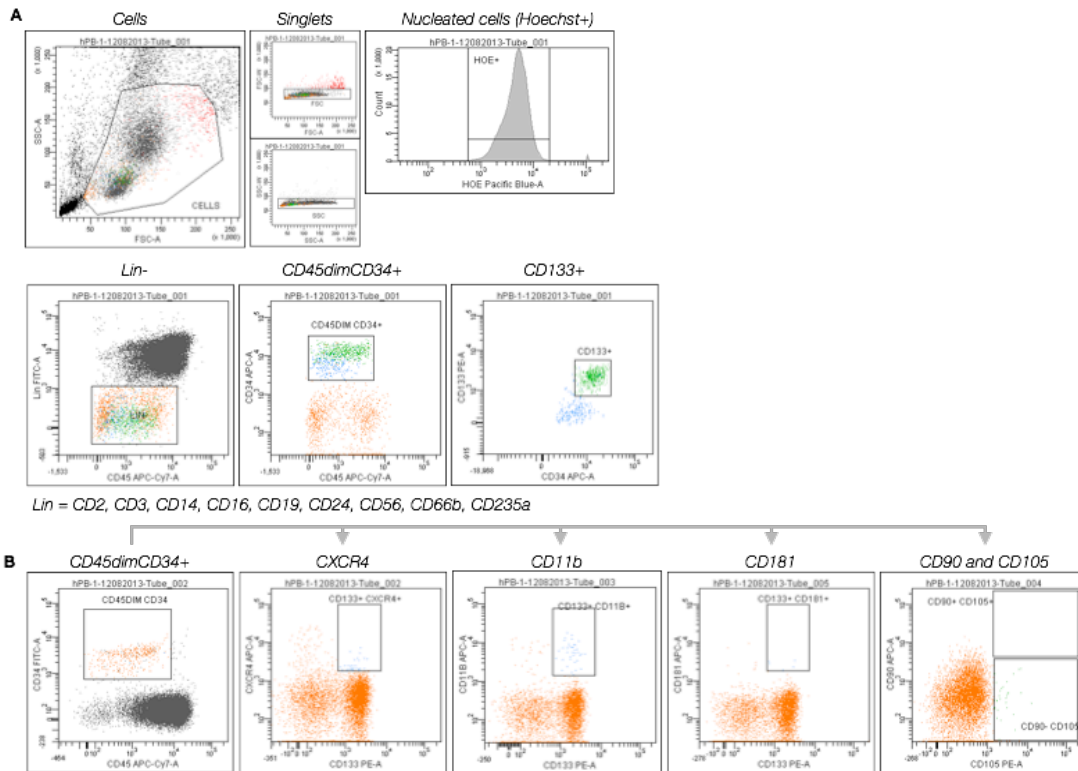
Figure 18. Phenotype of obtained peripheral blood mononuclear cells isolated with apheresis and further purified with centrifugation on ficoll gradient. **(A)** 96% of singlets are nucleated cells. Out of them, 2% is Lin⁻CD45^{dim}CD34$^+$. 67% of the latter cells co-express CD133$^+$. **(B)** Analysis of the expression of CXCR4, CD11b, CD181, CD90 and CD105 within the CD45^{dim}CD34$^+$ population. Phenotype of isolated cells was assessed with immunofluorescent staining and subsequent analysis on flow cytometer.

First, we assessed the phenotype of cells that were obtained with apheresis from a healthy donor who was administered with G-CSF for 5 consecutive days. 2 % of peripheral blood mononuclear cells, which were further purified from erythrocytes with the centrifugation on ficoll gradient, were CD45^{dim}CD34$^+$ (Figure 18A). What is more, 67% of CD45^{dim}CD34$^+$ expressed CD133/AC133, which is often found on stem and progenitor cells (Figure 18A). CD45^{dim}CD34$^+$ did not express mesenchymal stromal cell markers CD90 or CD105 (Figure 18B) and only small fractions of these cells were positive for CXCR4, CD184 or CD11b (Figure 18B).

Treatment of CD34$^+$-derived cells with atorvastatin enhanced their paracrine angiogenic activity. HAoEC cells were treated with conditioned media harvested from cells stimulated for 48 h with atorvastatin, acetylsalicylic acid, resveratrol, sulforaphane or metformin. Of note, conditioned media contained as well the stimulants. Therefore, to distinguish angiogenic activity of factors released by stimulated cells from effect of stimulants alone, we used non-conditioned media as controls. Non-conditioned media

contained the same concentrations of stimulants and were incubated for the same period in the cell culture incubator as conditioned media but without cells. HAoEC cells used in Matrigel assay *in vitro* formed tube-like structures characterized with higher number of meshes and junctions (Figure 19A-C) when conditioned media from CD34^{+}-derived cells stimulated with high doses of atorvastatin but not from cells treated with acetylsalicylic acid, resveratrol, sulforaphane or metformin. Interestingly, effect of conditioned media from atorvastatin-treated cells was stronger than that observed in cells treated with non-conditioned media.

Then, we assessed whether stimulation with any of used stimulants can affect expression of heme oxygenase-1 – known proangiogenic factor, that was crucial for the paracrine angiogenic activity of mouse PAC cells [4]. The only stimulant that enhanced *HMOX1* expression in CD34^{+}-derived cells was sulforaphane, known inductor of Nrf2 transcription factor activity (Figure 19D). However, sulforaphane did not affect paracrine angiogenic activity of tested population of CD34^{+}-derived cells. On the other hand, atorvastatin, which increased PAC angiogenic activity did not change *HMOX1* transcript levels (Figure 19D).
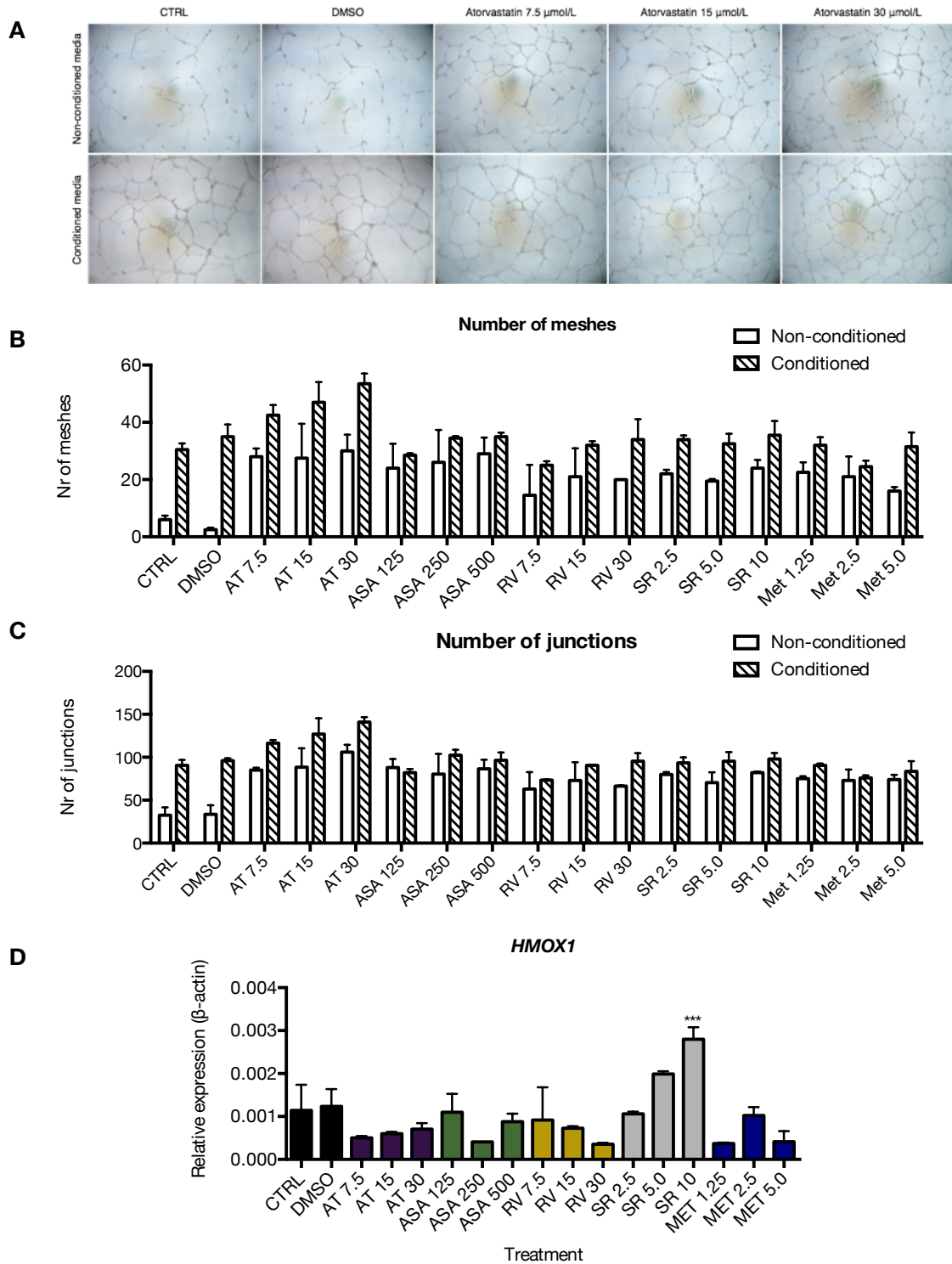
Figure 19. Effect of atorvastatin (AT), acetylsalicylic acid (ASA), resveratrol (RV), sulforaphane (SR), or metformin (Met) in control (CTRL) and conditioned media from CD34^{+}-derived cells on angiogenesis in Matrigel assay (**A,B,C**). Tube formation was assessed with ImageJ software with Angiogenesis macro. *HMOX1* expression in CD34^{+}-derived cells stimulated for 6 hours with 7.5, 15 or 30 μmol/L atorvastatin (AT), 125, 250 or 500 μmol/L acetylsalicylic acid (ASA), 7.5, 15 or 30 μmol\L resveratrol (RV), 2.5, 5.0 or 10 μmol\L sulforaphane (SR), or 1.25, 2.5 or 5 mmol\L metformin (MET) (**D**). *HMOX1* expression was measured with qRT-PCR, (n=2) [***]$p<0.001$, One-way Anova with Bonferroni post-test.

Then, paracrine angiogenic activity of CD34^{+}-derived cells stimulated with 30 μmol/L atorvastatin, the highest dose used *in vitro* which showed a proangiogenic effect, was tested in Matrigel plug assay *in vivo*. Similar to *in vitro* experiments, we used conditioned media from CD34^{+}-derived cells treated with atorvastatin, non-conditioned media containing 30 μmol/L atorvastin and incubated in cell culture incubator for the same period of time without cells. Additionally, we tested wheter paracrine angiogenic activity can be maintained in atorvastatin-treated cells after wash-out of the stimulant. Therefore, we harvested media from cells stimulated with atorvastatin for 48 hours, which were then washed and re-seeded in growth medium for additional 48 hours. Finally, to test the influence of the CD34^{+}-derived cells themselves we mixed atorvastatin-treated or control cells with Matrigel and injected them subcutaneously to nude mice.
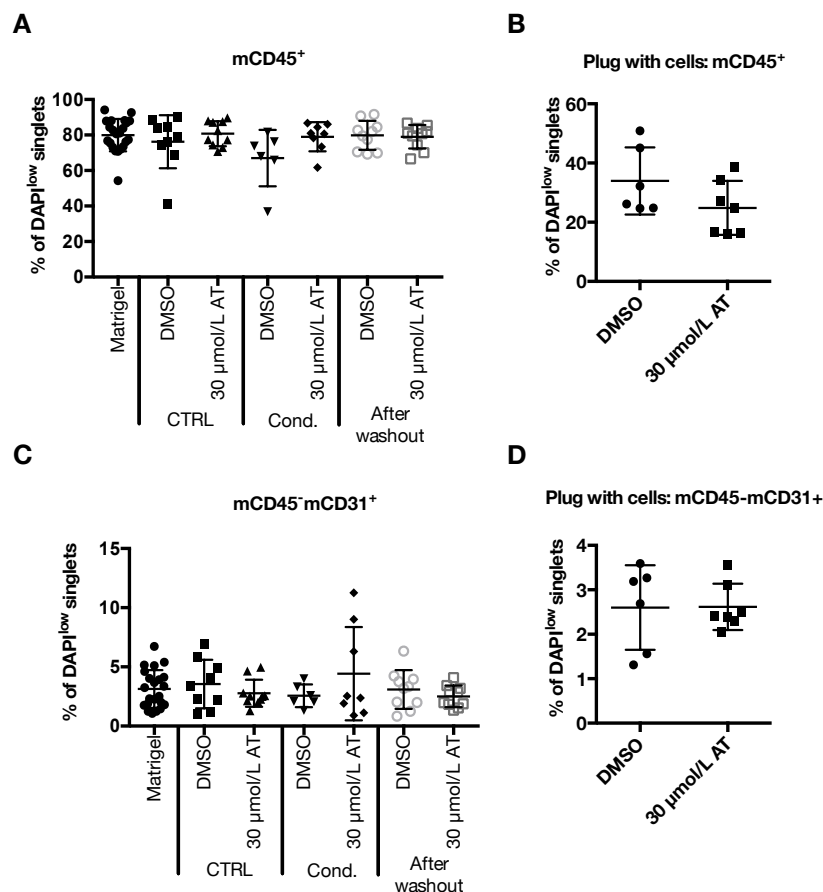
Figure 20. Infiltrating murine CD45^{+} cells in Matrigel plugs with control, conditioned media or media after stimulant washout from atorvastatin (N=10-22) **(A)** or in Matrigel plugs with atorvastatin-stimulated CD34^{+} derived cells (N=6-7) **(B)**. Mouse CD45^{-}CD31^{+} endothelial cells in Matrigel plugs with control, conditioned media or media after stimulant washout from atorvastatin (N=10-22) **(C)** or in Matrigel plugs with atorvastatin-stimulated CD34^{+} derived cells (N=6-7) **(D)**.

Angiogenic response to the media or cells was tested with flow cytometry. Numbers of infiltrating CD45^{+} immune cells as well as CD45^{-}CD31^{+} host endothelial cells was similar in all groups (Figure 20A,C). Similarly neither control cells, nor CD34^{+}-derived cells stimulated with atorvastatin induced angiogenesis or infiltration with CD45^{+} cells (Figure 20B,D).

In order to further study paracrine proangiogenic activity observed *in vitro* we tested concentration of 30 human growth factors and inflammatory mediators in conditioned media from CD34^{+}-derived cells stimulated with 7.5, 15 or 30 μmol\L atorvastatin. Only IL-8 seemed to increase upon stimulation with atorvastatin, however, it was not reproduced in further experiments (

Figure 21 and Supplementary Figures 1-3). When treatment was repeated two more times (altogether for 144 h, whereas initially cells were stimulated for 48) cells stimulated with atorvastatin produced less IL-10 and more MCP-1 (Figure 22).
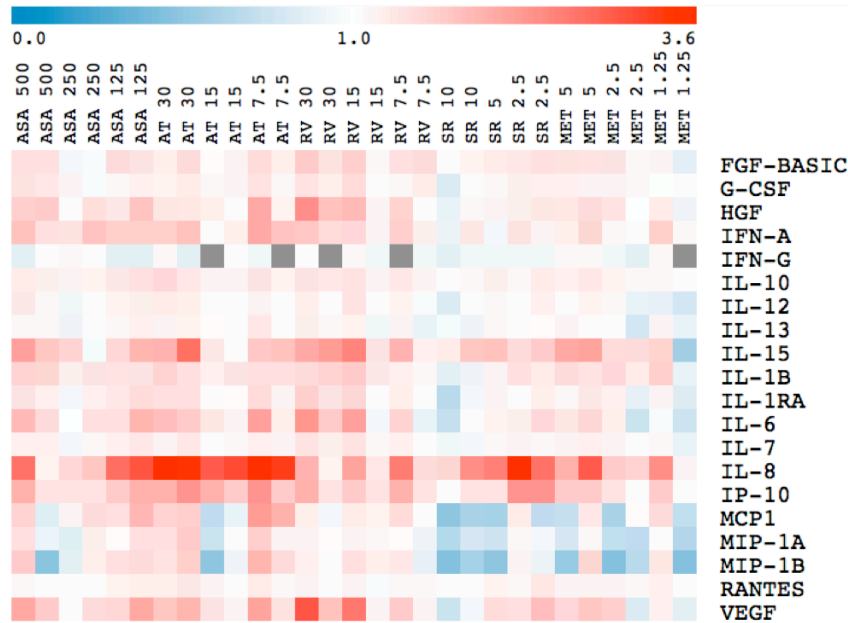
Figure 21. Changes in the levels of secreted factors in CD34^{+}-derived cells stimulated for 48 hours with 7.5, 15 or 30 μmol\L atorvastatin (AT), 125, 250 or 500 μmol/L acetylsalicylic acid (ASA), 7.5, 15 or 30 μmol\L resveratrol (RV) 2.5, 5 or 10 μmol/L sulphorafan (SR) and 1.25, 2.5 or 5 mmol/L metformin (MET) *versus* control cells stimulated with solvent (DMSO). Concentrations of cytokines were measured with Cytokine Human 30-Plex Panel (Invitrogen) on the Luminex *FlexMAP 3D* platform. Each square represents fold difference in cytokine concentration *versus* cells stimulated with solvent – DMSO.
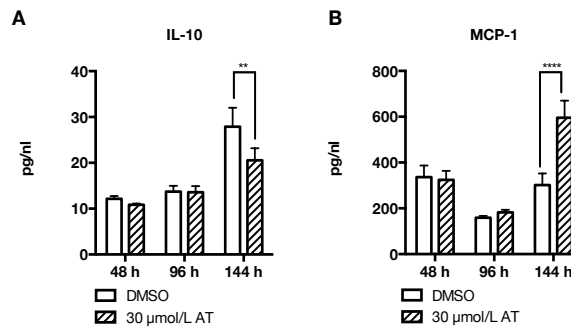
Figure 22. Concentration of IL-10 **(A)** and MCP-1 **(B)** in media from CD34^{+}-derived cells stimulated for 48, 96 or 144 hours with 30 μmol/L atorvastatin. Each bar represents mean + SD. **$p<0.01$, ****$p<0.0001$ 30 μmol/L AT vs DMSO, Two-way Anova with Bonferroni post-test, N=3.

However, the observed effect on tube formation *in vitro* was observed in media collected already after 48 hours of stimulation. Therefore, it cannot be explained with the increased levels of MCP-1.

8.9. Effects of *Hmox1* deletion in murine bone marrow mesenchymal stromal cells

Mesenchymal stromal cells are important for the proper function of stem cell niches in bone marrow. Therefore, the influence of various stress factors on their functions is of great interest. Lack of heme oxygenase-1, crucial cytoprotective enzyme, was shown to potently affect other bone marrow-derived cells *i.e.* proangiogenic cells [4]. Although role of heme oxygenase-1 in MSC is vastly studied, published results are often contradictory and numerous publications are based on the chemical inhibition or stimulation of heme oxygenase-1 activity, which can have many unspecific effects. Therefore, since we have access to the C57B:6 × FVB *Hmox1*^{-/-} mice, we decided to characterize murine bone marrow-derived mesenchymal stromal cells lacking functional *Hmox1* gene.

Hmox1^{+/+} or Hmox1^{-/-} bone marrow mesenchymal stromal cells do not differ in their phenotype

Bone marrow stromal cells isolated from long bones of mice *Hmox1*^{+/+} or *Hmox1*^{-/-} formed similar numbers of colonies that consisted of fibroblastoid cells (Figure 23A) or cells able to be differentiated to osteoblasts (Figure 23B).
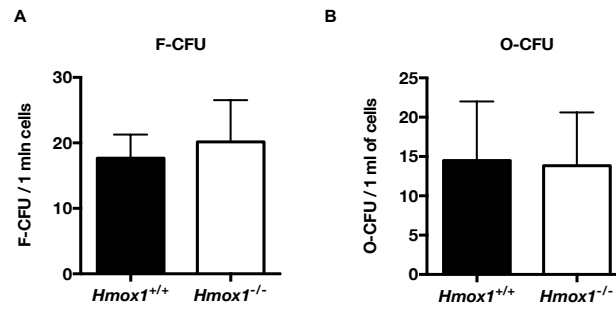
Figure 23. Ability to form fibroblastoid **(A)** or osteoblast **(B)** colonies by bone marrow cells isolated from $Hmox1^{+/+}$ or $Hmox1^{-/-}$ MSCs assessed with fibroblastoid or osteoblast colony forming unit assay, respectively. Data shown as mean + SD, N=3.

Phenotype of murine bone marrow stromal cells in culture was assessed with flow cytometry. Regardless of the genotype, 60% of the cells in culture were $CD45^-$ $CD31^-$ (Figure 24D). Therefore, cells used for all the experiments were purified from remaining $CD45^+$ fraction with MACS sorting. Obtained cells expressed positive markers attributed to the mesenchymal stem/stromal cells *i.e.* CD29, CD90, CD105, Ly-6A/E (Sca1) and CD140a (PDGFRα) (Figure 24B,C,E,F), and lacked expression of endothelial markers CD31 and CD34, while CD117 (ckit) was expressed only on small subfraction of cells (Figure 24A).
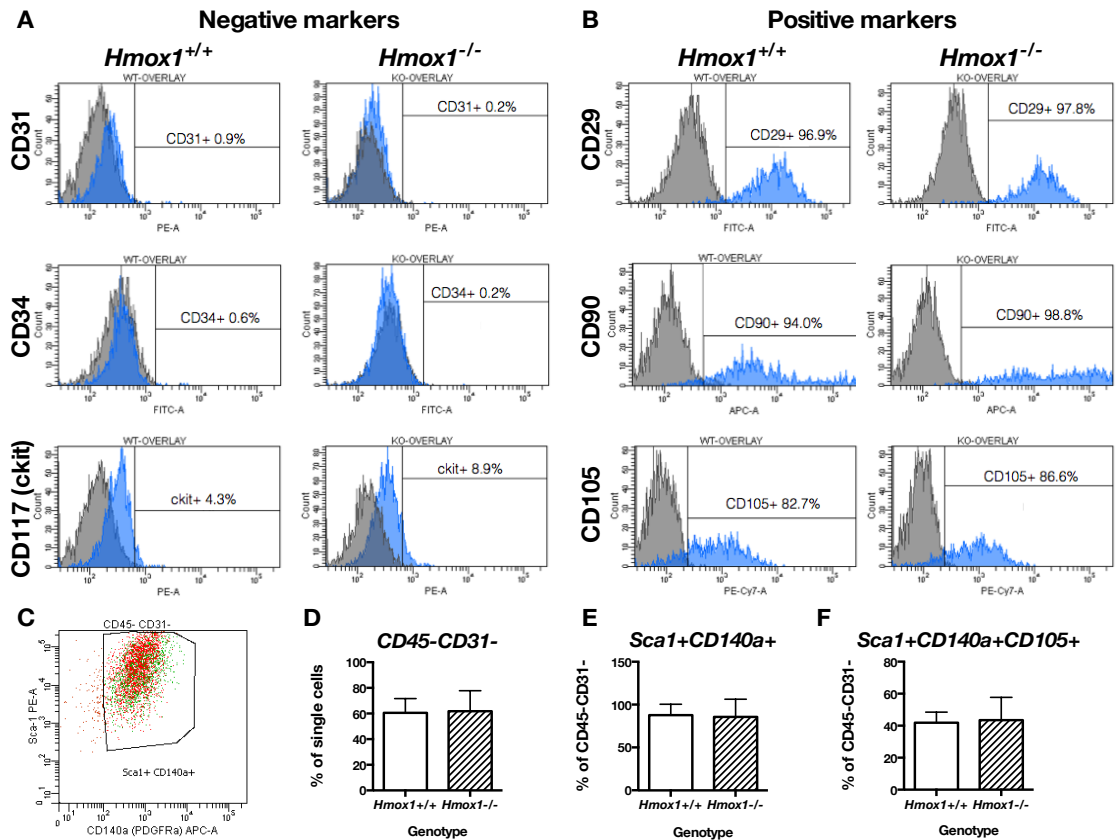
Figure 24. Expression of MSC negative markers CD31, CD34 and ckit **(A)**, MSC positive markers CD29, CD90 and CD105 **(B)** in $Hmox1^{+/+}$ or $Hmox1^{-/-}$ MSCs. Dot plot showing the expression of Ly-6A/E (Sca-1) and CD140a in cultured MSCs **(C)**. Phenotype of non-sorted bone marrow stromal cells in passage 4: fraction of $CD45^-CD31^-$ cells in culture **(D)**, fraction of $Sca-1^+CD140a^+$ cells within $CD45^-CD31^-$ population **(E)** or $Sca-1^+CD140a^+CD105^+$ cells within $CD45^-CD31^-$ population in $Hmox1^{+/+}$ or $Hmox1^{-/-}$ cells. (D-F, N=9-10).

$Hmox1^{+/+}$ or $Hmox1^{-/-}$ bone marrow mesenchymal stromal cells show similar ability to differentiate to osteoblasts and adipocytes

Mesenchymal stromal cells were shown to be able to differentiate to adipocytes, osteoblasts and chondrocytes [113]. Both $Hmox1^{+/+}$ and $Hmox1^{-/-}$ MSCs were able to differentiate to osteoblasts and adipocytes. Because HO-1 was suggested to play a role in regulation of adipogenesis [185], we focused on the effects of $Hmox1$ knockout on the genes associated with lipid metabolism. Interestingly, differentiation of murine bone marrow MSCs to adipocytes induced similar changes in the gene expression in both $Hmox1^{+/+}$ and $Hmox1^{-/-}$ cells (Figure 25).
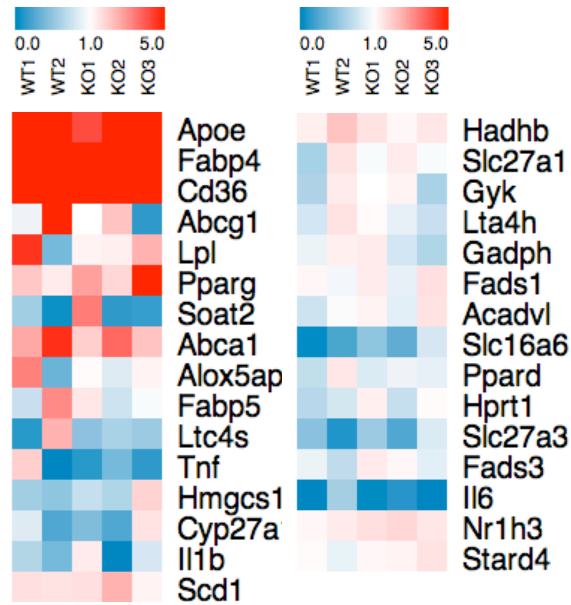
Figure 25. Changes in the expression of genes associated with lipid metabolism in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ bone marrow mesenchymal stromal cells. Expression of genes was assessed with qRT-PCR using TaqMan® Array Mouse Lipid Regulated Genes. Each square represents fold difference in MSC differentiated to adipocytes versus MSC cultured in growth medium. Blue squares show downregulation while red upregulation of gene expression.

However, *Hmox1*$^{+/+}$ MSCs were the only that increased expression of miR-21-5p, the microRNA which, via TGF-β signalling, regulates adipogenesis [203] (Figure 26A). Levels of other tested microRNAs, miR-31-5p, miR-150-5p, miR-301a-5p, miR-378a-3p or miR-378a-5p, remained unchanged in cells of both genotypes (Figure 26B-F). What is more, basal expressions of all tested microRNA, including miR-21-5p, which was induced during adipocyte differentiation only in *Hmox1*$^{+/+}$ cells, were similar in MSC *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ (data not shown).
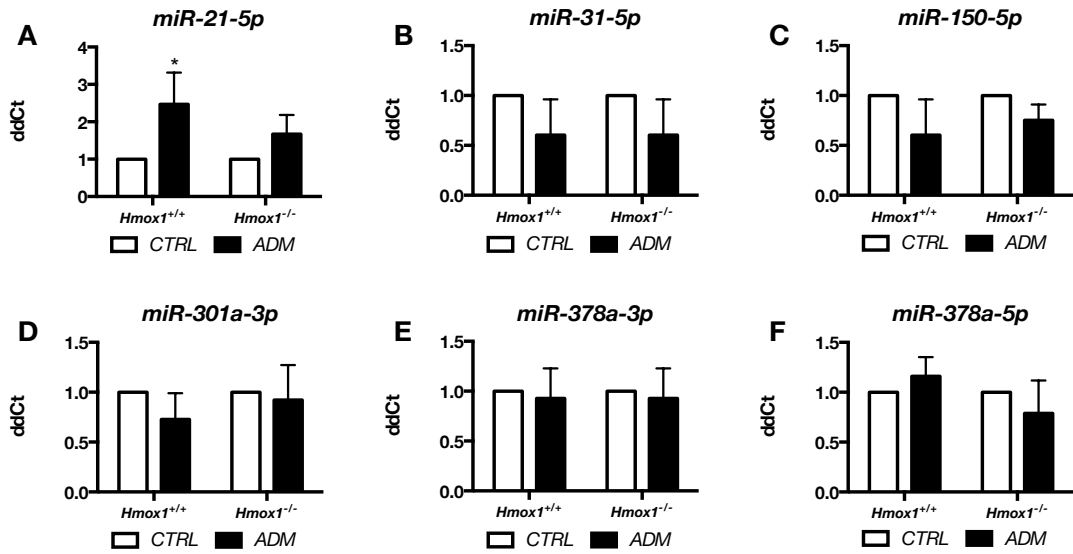
Figure 26. Expression of *miR-21-5p* **(A)**, *miR-31-5p* **(B)**, *miR-150-5p* **(C)**, *miR-301a-3p* **(D)**, *miR-378a-3p* **(E)**, *miR-378a-5p* **(F)**, in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells differentiated to adipocytes. N=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ ADM treated cells vs control. miRNA expression was assessed with qRT-PCR. Data are shown as mean + SD, N=3.

Effects of changed expression of *HMOX1* in human MSC on the adipogenesis or osteogenesis were stronger when cells were cultured in high glucose concentration [187]. Therefore, we tested some markers of adipogenesis and osteogenesis in MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ cultured in low or high glucose concentration. Interestingly, neither osteogenic nor adipogenic differentiation of murine bone marrow derived *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ MSCs were changed when cells were cultured in high glucose conditions (Figure 27). Both *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ MSCs upregulate fatty acid binding protein 4 – marker of adipocyte differentiation (Figure 27A). Levels of early marker of osteogenesis – Runx2 remain unchanged (Figure 27B). Nevertheless, Runx2 levels were assessed in the end of the experiment, and probably Runx2 expression was already decreased.

A

B
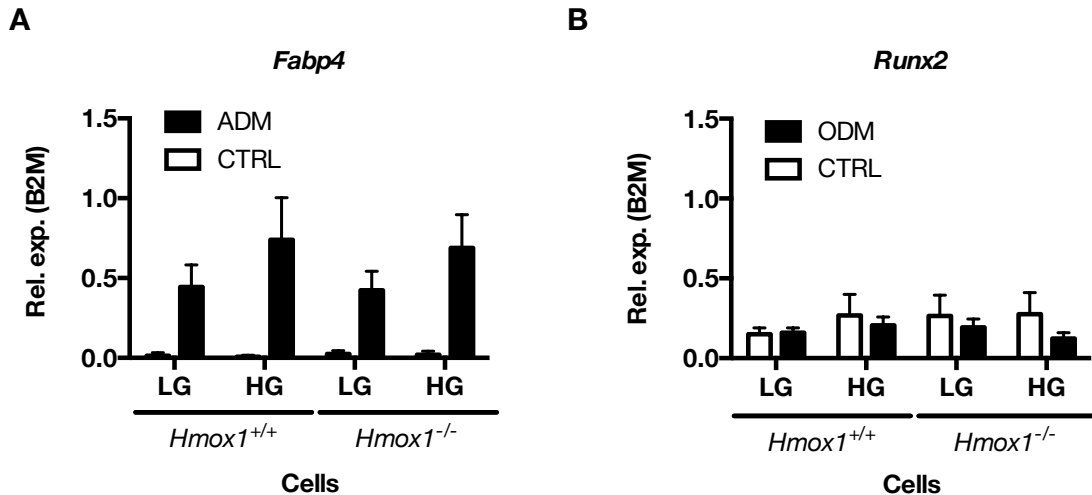
Fabp4

Runx2

Figure 27. Expression of *Fabp4* in *Hmox1*^{+/+} or *Hmox1*^{-/-} MSCs differentiated to adipocytes in low (LG) or high glucose (HG) conditions **(A)**. Expression of *Runx2* in *Hmox1*^{+/+} or *Hmox1*^{-/-} MSCs differentiated to osteoblasts in low (LG) or high glucose (HG) conditions **(B)** Gene expression was assessed with qRT-PCR. Data are shown as mean + SD, (N=4-7).

Mesenchymal stromal cells acquire some endothelial markers during differentiation

Mesenchymal stromal cells can affect formation of new blood vessels with paracrine stimulation of angiogenesis [204], support vasculature acting as pericytes [205] but were also shown to be able to be differentiated to endothelial cells [141]. On the other hand, heme oxygenase-1 is crucial for the vasculature [94] and angiogenic activity of bone marrow-derived proangiogenic cells [4]. Therefore, we decided to test the ability of mouse bone marrow-derived MSC to differentiate to endothelial cells and further the impact of *Hmox1* knock-out on their angiogenic potential.

Wild type mesenchymal stromal cells were differentiated for 7 days to endothelial cells with culture in EGM2-MV medium and in plates coated with fibronectin and gelatin. Differentiated cells (mesenchmal stromal cell-derived endothelial cells, MSC-ECs) showed enhanced staining with BS1 lectin and AcLDL binding (Figure 28A). What is more, they showed increased *Kdr* expression (Figure 28B) and decreased *Cox2* (Figure 28C), while levels of miR-21 remained unaffected (Figure 28D). Nevertheless endothelial differentiation did not result in change of MSC phenotype (Table 9).

Table 9. Phenotype of MSCs and MSC-ECs assessed with immunofluorescent staining and flow cytometry.

Antigen	MSC [%]	MSC-EC [%]
CD31$^+$	0.5	0.5
CD34$^+$	1.0	0.3
CD102$^+$	0.1	0.2
CD105$^+$	49.4	40.5
CD106$^+$	98.7	73.8
CD117$^+$	0.7	0.4
CD144$^+$	0.5	0.5
CD202b$^+$	0.4	0
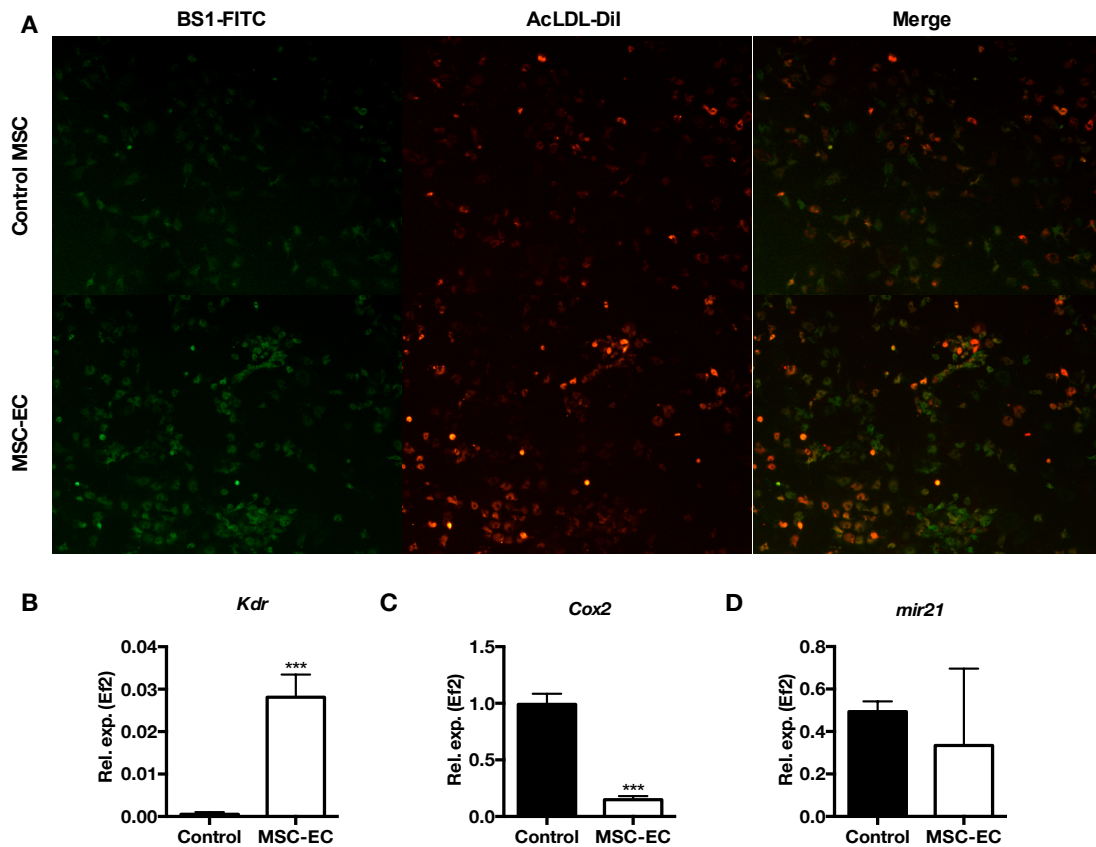
Figure 28. Staining with *Bandeiraea simplicifolia* lectin BS1-FITC and AcLDL-DiI in control MSCs and MSC-ECs on day 7 of endothelial differentiation **(A)**. Expression of *Kdr* **(B)**, *Cox2* **(C)** and *mir21* **(D)** assessed with qRT-PCR on day 7 of endothelial differentiation in control MSCs and MSC-ECs. Gene expression was assessed with qRT-PCR. Data are shown as mean + SD, ***$p<0.001$, Student *t*-test, N=3.

Moreover, differentiated cells upregulated von Willebrand factor and decreased expression of α smooth muscle actin (Figure 29). To sum up, mesenchymal stromal cells cultured in EGM2-MV medium, which is often used to induce endothelial differentiation, increased levels only of some endothelial markers *i.e.* von Willehrand factor while most of the classical markers, *i.e.* CD31, CD34 and Tie2 remained unaffected. Therefore, to further assess their endothelial character we decided to test their ability to form tube-like structures on Matrigel and sprouting spheres in 3D model.
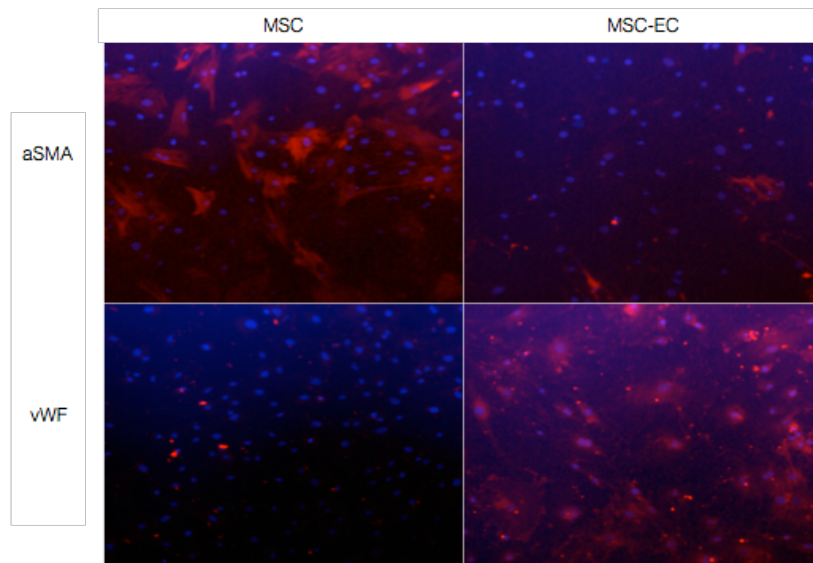
Figure 29. Expression of α smooth muscle actin (top two pictures) or von Willebrand factor (bottom two pictures)in MSCs and MSC-ECs assessed with immunocytological staining (representative pictures).

Surprisingly, undifferentiated control cells were able to form tube-like structures (Figure 30) and to form spheres that sprouted when stimulated with EGM-2MV (Figure 31). At the time when tube-like structures were already formed by control cells, MSC-ECs did not show angiogenic activity (Figure 30). Noteworthy, HAoEC cells, seeded with either control MSCs or MSC-ECs tended to adhere to structures formed by mesenchymal stromal cells both control and differentiated toward endothelial cells (Figure 30).
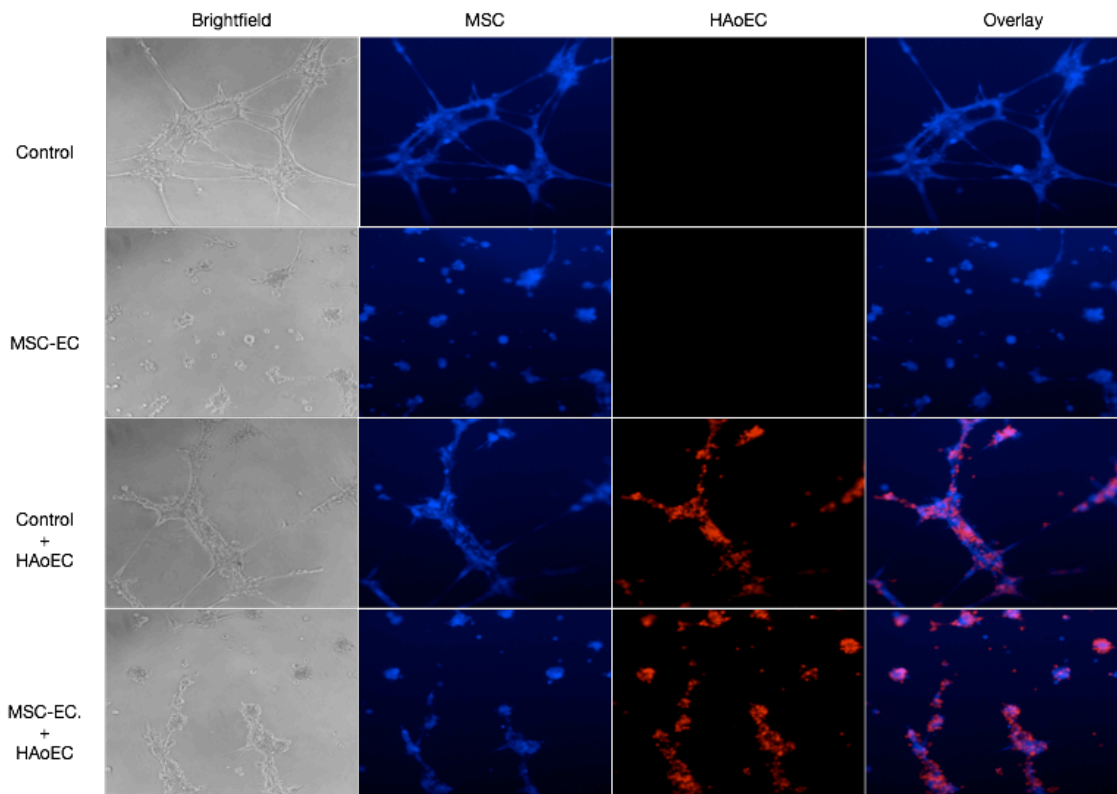
Figure 30. Formation of tube like structures by MSC or MSC-EC alone or in co-culture with adult human endothelial cells. Mesenchymal stromal cells (in blue), control or differentiated to endothelial-like cells (MSC-ECs) seeded on Matrigel with or without HAoEC endothelial cells (in red). Pictures were taken after 6 hours.

Spheres formed by MSC-ECs where bigger, even though the same numbers of control and differentiated cells were seeded (Figure 31). Similar effect on cell size was reported in human MSCs [141].
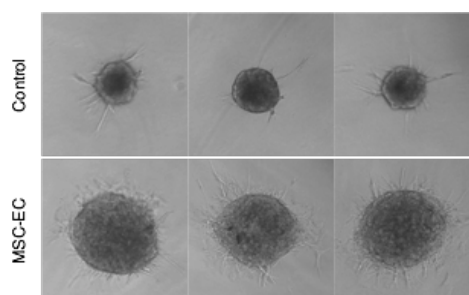
Figure 31. Sprouting of spheres formed by control MSCs and MSC-ECs formed in 10% methylcellulase by 750 cells each and stimulated with growth medium. Representative pictures taken 24 hours after sphere embedding in collagen.

Interestingly, mesenchymal stromal cells formed co-tubes when seeded on Matrigel with iPS-derived endothelial progenitor cells (Figure 32).
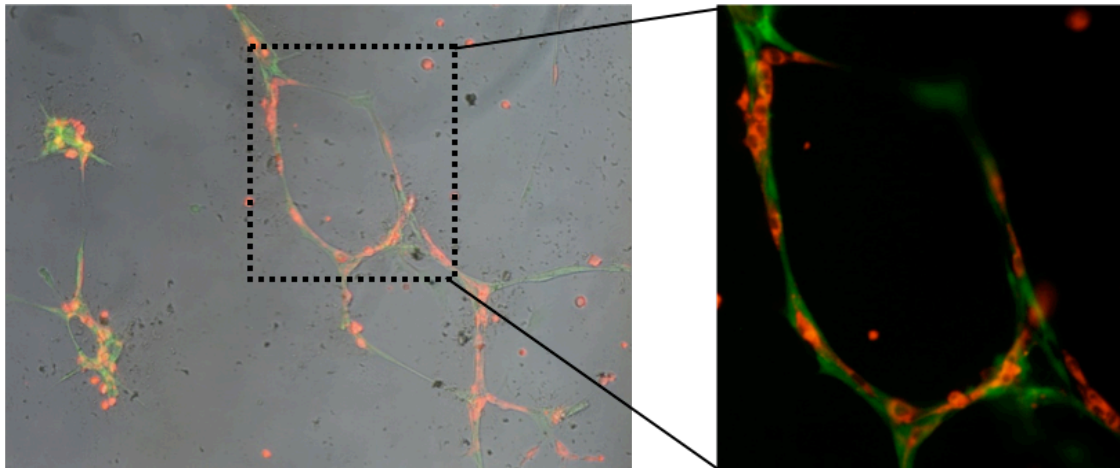
Figure 32. Mesenchymal stromal cells (in green) expressing GFP seeded on Matrigel with iPS-derived endothelial progenitor cells (in red) labelled with PKH26.

Then angiogenic activity of control MSCs and MSC-ECs was assessed *in vivo* in Matrigel plug assay. MSC or MSC-EC cells obtained from GFP$^+$ mice were mixed with Matrigel and injected subcutaneously to syngeneic C57BL6 mice. Presence of functional blood vessels with blood flow was assessed on day 7 and day 14 with ultrasonography while content of GFP$^-$ host endothelial cells was measured on day 14 using flow cytometry. Neither of cells tested showed enhanced vascularization in terms of vascularity in any tested timepoint (Figure 33A). Furthermore, tested cells had rather low and similar to each other ability to stimulate local angiogenesis since fraction of host CD31$^+$CD45$^-$ endothelial cells was similar in all groups (Figure 33B).
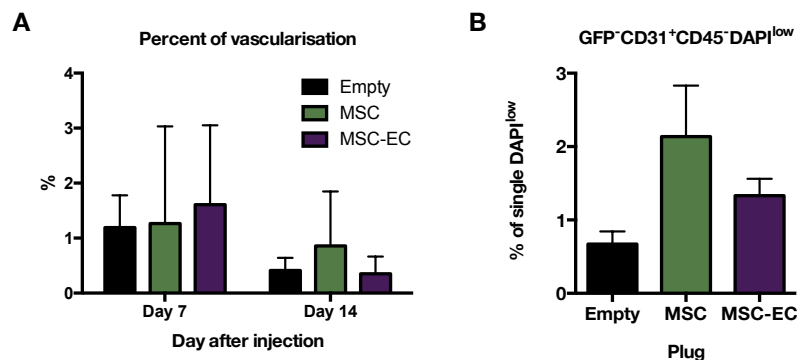
Figure 33. Vascularisation of Matrigel plugs: empty, with control MSCs or MSC-ECs assessed with VEVO 2100 ultrasonography on day 7 and 14 after subcutaneous injection **(A).** Data shown as mean + SD, Two-way Anova with Bonferroni post-test, N=3-6. Content of GFP$^-$CD31$^+$CD45$^-$DAPI^{low} host endothelial cells in plugs with MSC or MSC-EC assessed with immunofluorescent staining and flow cytometry on day 14 after subcutaneous injection **(B)** Data shown as mean + SD, One-way Anova with Bonferroni post-test, N=3-6.

Angiogenic activity of control MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$ tested *in vitro* with Matrigel assay showed no differences regardless of the concentration of glucose in the medium (Figure 34).
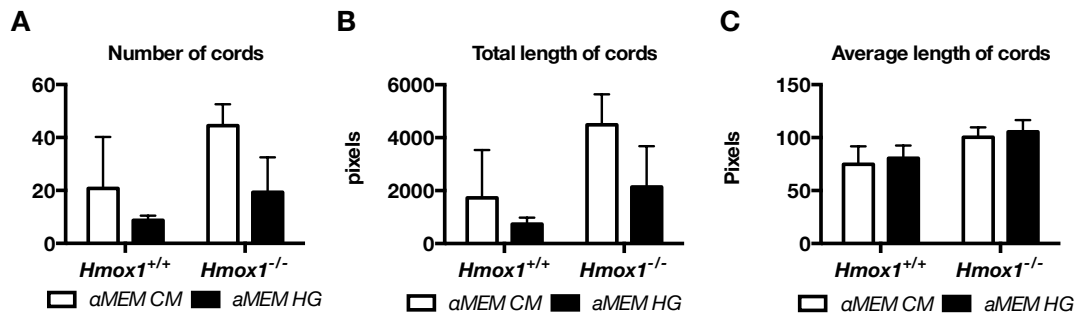
Figure 34. Influence of high glucose in medium on number of cords **(A)**, total length of cords **(B)** and average length of cords **(C)** formed on Matrigel in 6 hours by non-differentiated MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$, Data shown as mean + SD, One-way Anova with Bonferroni post-test, N=3.

$Hmox1^{+/+}$ or $Hmox1^{-/-}$ mesenchymal stromal cells show similar ability to form myofibroblasts

Mesenchymal stromal cells were shown to be precursors of fibroblasts and myofibroblasts and therefore contribute to the tumour stroma [206] or development of fibrosis [207]. Heme oxygenase-1 can affect both tumour microenvironment [208] and kidney fibrosis [209]. Therefore, we investigated whether lack of $Hmox1$ gene in MSC can affect their ability to form myofibroblasts.

$Hmox1^{+/+}$ and $Hmox1^{-/-}$ mesenchymal stromal cells were differentiated to myofibroblasts with TGFβ1 treatment for 6 days. Cells changed their morphology (Figure 35A) and upregulated α-smooth muscle actin (Figure 35B). Of note, upregulation of $Acta2$ was lower in myofibroblasts derived from $Hmox1^{-/-}$ cells.
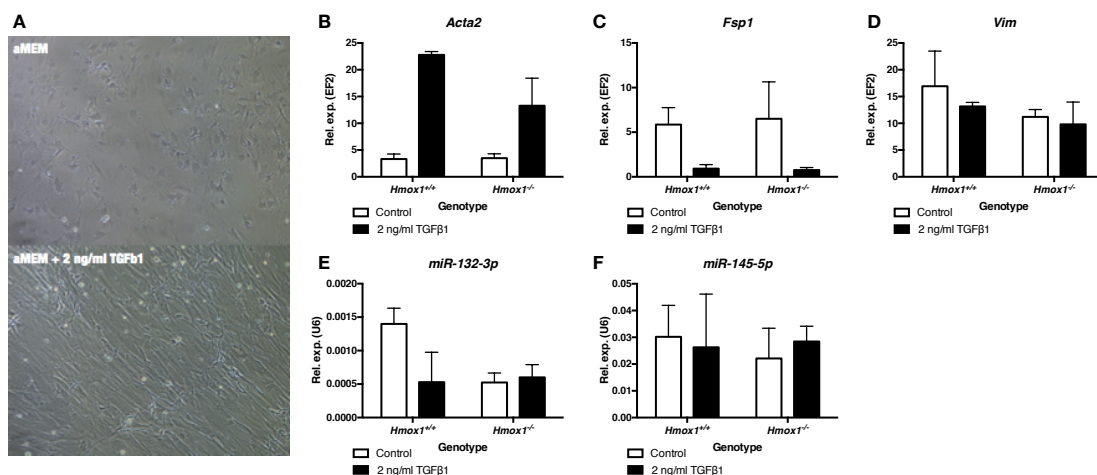
Figure 35. Morphology of mesenchymal stromal cells differentiated to myofibroblasts with TGFβ1 treatment **(A)**. Expression of $Acta2$ **(B)**, $Fsp1$ **(C)**. Vim **(D)** $miR-132-3p$ **(E)** and $miR-145-5p$ **(F)** measured with qRT-PCR in control

and differentiated MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$. Data shown as mean + SD, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ control vs TGFβ1; $^{.}p<0.05$, $^{..}p<0.01$ $Hmox1^{-/-}$ vs $Hmox1^{+/+}$, N=3.

Transcript levels of fibroblast specific protein 1 (Fsp1) as well as vimentin (Vim) remained unchanged in both $Hmox1^{+/+}$ and $Hmox1^{-/-}$ MSCs (Figure 35C,D). Then, expression of miR-132, important regulator of fibrogenesis, was decreased in differentiated MSC $Hmox1^{+/+}$ while in control undifferentiated $Hmox1^{-/-}$ cells was already lower than in $Hmox1^{+/+}$ cells (Figure 35E). Levels of miR-145-5p were not affected by TGFβ1 treatment in any of the cells (Figure 35F).

Allogeneic $Hmox1^{+/+}$ MSCs inhibit SCC VII tumour growth *in vivo*

Influence of mesenchymal stromal cells on the tumour growth is still a matter of debate since contradictory data were published. What is more, MSCs can affect immune response and therefore further change tumour microenvironment. Our data showed that $Hmox1^{-/-}$ mice developed more numerous but less malignant tumours when treated with carcinogen [208]. Therefore, we evaluated the effect of allogeneic $Hmox1^{+/+}$ or $Hmox1^{-/-}$ MSCs on the growth of squamous cell carcinoma syngeneic to inoculated mice.

$Hmox1^{+/+}$ or $Hmox1^{-/-}$ mesenchymal stromal cells were mixed with squamous cell carcinoma SCC VII-*luc* cells and injected subcutaneously in the Matrigel to form a round plug. Tumour volume in the end of experiment was lower in those with SCC VII-*luc* and MSC $Hmox1^{+/+}$ (Figure 36A). Luminescence of tumour cells was measured at day 3, 7 and 14 in order to assess tumour growth. Tumours with either SCC VII-*luc-HO1* cells or with the mix of SCC VII-*luc* and MSC $Hmox1^{-/-}$ showed higher relative luminescence than control tumours with SCC VII-*luc* cells only (Figure 36B).
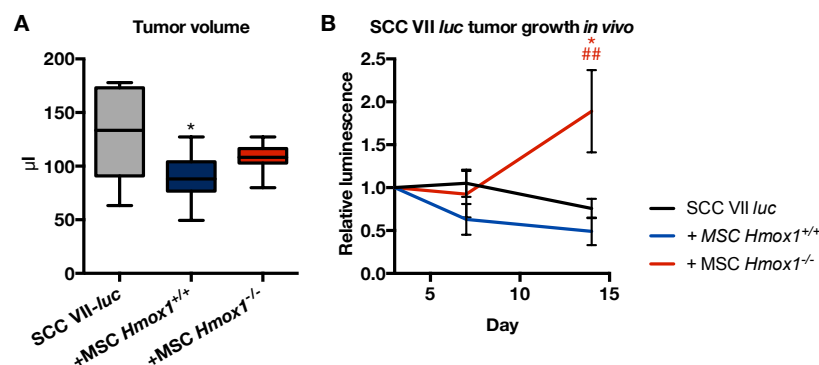
Figure 36. Volumes of tumours with SCC VII-*luc*, cells or mixed SCC VII-*luc* with MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$, data are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles **(A)**. Changes in luminescence of tumours with SCC VII-*luc*, SCC VII-*luc-HO1* cells or mixed SCC VII-*luc* with MSC

Hmox1$^{+/+}$ or *Hmox1*$^{-/-}$, data shown as mean ± SD **(B)** $^*p<0.05$, vs SCC VII-luc, $^{##}p<0.01$ vs SCC VII-*luc* + MSC *Hmox1*$^{+/+}$, One-way Anova with Bonferroni post-test, n=9-16.

In order to assess the direct effect of mesenchymal stromal cells on SCC VII carcinoma cell growth we co-cultured MSCs and tumour cells. Carcinoma cells were seeded in low number (10 per well) and formed colonies on the MSC feeder layer (Figure 37A). Interestingly, SCC VII-*luc* cells showed similar growth on both MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ *in vitro* (Figure 37B). Therefore, enhanced luminescence and thus growth in case of tumours with both SCC VII-*luc* and *Hmox1*$^{-/-}$ mesenchymal stromal cells *in vivo* is probably not caused by the direct influence on SCC VII cells.
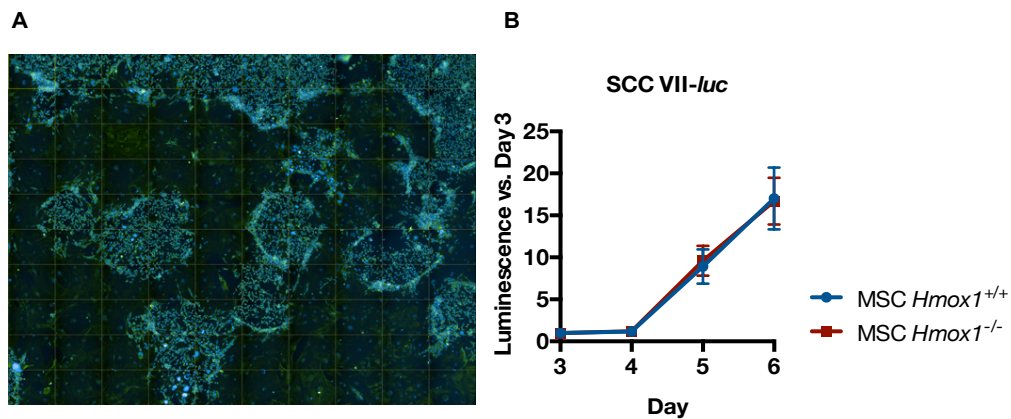
Figure 37. Colonies of SCC VII-*luc* cells seeded on the feeding layer of mesenchymal stromal cells (DAPI in blue, vimentin in green) **(A)**. Growth of SCC VII-*luc* **(B)** on the feeder layer of MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ (n=12).

Therefore, we assessed infiltrating cells that can affect tumour growth. Especially, since immunomodulatory properties are ascribed to mesenchymal stromal cells. Numbers of total CD45$^+$ immune cells were similar in all tumour types (Figure 38A). Tumours with mesenchymal stromal cells, regardless of MSC *Hmox1* expression, contained comparable numbers of CD11b$^+$ myeloid cells, which further did not differ in the content of Ly-6C^{hi} monocytes.
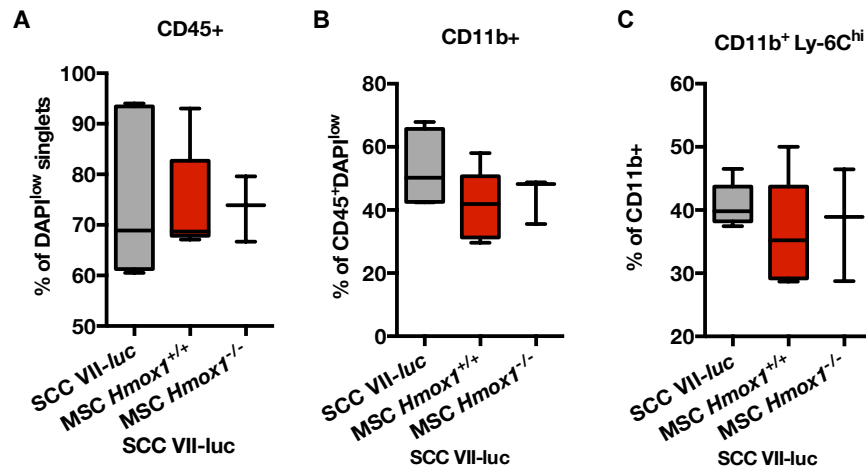
Figure 38. Fraction of CD45^{+} infiltrating cells **(A)**, CD11b^{+} myeloid cells **(B)**, or CD11b^{+}Ly-6C^{hi} macrophages in SCC VII-*luc* tumours with or without MSC *Hmox1*^{+/+} or *Hmox1*^{-/-} assessed with flow cytometry. Data are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles, One-way Anova with Bonferroni post-test, N=3-5.

Within total immune cells, similar fractions were characterized with macrophage phenotype CD11b^{+}Ly-6C^{hi}Ly-6G^{-}F4/80^{+} in all groups (Figure 39A). However, SCC VII-*luc* tumours with MSC *Hmox1*^{+/+} but not MSC *Hmox1*^{-/-} contained more CD11b^{+}Ly-6C^{hi}Ly-6G^{-}F4/80^{+}CD43^{+} macrophages within CD11b^{+} myeloid cell compartment than control SCC VII-*luc* tumours (Figure 39B). Fraction of Tie2^{+} macrophages in total infiltrating cells (Figure 39C) as well as fractions of CD11b^{+}Ly-6C^{hi}Ly-6G^{-}F4/80^{+}CD43^{-} cells (Figure 39D) within myeloid cells were similar in all groups.
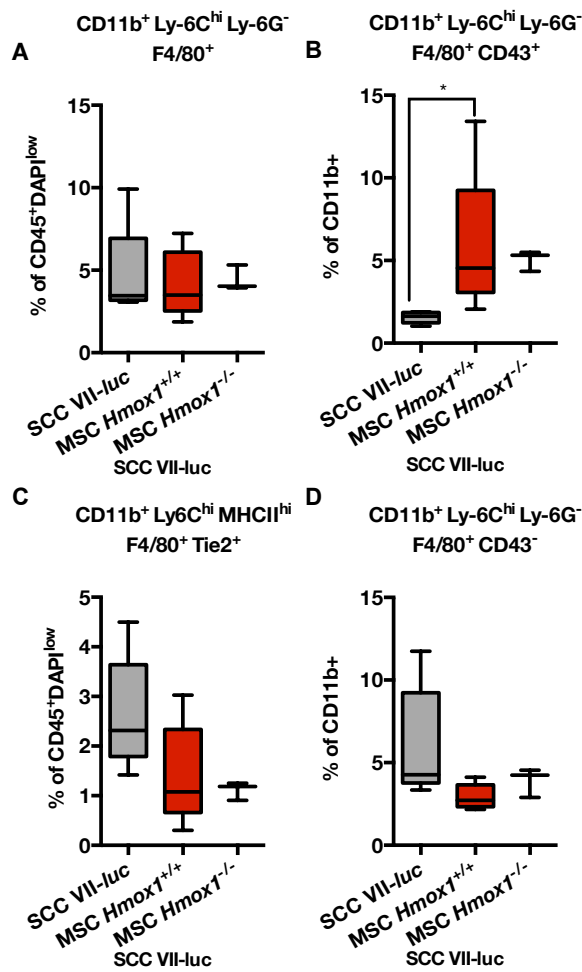
Figure 39. Fraction of CD11b$^+$Ly-6C^{hi}Ly-6G$^-$F4/80$^+$ macrophages **(A)**, CD11b$^+$Ly-6C^{hi}Ly-6G$^-$F4/80$^+$CD43$^+$ **(B)** CD11b$^+$Ly-6ChiMHCIIhiF4/80$^+$Tie2$^+$ **(C)** or CD11b$^+$Ly-6C^{hi}Ly-6G$^-$F4/80$^+$CD43$^-$ **(D)** macrophages in SCC VII-*luc* tumours with or without MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ assessed with flow cytometry. Data are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles. One-way Anova with Bonferroni post-test, N=3-5.

Numbers of general CD11c$^+$MHCII$^+$ dendritic cells as well as subpopulations of classical and plasmocytoid dendritic cells were similar in all groups (Figure 40A-C).
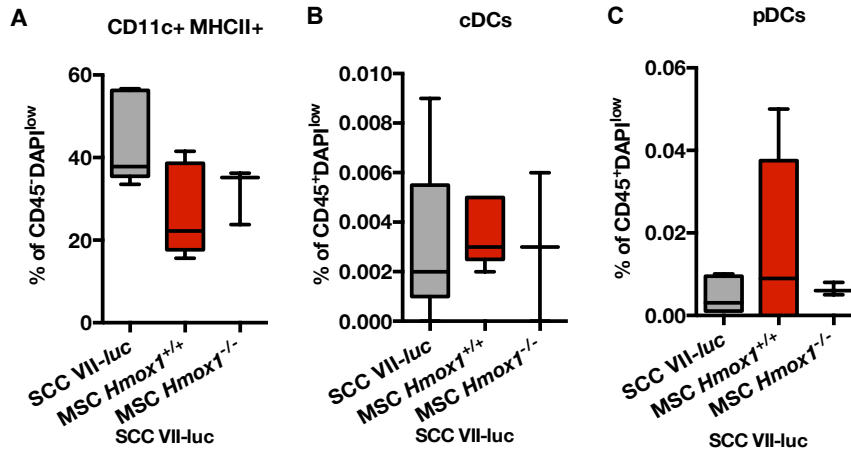
Figure 40. Fraction of CD11c^{+}MHC II^{+} total dendritic cells **(A)**, classical dendritic cells **(B)** and plasmocytoid dendritic cells **(C)** in SCC VII-*luc* tumours with or without MSC *Hmox1*^{+/+} or *Hmox1*^{-/-} assessed with flow cytometry. Data are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles. One-way Anova with Bonferroni post-test, N=3-5.

Furthermore, conditioned media from *Hmox1*^{+/+} and *Hmox1*^{-/-} MSCs contained similar concentrations of G-CSF, IL-6, LIF, LIX, CXCL1, MCP-1, RANTES and VEGF (Figure 41). Levels of eotaxin, GM-CSF, IGNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, MIP-1α, MIP-1β, M-CSF, MIP-2, MIG and TNFα were under the threshold of detection.
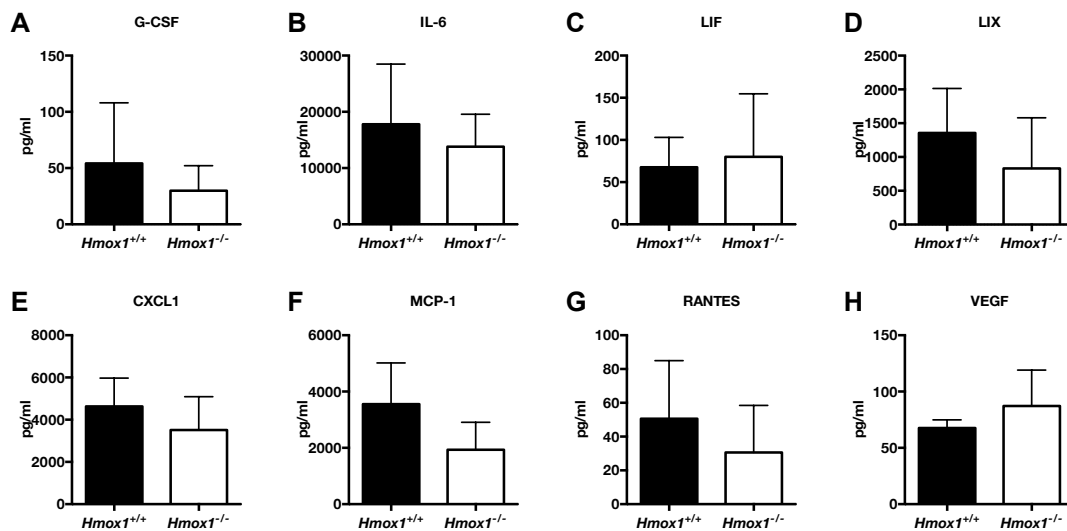
Figure 41. Concentration of G-CSF **(A)**, IL-6 **(B)**, LIF **(C)**, LIX **(D)**, CXCL1 **(E)**, MCP-1 **(F)**, RANTES **(G)**, VEGF **(H)** in conditioned media from *Hmox1*^{+/+} or *Hmox1*^{-/-}MSC assessed with multiplex assay on Luminex platform. Data shown as mean + SD, Mann-Whitney, N=4.

SCC VII-*luc* tumours containing MSC *Hmox1*^{-/-} were the only to increase their luminescence over the course of the experiment. What is more, contrary to tumours with MSC *Hmox1*^{+/+}, they did not decrease their size. However, effect of MSCs on tumour growth probably is not related to the direct influence on SCC VII-*luc* proliferation, secretion of tested cytokines or impact on analysed populations of myeloid cells. On the other hand, SCC VII-*luc* tumours contanining MSC *Hmox1*^{+/+} were smaller and did not increase their luminescence what may sugest the role of *Hmox1* played in the MSC-mediated tumour inhibition.

Hmox1^{+/+} and *Hmox1*^{-/-} MSCs show high resistance to oxidative stress induced with H$_2$O$_2$ or hemin

Bone marrow derived proangiogenic cells lacking *Hmox1* gene were characterized with higher sensitivity to oxidative stress, i.e. induced with hemin, and lower proliferation [4]. Therefore, we decided to test, whether it is also true for mesenchymal stromal cells. Surprisingly, *Hmox1*^{+/+} and *Hmox1*^{-/-} MSCs showed no difference in the sensitivity to either H$_2$O$_2$ or 50 μmol/L hemin in both low and high glucose conditions (Figure 42A,B). What is more, cells with or without functional *Hmox1* gene were characterized with similar proliferation (Figure 42C).

Subsequently, we checked the toxic concentrations of hemin for MSC *Hmox1*^{+/+} and *Hmox1*^{-/-}. Hemin was more toxic for *Hmox1*^{-/-} MSCs than to *Hmox1*^{+/+} cells not until at concentration of 200 μmol/L (Figure 42D).
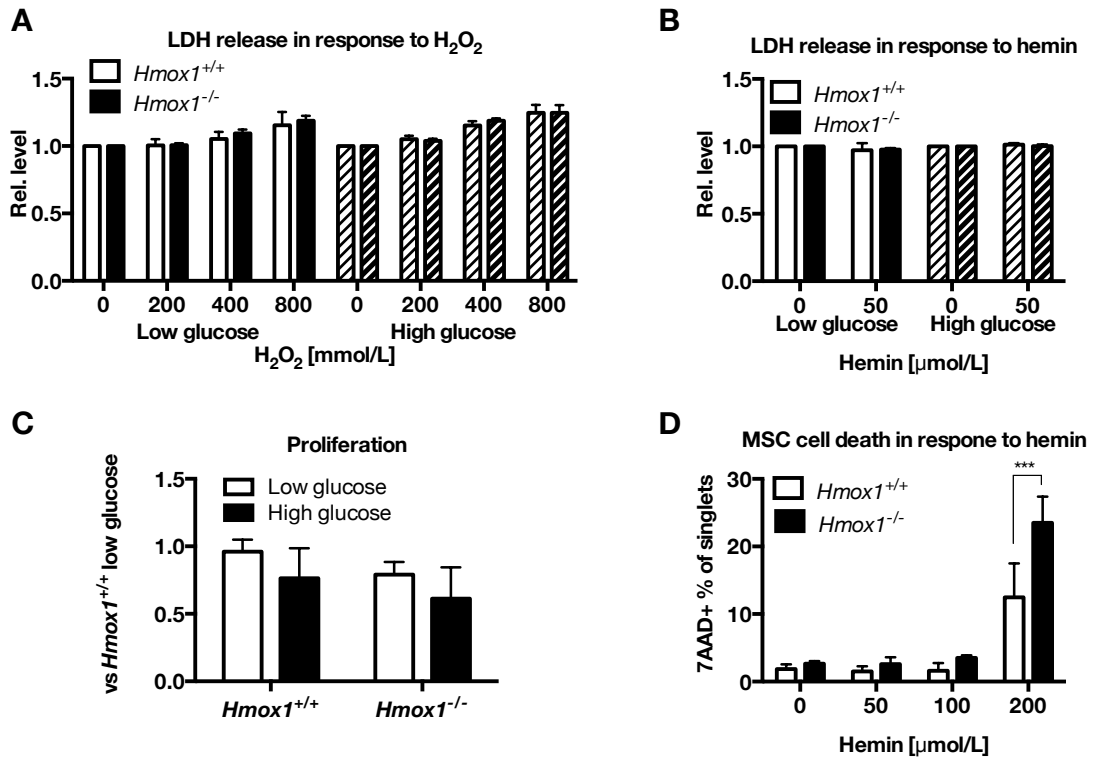
Figure 42. LDH release in MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$ treated with H_2O_2 **(A)** or hemin **(B)** in low or high glucose medium. Proliferation of MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$ in low or high glucose medium assessed with BrDU assay **(C)**. Cell death in MSC $Hmox1^{+/+}$ or $Hmox1^{-/-}$ treated with increasing concentrations of hemin for 6 hours, assessed with 7-AAD staining and flow cytometry **(D)**. Data shown as mean + SD. ***$p<0.001$ Two-way ANOVA with Bonferroni post-test, N=3.

Then, we supposed that low sensitivity of $Hmox1^{+/+}$ and $Hmox1^{-/-}$ MSCs to hemin, especially in comparison to bone marrow-derived proangiogenic cells can be related to the high expression of heme oxygenase-2. Noteworthy, expression of $Hmox1$ was lower in MSCs $Hmox1^{+/+}$ than in PACs $Hmox1^{+/+}$ (Figure 43A). Similarly, expression of $Hmox2$ was lower in MSCs than in PAC cells and similar in $Hmox1^{+/+}$ and $Hmox1^{-/-}$ cells (Figure 43B).
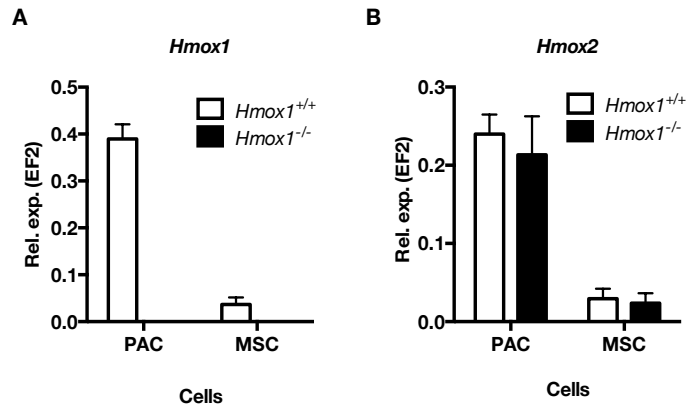
Figure 43. Expression of *Hmox1* **(A)** and *Hmox2* **(B)** in bone marrow derived proangiogenic cells (PAC) and *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells (MSC). Expression of genes was assessed with qRT-PCR. Data shown as mean + SD. N=3

Low sensitivity of *Hmox1*$^{-/-}$ MSC cells could be also caused by low uptake of heme from the culture medium. Therefore, we analysed heme uptake with two methods – direct measurement of heme in cells stimulated for two hours with 50 µmol/L hemin and then after 2 hours in fresh medium (Figure 44A) and with the measurement of autofluorescence of tin protoporphyrin IX. *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ MSCs displayed similar levels of cellular heme in all timepoints tested (Figure 44B). Moreover, both cell genotypes showed comparable SnPP autofluorecence proportional to the concentration of SnPP in the culture medium (Figure 44C).
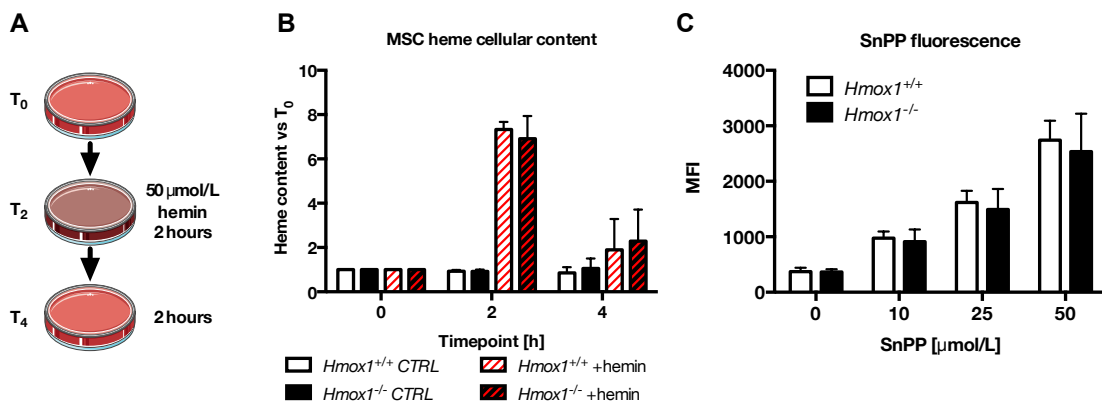
Figure 44. Scheme of the experiment **(A)**. Heme cellular content measured with spectrophotometry in MSC *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ stimulated for 2 hours with hemin (T$_2$) and then after 2 hours in hemin-free medium (T$_4$), N=3 **(B)**. SnPP fluorescence in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ MSCs assessed with flow cytometry, N=3 **(C)**.

Hemin increases cellular H₂O₂ in Hmox1⁻/⁻ mesenchymal stromal cells and fibroblasts

In order to assess the effects of hemin on the $Hmox1^{+/+}$ and $Hmox1^{-/-}$ mesenchymal stromal cells we analysed the levels of cellular hydrogen peroxide using H₂DCFDA staining. Tail tip fibroblasts isolated from $Hmox1^{+/+}$ or $Hmox1^{-/-}$ mice were used as non-progenitor control cells. After 6 and 16 hours of incubation with hemin (50 µmol\L) levels of H_2O_2 were increased in $Hmox1^{-/-}$ MSCs and fibroblasts and higher than in respective wild type cells (Figure 45A,B). After a 24 hour incubation period levels of H_2O_2 remained low in $Hmox1^{+/+}$ cells and were higher in $Hmox1^{-/-}$ fibroblasts than in $Hmox1^{-/-}$ MSCs (Figure 45C). After 48 hours of stimulation with hemin both $Hmox1^{-/-}$ MSCs and $Hmox1^{-/-}$ fibroblasts were dead. Interestingly, levels of cellular H_2O_2 were higher in hemin-treated $Hmox1^{+/+}$ fibroblasts than in $Hmox1^{+/+}$ MSCs (Figure 45D).
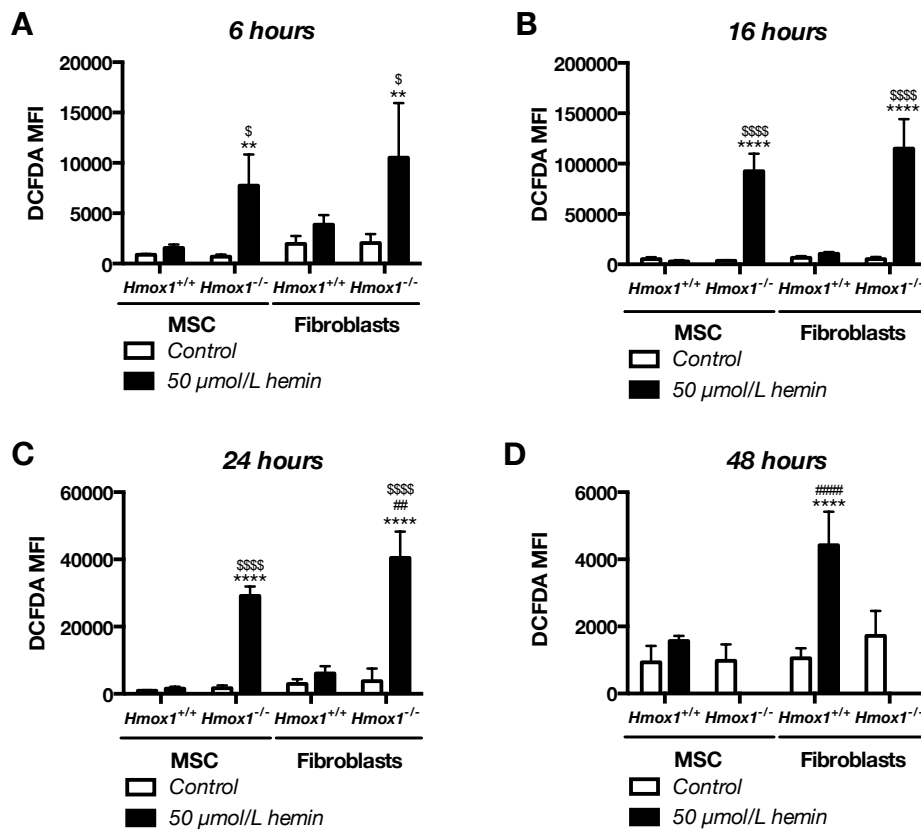
Figure 45. Levels of H_2O_2 measured with H₂DCFDA after 6 **(A)**, 16 **(B)**, 24 **(C)** or 48 hours **(D)** in $Hmox1^{+/+}$ or $Hmox1^{-/-}$ mesenchymal stromal cells (MSCs) or fibroblasts stimulated with hemin (50 µmol/L). Data shown as mean + SD, [*]$p<0.05$, [**]$p<0.01$, [***]$p<0.001$, [****]$p<0.0001$ hemin treated cells vs control; [#]$p<0.05$, [##]$p<0.01$, [###]$p<0.001$, [####]$p<0.0001$ MSC vs fibroblasts, [$]$p<0.05$, [$$]$p<0.01$, [$$$]$p<0.001$, [$$$$]$p<0.0001$ $Hmox1^{+/+}$ vs $Hmox1^{-/-}$. Two-way Anova with Bonferroni post-test, N=3.

Hmox1$^{+/+}$ and *Hmox1*$^{-/-}$ MSC cells showed higher resistance to 50 μmol/L hemin than respective tail tip fibroblasts. To further elucidate the mechanisms that may be responsible for this phenomenon we analysed genes involved in heme synthesis and catabolism, heme transporters as well as genes involved in the antioxidant response in *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ MSCs and fibroblasts.

Treatment with hemin (50 μmol/L) decreased *Alas1* expression in all treated cells. In *Hmox1*$^{-/-}$ fibroblasts already control cells were characterised with lower *Alas1* levels than *Hmox1*$^{+/+}$ cells (Figure 46A). Expression of *Uros* in hemin-treated *Hmox1*$^{-/-}$ fibroblasts was lower than in corresponding MSC cells (Figure 46B). What is more, fibroblasts *Hmox1*$^{-/-}$ treated with hemin decreased *Cpox* expression while it remained unchanged in other cell types (Figure 46C).
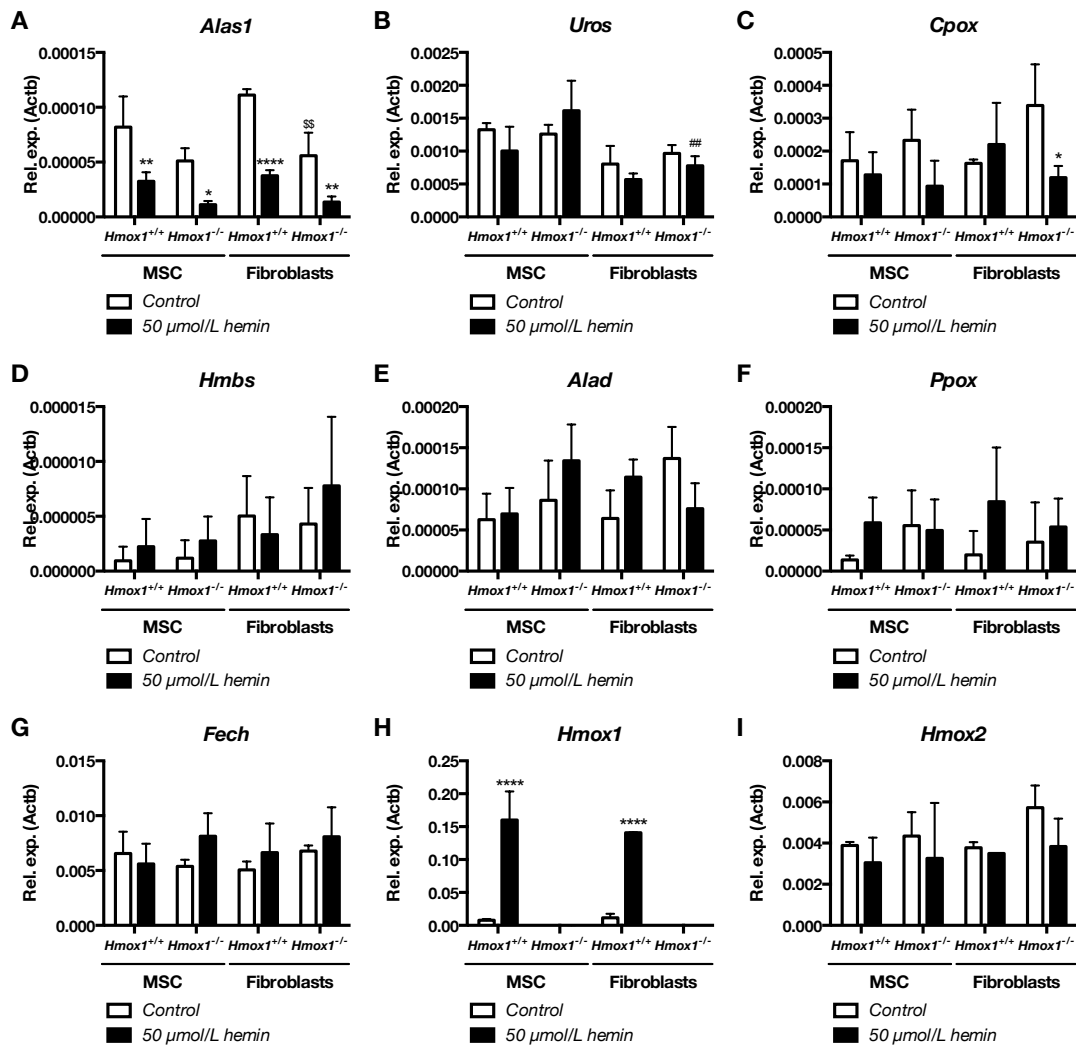
Figure 46. Expression of *Alas1* **(A)**, *Uros* **(B)**, *Cpox* **(C)**, *Hmbs* **(D)**, *Alad* **(E)**, *Ppox* **(F)**, *Fech* **(G)**, *Hmox1* **(H)**, *Hmox2* **(I)** in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells (MSCs) or fibroblasts stimulated for 6 hours with hemin (50 μmol/L). Data shown as mean + SD, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ hemin treated cells vs

control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC *vs* fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1*$^{+/+}$ vs *Hmox1*$^{-/-}$. Two-way Anova with Bonferroni post-test, N=3.

There were no differences in the expression of *Hmbs, Alad, Ppox* and *Fech* (Figure 46D-G). *Hmox1* was similarly upregulated in both *Hmox1*$^{+/+}$ MSCs and fibroblasts (Figure 46H). Expression of *Hmox2* was not affected with hemin treatment and did not differ in any of tested grups of cells (Figure 46I).
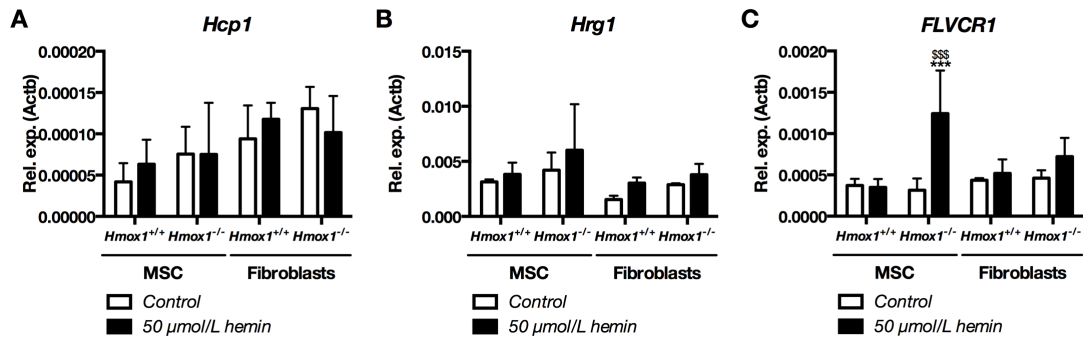
Figure 47. Expression of *Hcp1* **(A)**, *Hrg1* **(B)**, *FLVCR1* **(C)** in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells (MSCs) or fibroblasts stimulated for 6 hours with 50 μmol/L hemin. Data shown as mean + SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 hemin treated cells vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC *vs* fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1*$^{+/+}$ vs *Hmox1*$^{-/-}$. Two-way Anova with Bonferroni post-test, N=3.

All cell types tested were characterised with similar expression of heme transporters *Hcp1* (*Slc46a1*) and *Hrg1* (*Slc48a1*) (Figure 47A,B). *Hmox1*$^{-/-}$ MSCs were the only to increase the expression of heme exporter *FLVCR1* in response to hemin treatment and *FLVCR1* level was then higher than in *Hmox1*$^{+/+}$ MSC cells (Figure 47C).
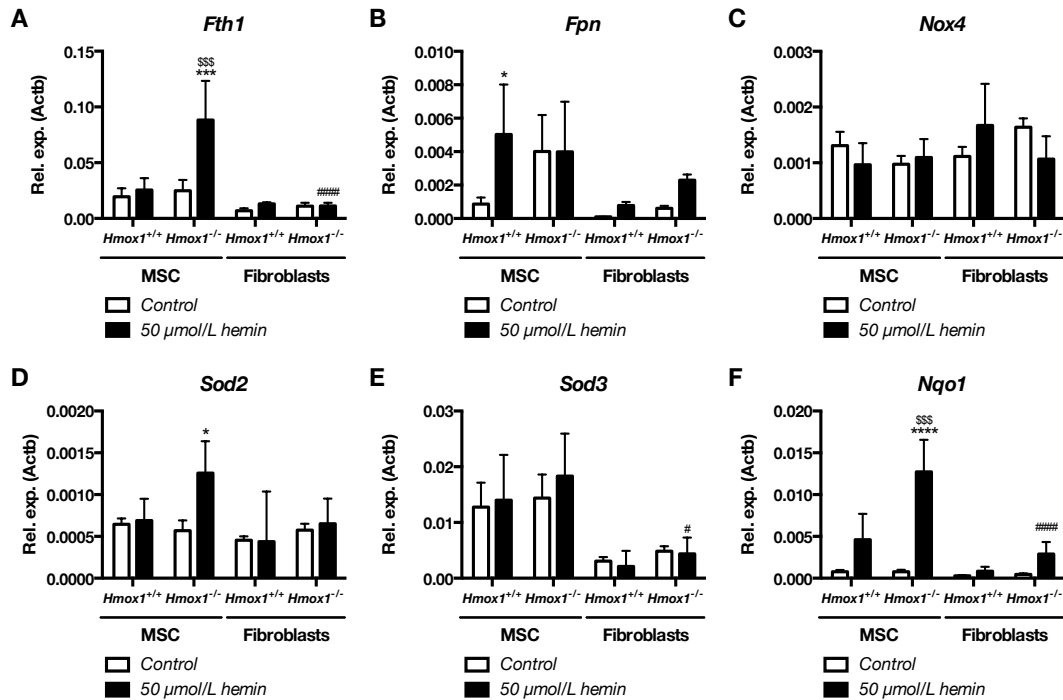
Figure 48. Expression of *Fth1* **(A)**, *Fpn* **(B)**, *Nox4* **(C)**, *Sod2* **(D)**, *Sod3* **(E)**, *Nqo1* **(H)** in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells (MSCs) or fibroblasts stimulated for 6 hours with hemin (50 μmol/L). Data shown as mean + SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 hemin treated cells vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC vs fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1*$^{+/+}$ vs *Hmox1*$^{-/-}$, Two-way Anova with Bonferroni post-test, N=3.

Hmox1$^{-/-}$ mesenchymal stromal cells were the only that up-regulated ferritin heavy chain 1 (*Fth1*) expression in response to the hemin treatment (Figure 48A). Levels of *Fpn* were, on the other hand, increased only in *Hmox1*$^{+/+}$ MSCs (Figure 48B). Hemin did not affect *Nox4* expression in any cells (Figure 48C). Furthermore, expression of *Sod2* was changed only in *Hmox1*$^{-/-}$ MSC (Figure 48D) while hemin-treated *Hmox1*$^{-/-}$ fibroblasts had lower *Sod2* levels than corresponding MSC cells (Figure 48E). Then, *Hmox1*$^{-/-}$ fibroblasts treated with hemin had lower expression of *Nqo1* than *Hmox1*$^{-/-}$ MSCs, the only cells to upregulate *Nqo1* and with its higher expression than *Hmox1*$^{+/+}$ MSCs (Figure 48F).
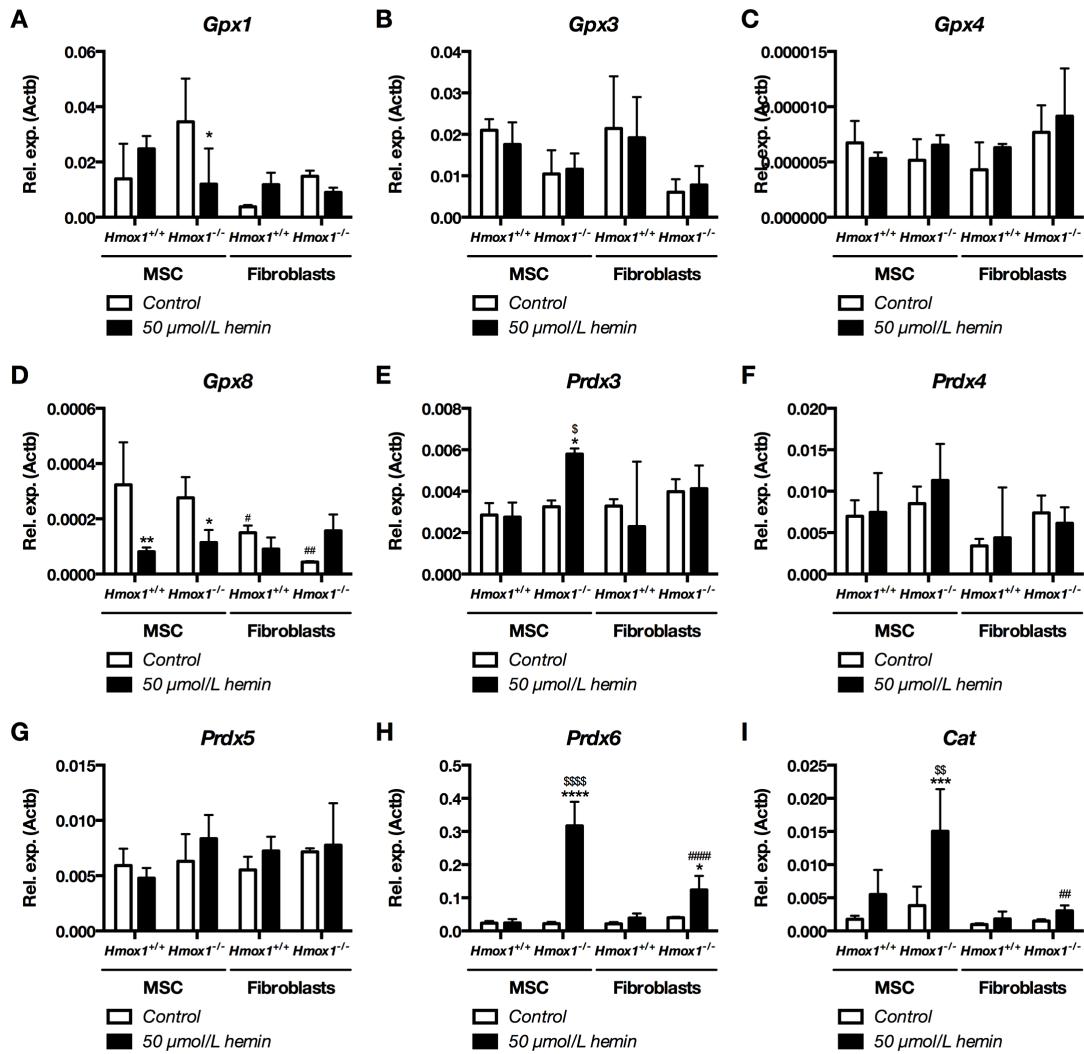
Figure 49. Expression of *Gpx1* **(A)**, *Gpx3* **(B)**, *Gpx4* **(C)**, *Gpx8* **(D)**, *Prdx3* **(E)**, *Prdx4* **(F)**, *Prdx5* **(G)**, *Prdx6* **(H)**, and *Cat* **(I)** in *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mesenchymal stromal cells (MSCs) or fibroblasts stimulated for 6 hours with hemin (50 μmol/L). Data shown as mean + SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 hemin treated cells vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC vs fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1*$^{+/+}$ vs *Hmox1*$^{-/-}$. Two-way Anova with Bonferroni post-test, N=3.

Interestingly, only *Hmox1*$^{-/-}$ MSCs decreased *Gpx1* expression in response to hemin (Figure 49A), while expressions of *Gpx3* and *Gpx4* were unchanged (Figure 49B,C), and expression of *Gpx8* was decreased in both MSC cell types (Figure 49D). On the other hand, control fibroblasts expressed lower levels of *Gpx8* than respective MSCs (Figure 49D). Expression of *Prdx3* was enhanced with hemin only in *Hmox1*$^{-/-}$ MSC cells (Figure 49E) while *Prdx4* was not affected (Figure 49F). Interestingly, the only 1-Cys member of peroxiredoxin family – *Prdx6* – was potently upregulated in hemin-treated *Hmox1*$^{-/-}$ MSC. *Prdx6* expression was then higher in MSC *Hmox1*$^{-/-}$ than

in all other cells (Figure 49G). Similarly, expression of *Cat* was the highest in hemin-treated MSC *Hmox1$^{-/-}$*, the only to change its levels (Figure 49H).
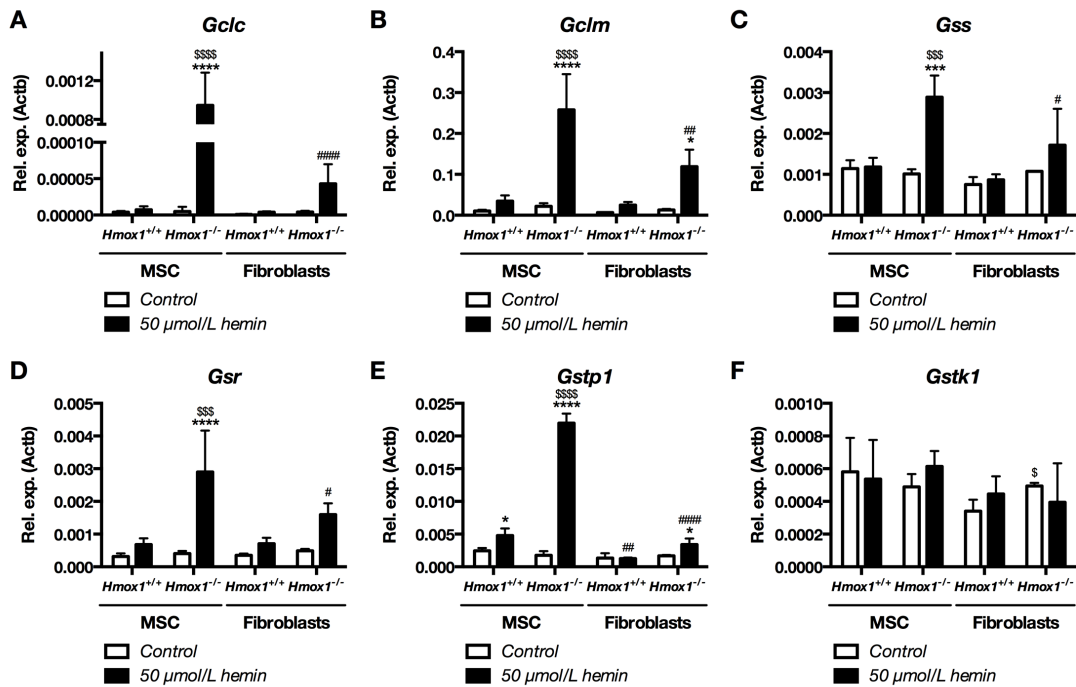
Figure 50. Expression of *Gclc* **(A)**, *Gclm* **(B)**, *Gss* **(C)**, *Gsr* **(D)**, *Gstp1* **(E)**, *Gstk1* **(F)** in *Hmox1$^{+/+}$* or *Hmox1$^{-/-}$* mesenchymal stromal cells (MSCs) or fibroblasts stimulated for 6 hours with hemin (50 µmol/L). Data shown as mean + SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 hemin treated cells vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC vs fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1$^{+/+}$* vs *Hmox1$^{-/-}$*, Two-way Anova with Bonferroni post-test, N=3.

Importantly, in response to hemin the *Hmox1$^{-/-}$* MSC cells potently upregulated genes involved in glutathione pathway, namely *Gclc, Gclm, Gss, Gsr,* and *Gstp1* (Figure 50A-E), while levels of *Gstk1* remained unchanged in all cell types (Figure 50F). Levels of the up upregulated genes were higher in the hemin-treated *Hmox1$^{-/-}$* MSC cells than in hemin-treated *Hmox1$^{+/+}$* MSCs and *Hmox1$^{-/-}$* fibroblasts. The latter ones increased expression only of *Gclm* and *Gstp1* (Figure 50B,F).

In order to functionally confirm the influence of hemin on glutathione pathway genes in *Hmox1$^{-/-}$* MSCs we analysed GSH to GSSG ratio, changes in total GSH and GSSG in MSCs and fibroblasts of both *Hmox1$^{+/+}$* and *Hmox1$^{-/-}$* phenotypes. Cells were treated with hemin (50 µmol/L) for 6 hours, like in gene expression experiments. However, we then incubated the cells for 2 more hours in hemin-free complete medium to allow for GSH recovery.

The GSH/GSSG ratio, that allows to assess cellular oxidative stress, was decreased in both *Hmox1*^{+/+} and *Hmox1*^{-/-} MSCs as well as in *Hmox1*^{-/-} fibroblasts. What is more, *Hmox1*^{-/-} fibroblasts were characterised with lower GSH/GSSG ratio than *Hmox1*^{-/-} MSC cells, which additionally had slightly lower ratio than *Hmox1*^{+/+} MSCs (Figure 51A). Furthermore, *Hmox1*^{-/-} MSCs were the only that increased total GSH in response to hemin, while it caused decrease in total GSH in *Hmox1*^{+/+} MSCs (Figure 51B). Levels of GSSG were increased in *Hmox1*^{+/+} MSCs, *Hmox1*^{-/-} MSCs and in *Hmox1*^{-/-} fibroblasts. They were higher in both *Hmox1*^{-/-} cell types than in respective *Hmox1*^{+/+} counterparts. However, levels of oxidized glutathione increased more in *Hmox1*^{-/-} fibroblasts than in *Hmox1*^{-/-} MSCs (Figure 51C).
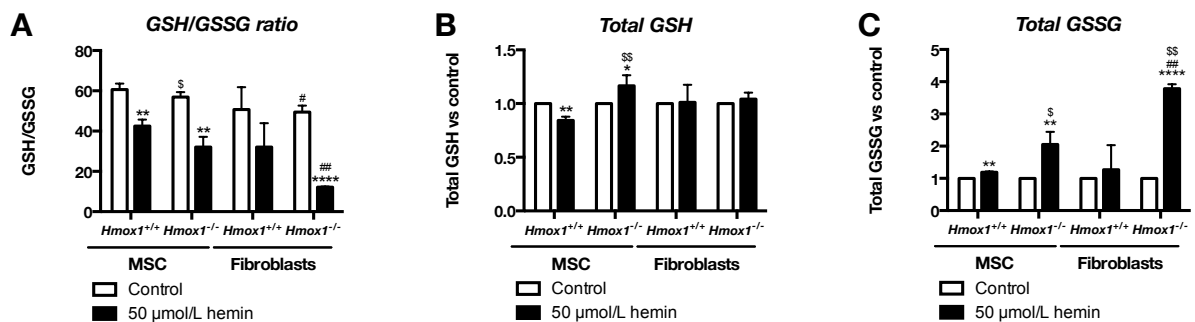
Figure 51. Ratio of GSH to GSSG **(A)**, changes in total GSH **(B)** and GSSG **(C)** in *Hmox1*^{+/+} or *Hmox1*^{-/-} mesenchymal stromal cells (MSCs) and fibroblasts stimulated for 6 hours with hemin (50 μmol/L) and then kept in fresh medium for 2 hours. Levels of GSH and GSSG were measured with GSH/GSSG-Glo™ Assay. Data shown as mean + SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 hemin treated cells vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 MSC vs fibroblasts, $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *Hmox1*^{+/+} vs *Hmox1*^{-/-}, N=3.

Mesenchymal stromal cells isolated from *Hmox1*^{-/-} mice showed relatively high resistance to hemin regardless of the absence of the most important enzyme degrading heme. Although hemin increases cellular concentration of H_2O_2 in both MSCs and fibroblasts *Hmox1*^{-/-}. The latter cells could better induce antioxidant gene response, especially genes involved in the glutathione pathway. One may speculate that such fast response to stress factors can in part contribute to the presence of only minor effects of *Hmox1* knockout on other functions of murine bone marrow-derived mesenchymal stromal cells.

9. Discussion

The current work aimed to elucidate the influence of various stress factors on populations of bone marrow-derived stem and progenitor cells. We have shown that complications of type 2 diabetes, especially if made more complex by infection, can potently change numbers of circulating proangiogenic cells, but also hematopoietic stem and progenitor cells and cells with MSC phenotype. We have also demostrated that single exercise in non-trained patients with intermittent claudication could increase numbers of circulating prangiogenic cells. Such effect was not observed after the completion of treadmill training programme. Both complications of diabetes in T2DM patients as well as exercise and training in intermittent claudication patients affected profiles of plasma cytokines and expression of antioxidant genes, including expression of heme oxygenases.

We tried to improve the proangiogenic potential of circulating progenitor cells by pharmacological stimulation. We found that paracrine angiogenic function of human peripheral blood CD34$^+$-derived cells could be enhanced by high concentration of atorvastatin, but the effect was observed only *in vitro* and not *in vivo*. Furthermore, none of the tested cytokines changed in tested conditioned media that had increased angiogenic activity. Improved function of tested cells presumably was not related to heme oxygenase-1.

Last but not least, mesenchymal stromal cells, an important component of bone marrow stem cell niche, were shown to be rather insensitive to the absence of heme oxygenase-1. Lack of *Hmox1* did not affect their phenotype, differentiation and other tested functions. However, MSC *Hmox1$^{-/-}$* were characterized with high and efficient antioxidant gene response, which may have contributed to their high resemblance to wild type cells.

9.1. Number of circulating pro-angiogenic cells, growth factor and anti-oxidative gene profiles might be altered in type 2 diabetes with and without diabetic foot syndrome [24]

The most consistent finding of the study on consequences of simple and complicated diabetes shows that all groups of diabetic patients, with and without diabetic foot syndrome, had an elevated concentration of plasma TNF-α and decreased

expression of catalase in peripheral blood nucleated cells. This may indicate a chronic inflammatory reaction and reduced cellular antioxidative capabilities. A low level of catalase in the blood cells of diabetic patients, accompanied by enhanced oxidative stress, has been already reported [12]. Of note, catalase expression is regulated by peroxisome proliferator-activated receptor-γ (PPARγ), the target for antidiabetic thiazolidinediones [210]. Moreover, the inherited catalase deficiency has been reported as a risk factor for the development of type 2 diabetes [211].

Type 2 diabetes patients without diabetic foot syndrome had also less circulating EPCs, MSCs and HSCs, when compared with healthy subjects. The decrease found in EPCs confirms the previously published data [8,212], while HSCs and MSCs in diabetes have not been thoroughly studied hitherto. Interestingly, only the $CD45^{dim}CD31^+CD34^+KDR^+$ subpopulation of EPCs correlated negatively with $Hb1_A$. We can suggest that the simultaneous decrease in circulating EPCs, MSCs and HSCs may reflect the impaired stem cell niche function in type 2 diabetes [51].

In contrast, $Lin^-CD45^-CD34^+$ and, to a lower extent, $Lin^-CD45^-CD133^+$ populations, both described as VSEL pluripotent cells [42,213], were elevated in type 2 diabetes patients. The latter population was less numerous in groups with diabetic foot syndrome than in patients without these complications. One could speculate that VSEL cell mobilization is a defensive mechanism, less effective in subjects with diabetic complications. On the other hand, a recent study demonstrated that such populations ($Lin^-CD45^-CXCR4^+CD34^+$ among them) do not express pluripotency markers, including *POU5F1*, and contain many aneuploid cells and products of defective cell divisions [214]. Accordingly, although using meticulously designed primers, we were unable to detect any *POU5F1* transcript in peripheral blood nucleated cells. Moreover, the number of $Lin^-CD45^-CD34^+$ cells correlated positively with $Hb1_{Ac}$, and negatively with the expression of cytoprotective *HMOX1*. This might suggest that the elevation of these cell subsets results from hyperglycemia-induced tissue injury.

Apart from changes in TNF-α and catalase levels, type 2 diabetes patients displayed a decreased concentration of EGF, and reduced expression of *HMOX1*, compensated by an upregulation of *HMOX2*. In mice, a decrease in EGF or *HMOX1* enhances the risk of diabetic complications and impairs wound healing [215,216]. Moreover, injection of MSC cells pretreated with EGF significantly improves neovascularization in ischemic limbs of diabetic *db/db* mice [217]. Therefore, one may speculate that low levels of mesenchymal stromal cells observed in our study conjoint

with low plasma of EGF can represent novel mechanism that can potentially contribute to the manifestation of diabetic foot syndrome.

The parameters, which were different in patients with the diabetic foot syndrome, even with ulceration already healed, than in those without this complication included a lower number of VSEL cells, higher CD45$^-$CD29$^+$CD90$^+$ MSCs, and augmented expression of *HMOX1*. Interestingly, intramuscular injection and topical administration of autologous MSCs accelerated ulcer healing in the clinical study [218]. Of note, number of circulating EPCs and HSPCs was not different between DFU group and healthy controls. Interestingly, in db/db diabetic mice, both EPCs and HSPCs are normally released from the bone marrow, but poorly home to the site of injury [219].

Noteworthy, infected ulcers were additionally associated with increased CD45^{dim}CD31$^+$CD34$^+$KDR$^+$ endothelial progenitors and CD45$^-$CD29$^+$CD90$^+$ mesenchymal stromal cells while numbers of hematopoietic stem and progenitor cells as well as VSEL cells remained low. We also found that the characteristic feature of patients with peripheral Charcot osteoneuropathy was a lowered level of plasma FGF-2, which may be related to the decreased number of endothelial progenitors preventing neuropathy in mice [220]. Noteworthy, FGF-2 has been found to be neuroprotective in the rat model of diabetic neuropathy [221] also when secreted by intramuscularly injected mesenchymal stromal cells [222].

Numbers of circulating stem and progenitor cells have been proposed as marker of disease progression [223]. However, our results suggest that when complications of type 2 diabetes, *i.e.* diabetic foot syndrome are more complex or healed the numbers of circulating cells also change and can reach levels observed in helathy controls. Therefore, they cannot be considered as a robust and specific marker of high prognostic value.

9.2. Exercise training in intermittent claudication: effects on antioxidant genes, inflammatory mediators and proangiogenic progenitor cells [73]

Intermittent claudication remains one of the major problems related to the POAD. Since the effectiveness of most pharmacotherapies, except for aspirin, pentoxyfilline and dipyridamole, remains low, training is the treatment of choice to improve the quality of life of patients with IC [84]. We have confirmed that treadmill training improves both PWFT and MWT in claudicant subjects. Furthermore, exercise

training tended to improve endothelial function measured with the FMD what corresponds to the published data [224]. Increase in FMD would probably reach the statistical significance in the larger group of patients [198]. Treadmill training did not change the influence of IC patients' plasma on the NOS3 Ser1177 phosphorylation. However, increased shear stress might have induced NOS3 activity *in situ* in the patients [225].

We demonstrated that number of CD45^{dim}CD34^{+}CD133^{-}KDR^{+} PACs was increased 3 h after the single exercise at the beginning of the training, but remained stable at day 90. This might suggest the attenuation of ischemic stress response induced by a single exercise after the training. PACs, enriched in the endothelial lineage committed cells, can be mobilized in response to ischemia and can incorporate to the vessels and take part in the vessel regeneration. On the other hand, increased number of circulating proangiogenic cells could have resulted from the shear stress induced differentiation of earlier progenitors [226], especially with no difference we observed in CD45^{-}CD133^{-}CD31^{bright} circulating endothelial cells (CEC), which are markers of vascular damage (data not shown).

Number of circulating CD3^{-}CD19^{-}CD33^{-}CD34^{+}CD133^{+}KDR^{+} cells tended to increase 10 minutes after the exercise in PAD patients who walked until the claudication-limiting symptoms occurred [227]. In other study, treadmill training augmented number of circulating CD45^{-}CD34^{+}CD133^{+}KDR^{+} cells after the 6 month long series of trainings. After 3 months, number of circulating latter cells tended to increase [228]. Our results suggest that mobilization of bone marrow derived PACs may require strong stimulus associated with ischemia during the intensive training. This might be the reason for the relatively weak influence of the exercise model applied in our study, which was limited to particular subpopulation of PACs.

Exhaustive and acute training increase the oxidative stress and may subsequently lead to the vascular damage. In our study, single exercise increased level of *SOD1* mRNA in peripheral blood TNCs both at the beginning of training and at day 90. On the other hand, single exercise decreased *HMOX1* expression in POAD patients on day 0, but not after training on day 90. Low level and reduced induction of HO-1 results in decreased antioxidative protection of cells, including endothelial cells [3]. Interestingly, treadmill exercise was shown to induce *HMOX1* expression in lymphocytes in young, healthy individuals in controlled treadmill test [229]. In other study, expression of *HMOX1* increased after the half-marathon run in granulocytes,

monocytes and lymphocytes of male trained subjects [230]. We may speculate, that increase in the ROS production caused by exercise could enhance the expression of antioxidant *SOD1*. Nevertheless, even in healthy individuals increase in lymphocyte antioxidant defence may be not sufficient to prevent oxidative damage [231]. Our results suggest that efficacy of the protective mechanisms can be reduced by exercise-induced downregulation of *HMOX1* in IC patients. Three-month training may improve the antioxidative response, through preventing the decrease in *HMOX1*. Additionally we observed a trend toward increased expression of *HMOX2* at day 90, whose cytoprotective functions are similar to *HMOX1*.

Pro-inflammatory IL-6, one of the biomarkers associated with the PAD [232,233] which was reported to correlate with the poorer walk performance [234], was decreased in response to the treadmill training in analysed group of IC patients. On the other hand, plasma level of MCP-1, potent monocyte chemoatractant associated with the PAD [235], was increased in the same trained subjects. It is notable that inflammatory mediators, especially MCP-1, has been shown to be involved in the arteriogenesis [236,237]. Interestingly, single exercise on day 0 decreased plasma MCP-1. Moreover, in our group of patients there was a positive correlation between MWT or PFWT at day 90 and concentration of IL-6, accompanied by a negative correlation with expression of anti-inflammatory miR-146a in TNC.

Plasma myeloperoxidase is up-regulated in healthy individuals subjected to the severe exercise *e.g.* marathon [238] or half-marathon [239]. MPO is also a biomarker associated with a higher risk of cardiovascular events in the PAD patients [240]. We found that plasma level of myeloperoxidase in IC subjects tended to increase in response to the single exercise at day 0, but remained stable over the trainings. Lack of MPO increase after the single exercise in trained IC patients can be related to the blunted neutrophil activation and degranulation [241]. Decreased level of total PAI-1 in response to the exercise after the training may suggest pro-fibrinolytic effect of the single exercise after the training. It was shown that PAD patients have disturbed fibrinolytic activity that is improved with the training [242]. Finally, plasma levels of sVCAM-1, sICAM-1, adiponectin, IL-1β, IL-8, IL-10, IL-12 IL-17, VEGF, IFN-γ, SDF-1α+β, G-CSF, Flt3 ligand, FGF-2, TPO, SCF and LIF remained stable after the single exercise and over the series of trainings. However, levels of sVCAM-1, sICAM-1 [243], IL-1β, IL-6 [244] might have increased at peak exercise and decrease after the

short rest (< 1 h), while IL-8 might have decreased [245]. Lack of changes in the plasma VEGF and FGF-2 is in concordance with previously published data [246].

In spite of the low number of patients analysed we have been able to detect changes in the number of circulating PACs, antioxidant gene response, and inflammatory mediators. Neither smoking nor comorbidities influenced the measured parameters. However, larger group of the claudicants would be necessary to confirm the effects of exercise on the mobilization of PAC subpopulations.

9.3. Atorvastatin enhances paracrine angiogenic activity of peripheral blood CD34^{+}-derived cells *in vitro*

Human peripheral blood CD34^{+}-derived cells stimulated with atorvastatin were characterized with enhanced paracrine activity *in vitro* but not in *in vivo*. However, this effect was rather not mediated by heme oxygenase-1 since *HMOX1* expression level was not affected by atorvastatin. On the other hand, increased expression of *HMOX1* induced in sulforaphane-treated cells did not lead to any changes in paracrine pro-angiogenic activity. Interestingly, conditioned media from C2C12 myoblast cells overexpressing heme oxygenase-1 had higher proangiogenic activity *in vivo* in ischemic hind limbs of *db/db* diabetic mice [247]. Media from the latter cells were devoid of anti-angiogenic PEGF and BGN, which were present in media from control cells, and contained more pro-angiogenic PPlase A, MIF, haptoglobin and galectin-1 [247].

High concentrations of atorvastatin were previously shown to inhibit HUVEC sprouting and growth factor-induced proliferation *in vitro* [248]. What is more, atorvastatin increased heme oxygenase-1 mRNA but did not influence HMOX1 protein levels [248]. Levels of HMOX1 were also unaffected in human microvascular endothelial cells (HMEC1) treated with clinically relevant concentration of atorvastatin (0.1 μmol/L) [249]. On the other hand, atorvastatin (and other statins) increased heme oxygenases' activity and heme oxygenase-1 but not 2 mRNA and protein levels in murine hearts and lungs *in vivo* [250]. In other experimental setting high doses of simvastatin and lovastatin were able to increase heme oxygenase-1 levels in HUVEC independently of NFκB and Nrf2 signalling [251]. Furthermore, *Hmox1* was also up-regulated by statins in RAW264.7 macrophages and its induction was mediated by p38 MAPK, ERK and protein kinase G [110].

Enhanced angiogenic properties of atorvastatin-stimulated cells observed *in vitro* in Matrigel tube formation assay were not confirmed with *in vivo* Matrigel plug

assay. What is more, conditioned media from CD34^{+}-derived cells stimulated with all compounds contained similar levels of growth factors and inflammatory mediators. Prolonged stimulation with atorvastatin increased levels of MCP-1, which can induce arteriogenesis [236,237]. However, atorvastatin was shown previously to inhibit MCP-1 production in rabbit vascular smooth muscle cells [252] and U937 monocytic cell line [253]. Finally, CD34^{+} monocytes can exert their proangiogenic activity with the release of miR-126 that is reduced in diabetic patients [254]. However, although miR-126 was decreased in EPCs isolated from peripheral blood of diabetic patients, it was not affected with atorvastatin treatment [255].

In our study the effects of atorvastatin on paracrine angiogenic activity of CD34^{+}-derived cells could be observed only *in vitro* and not *in vivo*. Levels of tested cytokines in media that enhanced angiogenesis were not changed. Therefore, the mechanism that is responsible for the observed phenomenom should be further investigated. Nevertheless, one may speculate that prolonged treatment of hypercholesterolemia with statins can also improve functions of local or circulating proangiogenic cells.

9.4. Mesenchymal stromal cells lacking *Hmox1* gene are similar to wild type cells but show enhanced antioxidant gene response

Murine bone marrow derived mesenchymal stromal cells isolated from *Hmox1*^{+/+} or *Hmox1*^{-/-} mice showed similar phenotype, proliferation, differentiation and ability to form tube-like structures on Matrigel. Neither proliferation nor angiogenic activity nor differentiation to basic lineages were affected with increased glucose concentrations in the culture media.

Lack of heme oxygenase-1 in murine bone marrow-derived mesenchymal stromal cells does not affect their phenotype or function

Unaffected phenotype of *Hmox1*^{-/-} MSCs as well as no changes in the differentiation to adipocytes, osteoblasts and chondrocytes was previously showed by Zarjou and co-workers [188]. On the other hand, Barbagallo and co-workers reported that expression of heme oxygenase-1 changes during the differentiation of human MSCs to osteoblasts [187] and treatment with osteogenic growth peptide increases *HMOX1* expression in human bone marrow MSC [184]. Moreover, human MSCs stimulated with CoPP during the osteogenic differentiation were characterized with up-regulated osteonectin, osteogenic growth peptide and osteocalcin, which were slightly

decreased when cells were cultured in high glucose concentration. Interestingly, CoPP decreased adipogenic differentiation of MSCs [184,187], while downregulation of *HMOX1* with siRNA resulted in enhanced adipogenesis.

What is more, human bone marrow MSCs treated with epoxyeicosatreinoic acid displayed the decreased levels of Bach1, repressor of *HMOX1* expression [256], and increased *HMOX1* mRNA while levels of PPARγ and C/EBPα, involved in adipogenesis, were decreased [183]. However hemin, heme oxygenase-1 substrate and potent inductor, surprisingly increased adipogenesis in murine 3T3L1 pre-adipocytes and human bone marrow MSCs [257]. Also, hemin (10 μmol/L) increased oxidative stress and induced DNA damage in murine pre-adipocytes. In accordance, cells treated with hemin expressed higher levels of PPARγ and aP2 while sirtuin 1 was decreased. All observed changes in gene expression induced with hemin could be reversed with anti-oxidant tempol therefore authors concluded, that oxidative stress is the key factor that regulates differentiation patterns in hemin-treated cells [257].

Vanella and co-workers reported later on that inhibition of adipogenesis induced with CoPP and increase with SnMP, could be linked to modulation of the canonical Wnt signalling [185]. Cells treated with CoPP were characterized with lower number of large fat droplets and higher numbers of small fat droplets. CoPP decreased production of TNF-α and increased adiponectin. Then, siRNA for HO-1 enhanced expression of aP2, C\EBPα and PPARγ while Shh, Pref-1, Wnt-10, β-catenin were decreased. Moreover, siRNA for Wnt10b increased lipid formation and inhibition of Dkk1, antagonist of Wnt, reduced lipid accumulation. Opposite effects were observed with CoPP treatment [185].

Nevertheless, the influence of heme oxygenase-1 on the adipogenesis and osteogenesis still seems to be controversial. Human bone marrow MSCs transduced with adenoviral vectors encoding for *Hmox1* showed no changes in differentiation pattern but improved viability in hypoxia [189,190]. However, adenoviral vectors give only transient expression of the transgene, which was lost after 2 weeks of culture [189]. Primary rat osteoblasts isolated from calvaria showed reduced expression and activity of alkaline phosphatase and reduced osteocalcin and Runx2 expression levels when treated with hemin or when transduced with adenoviral vectors harbouring *Hmox1* gene [191]. Similar results were obtained with carbon monoxide releasing molecule CORM-2 or bilirubin or when *Hmox1* expression was induced with prostaglandin J2 (PGJ2). Effects of hemin or PGJ2 could be reversed with heme

oxygenase-1 inhibitor zinc protoporphyrin [191]. In other study, calcification of human smooth muscle cells was inhibited when cells were stimulated with heme, what induced heme oxygenase-1 transcription, translation and enhanced its activity [258]. Moreover, iron and to lesser extent biliverdin but not carbon monoxide affected calcification. Importantly, effect of heme and heme oxygenase-1 was mediated by ferritin [258].

In our study mesenchymal stromal cells isolated from $Hmox1^{-/-}$ mice were characterized with basal levels of H_2O_2 similar to that of wild type cells. Furthermore, $Hmox1^{-/-}$ MSC were able to induce efficient response to hemin, which is a strong stress factor for cells lacking heme-degrading enzyme. One may speculate that relatively high resistance to oxidative stress observed in MSC $Hmox1^{-/-}$ can at least in part explain observed lack of differences in the pattern of differentiation to basic lineages.

Finally, conflicting results were published as well for the influence of Nrf2 and its other than $Hmox1$ target genes on osteogenesis and adipogenesis. Overexpression of Nrf2 in MC3T3-E1 osteoblastic cell line resulted in increased activity of $Hmox1$ promoter but also decreased Runx2-mediated stimulation of osteocalcin expression [259]. Nrf2 did not affect Runx2 expression but its inhibition was rather caused by physical interaction of both transcription factors. Moreover, Nrf2 negatively regulated osteocalcin promoter, which contains antioxidant response element [259].

$Nrf2$-deficient mice were characterized with lower amount of fat tissue and they were more resistant to fat induced obesity [260]. Murine embryonic fibroblasts isolated from $Nrf2^{-/-}$ individuals showed impaired adipogenesis. Moreover, stable knockout of $Nrf2$ in 3T3-L1 cells and human preadipocytes decreased expression of adipocyte markers $i.e.$ PPARγ and C\EBPα while knockout of Keap1, imhibitor of Nrf2, gave opposite result [260]. However, in other study $Nrf2^{-/-}$ murine embryonic fibroblasts showed increased capacity to differentiate to adipocytes what could be reversed with the dominant-positive Nrf2 [261]. Then, $Keap1^{-/-}$ cells displayed slower adipose differentiation [261].

One of the most important targets of Nrf2 is Nqo1. Levels of its mRNA increased while protein decreased during the adipocyte differentiation of 3T3-L1 cells [262]. Treatment with sulforaphane decreased triglyceride accumulation in 3T3-L1 cells and inhibited their differentiation [262]. Mice devoid of another target of Nrf2 – glutathione peroxidase 1 where protected from high fat diet-induced obesity and insulin resistance. What is more, antioxidant N-acetylcysteine decreased the enhanced insulin sensitivity, which was attributed to the PI3K/Akt signalling [263]. Then, reduction of

glutathione with buthionine-(S,R)-sulfoxime (BSO) in 3T3-L1 preadipocytes decreased adipogenesis and expression of aP2 while levels of cellular H_2O_2 were increased [264]. Moreover, mice devoid of *Gclm* subunit of glutamate-cystein ligase were characterized with reduced glutathione levels and higher resistance to high fat diet-induced obesity [265]. They had also higher H_2O_2 production in liver and up-regulated Nrf2-dependent genes such as *Hmox1*, *Gpx1*, and *Gsr*. Interestingly, *Gclm*$^{-/-}$ mice had higher blood insulin and better dealt with increased blood glucose levels [265].

In our hands, MSC *Hmox1*$^{-/-}$ showed rather fast and efficient activation of Nrf2-dependent genes, especially, *Nqo1* but, most importantly, genes responsible for the maintenance of total glutathione levels as well as reduction of GSSG. Therefore, we may make supposition that this compensatory mechanism can protect MSC *Hmox1*$^{-/-}$ from the detrimental effect that can be caused by reactive oxygen species.

Mesenchymal stromal cells can acquire *in vitro* some features of endothelial cells but did not enhance angiogenesis *in vivo*

Mesenchymal stromal cells were shown to be able to differentiate to endothelial cells, namely to acquire endothelial phenotype and ability to form tube-like structures in Matrigel assay [266]. In our experimental setting, bone marrow mesenchymal stromal cells were differentiated on dishes coated with gelatin and fibronectin in EGM-2MV medium. Indeed, MSCs up-regulated some endothelial markers such as *Kdr* and von Willebrand factor, increased binding of acetylated LDLs and *Bandeiraea simplicifolia* lectin. However, differentiated cells did not change their phenotype, especially they did not change levels of CD31, CD34 and Tie2 which are considered as *bona fide* endothelial markers. Furthemore, MSC-EC did not show enhanced angiogenic activity *in vivo*. It should be kept in mind that expression of CD106 and CD105 is not specific for endothelial cells and is commonly reported in mesenchymal stromal cells [115,116,267] while Tie-2 (CD202b) was also described as a marker of stromal cells with higher endothelial and adipocyte differentiation capacity [125]. Hamou and co-workers reported significant increase of CD31$^+$ bone marrow mesenchymal stromal cells when co-cultured with endothelial cells. However, starting population of cells contained already high fraction of CD31$^+$ [268]. One may speculate that observed increase could result rather from the expansion of pre-existing endothelial fraction than the endothelial differentiation. Authors reported as well that fraction of cultured mesenchymal stromal cells bound acetylated LDLs. Furthermore, there was an increase

of $CD31^+Kdr^+$ cells in adipose tissue-derived MSCs when cells were cultured in presence of VEGF and in low oxygen pressure. However, the fraction of double positive cells was only 1.5% [268] and could also result from enhanced growth of contaminating endothelial cells. Silva and co-workers showed that allogeneic canine mesenchymal stromal cells injected to the ischemic canine hearts contributed to the vascular endothelial cells and smooth muscle cells [269]. However, one must be aware that presence of double-positive cells can result not only from trans-differentiation and incorporation to the host tissue also from cell fusion [270].

Janeczek Portalska and co-workers reported, that human bone marrow MSCs do not up-regulate CD31, KDR and von Willebrand factor, unless additionally cultured for 24 hours on Matrigel and when treated with sheer stress (EL-MSC) [141]. Moreover, naïve, non-differentiated mesenchymal stromal cells were able to form tube-like structures on Matrigel faster than cells differentiated in EGM-2MV medium. EL-MSC cells stained as well with Ac-LDLs and enhanced vascularization of polymeric constructs *in vivo* [141]. On the other hand, also cardiac fibroblasts were able to acquire expression of VE-cadherin, endothelial nitric oxide synthase and junction protein claudin-5 in response to ischemia-reperfusion injury [271]. What is more, such cells did not express pericyte markers CD146 and NG2. Endothelial phenotype of fibroblast-derived endothelial cells was then confirmed with perfusion of animals with DiO fluorescent dye or acetylated LDLs that stained VE-cadherin cells [271].

Knockout of *Hmox1* does not affect differentiation of MSC to myofibroblasts

Mesenchymal stromal cells were shown to be derived from pericytes [272] that support and stabilize blood vessels [273]. Bone marrow MSCs can also act as pericytes [274] and form tube like structures on Matrigel alone [275] or when seeded together with endothelial cells [276]. In our experiments, bone marrow mesenchymal stromal cells formed both tube like structues in Matrigel assay and sprouts when seeded to form spheres. Lack of heme oxygenase-1 did not have significant influence the angiogenic properties of MSCs. However, it was previously shown that $Hmox1^{-/-}$ MSCs cultured for 9 days on Matrigel in EBM-2 medium had worse angiogenic properties [188] while overexpression of *Hmox1* in porcine bone marrow cells resulted in enhanced angiogenic activity [179].

We demonstrated that $Hmox1^{+/+}$ and $Hmox1^{-/-}$ mesenchymal stromal cells similarly reacted to the treatment with TGFβ1 and up-regulated α-smooth muscle actin.

However, its induction was lower in *Hmox1*^{-/-} MSC cells. Of note, expression of α-smooth muscle actin was present in some of non-differentiated cells (Figure 29). Moreover, miR-132 was lower in control non-differentiated *Hmox1*^{-/-} MSCs cultured in growth medium and decreased in differentiated *Hmox1*^{+/+} MSCs. MiR-132 inhibits vascular smooth muscle proliferation [277] but also influences myofibroblasts since it targets pro-fibrotic MeCP2 [278]. Myofibroblast transformation, which leads to α-smooth muscle actin *de novo* synthesis was associated with decrease in miR-132 expression [278]. Interestingly, miR-132 was an important factor that decreased Nrf2 and then heme oxygenase-1 expression in kidneys of mice treated with pro-fibrotic ochratoxin A [209]. Heme oxygenase-1, which is a direct target of miR-24, was shown to mediate some of the miR-24 effects on smooth muscle cell proliferation [279]. Overexpression of *HMOX1* in smooth muscle cells to some extent reduced smooth muscle cell autophagy, which was induced by miR-24 [279].

In our hands lack of heme oxygenase-1 in murine bone marrow-derived mesenchymal stromal cells had little or no effect on expression of fibroblast and myofibroblast markers in cells treated with TGFβ1. Again, lack of effect can be probably linked to relatively high resistance of MSC *Hmox1*^{-/-} to oxidative stress.

Mesenchymal stromal cells *Hmox1*^{-/-} do not inhibit SCC VII-*luc* tumour growth *in vivo*

Mesenchymal stromal cells and heme oxygenase-1 were shown to influence tumour growth. Interstingly, SCC VII tumours with *Hmox1*^{+/+} but not *Hmox1*^{-/-} MSCs were smaller. The latter ones were the only to increase luminescence over the course of the experiment. Although MSCs were allogeneic (from C57BL6 × FVB mice) to recipient mice and SCC VII-luc cells (both C3H strain), they did not induce infiltration with immune cells. The only population that was different in tested tumours was CD11b^{+}Ly-6C^{hi}Ly-6G^{-}F4/80^{+}CD43^{+} fraction of macrophages which was more abundant in tumours with MSC *Hmox1*^{+/+}. CD43 is considered as a marker of more proinflammatory macrophages that can give rise of dendritic cells but also is highly expressed in Tie2^{+} macrophages [280].

A CD45$^-$CD31$^+$

B Percent of vascularisation
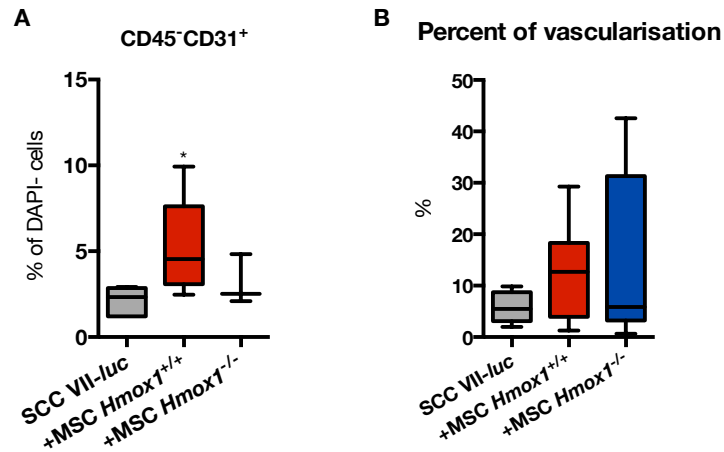
Figure 52. Fraction of CD45$^-$CD31$^+$ endothelial cells assessed with flow cytometry (N=3-5) **(A)** and perfusion measured with VEVO2100 ultrasonography system with 3D and PowerDoppler modes (N=12-15) **(B)** in tumours with or without $Hmox1^{+/+}$ or $Hmox1^{-/-}$ MSCs. Data shown as mean + SD, $^*p<0.05$, One-way Anova. Courtesy of mgr Joanna Markiewicz.

The latter population is often considered crucial for enhancement of the tumour angiogenesis [281]. However, fraction of Tie2$^+$ macrophages was similar in tumours with either $Hmox1^{+/+}$ or $Hmox1^{-/-}$ MSCs. Mesenchymal stromal cells could also influence tumour vascularization. However, analyses performed by mgr Joanna Markiewicz showed, that SCC VII tumours with $Hmox1^{+/+}$ MSCs contained more endothelial cells but similar numbers of blood vessels and similar perfusion (Figure 52).

Mesenchymal stromal cells $Hmox1^{-/-}$ show high resistance to hemin regardless of increased H$_2$O$_2$

Surprisingly, both $Hmox1^{+/+}$ and $Hmox1^{-/-}$ MSCs showed high resistance to H$_2$O$_2$ or, even more unexpectedly, to hemin, regardless of the concentration of glucose in the medium. Cells devoid of the heme oxygenase-1, enzyme degrading heme, were supposed to be highly sensitive to hemin. Free heme is toxic to the cells and increases oxidative stress that may lead to lipid peroxidation, DNA damage and protein aggregation (reviewed in [282]). Interestingly, $Hmox1^{-/-}$ mesenchymal stromal cells were resistant to hemin concentrations that potently induced cell death in bone marrow PAC cells [4]. Moreover, $Hmox1^{-/-}$ MSCs expressed lower levels of heme oxygenase-2 than PAC cells, therefore it cannot be considered as a rescue pathway. Finally, $Hmox1^{+/+}$ MSCs were characterized with lower $Hmox1$ levels than PAC cells and had similar $Hmox1$ expression in comparison to fibroblasts.

Although 50 μmol/L hemin showed no toxicity in $Hmox1^{-/-}$ MSCs it was not harmless for the cells since it increased concentration of hydrogen peroxide in both tested $Hmox1^{-/-}$ cell types. Of note, levels of H$_2$O$_2$ were higher in $Hmox1^{-/-}$ fibroblasts

than in $Hmox1^{-/-}$ MSCs after 24 hours of treatment with hemin and in $Hmox1^{+/+}$ fibroblasts than in $Hmox1^{+/+}$ MSCs after 48 of culture. However, neither in fibroblasts nor in MSCs we found any differences in basal levels of H_2O_2. Previously, higher H_2O_2 levels were reported in $Hmox1^{-/-}$ than $Hmox1^{+/+}$ iPS cells [283].

Increased protein carbonylation and lipid peroxidation was also reported in livers and kidneys of $Hmox1^{-/-}$ mice [284]. Interestingly, basal intracellular ROS levels in human MSCs and fibroblasts were similar and lower than ROS in INS-1 insulinoma [285]. Both MSCs and fibroblasts were characterized with similar levels of *SOD1*, *SOD2, CAT* and *GPX1* mRNA, and higher activities of catalase and glutathione peroxidase-1 than in INS-1 cells [285]. Of note, the concentration of hemin, which was toxic for MSC $Hmox1^{-/-}$ cells, caused also some increase in cell death in $Hmox1^{+/+}$ MSCs.

So far, hemin toxicity was studied mainly in neurons, especially in the context of hemorrhages. Hemin toxicity in astrocytes was shown to involve phosphorylation of ERK1/2 and was attenuated with MEK inhibition [286]. Furthermore, cortical cells isolated from mice devoid of heme oxygenase-2, which is highly abundant in neurons and crucial for their survival in ischemic conditions [287], were more resistant to hemin-induced oxidative stress and cell death [288]. Similar effect was obtained when neurons were co-treated with iron chelator. Moreover, valproic acid decreased heme oxygenase-1 levels in cortical neurons and prevented hemin-induced cytotoxicity [289].

We tested if high MSC resistance to hemin can result from low import of free heme. Interestingly, heme uptake in $Hmox1^{+/+}$ and $Hmox1^{-/-}$ MSCs did not differ. The latter results were mirrored by the expression of *Slc46a1* and *Slc48a1* − heme transporters. Expression of *Slc48a1* was shown, however, to be regulated by Bach1 [290], which represses *Hmox1* and responds to increased heme concentration. On the other hand, hemin increased expression of heme exporter *FLVCR1* only in $Hmox1^{-/-}$ MSCs but not in $Hmox1^{+/+}$ MSCs or in fibroblasts, regardless of their genotype. Increased *FLVCR1* was previously reported in kidneys of $Hmox1^{-/-}$ mice [291]. *FLVCR1* has a high specificity for planar porphyrins but does not transport bilirubin [292].
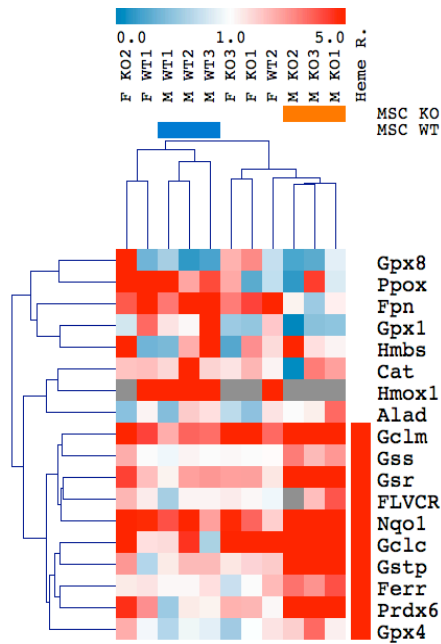
Figure 53. Changes in antioxidant gene response in fibroblasts and *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ MSCs treated for 6 hours with 50 μmol/L hemin. Each square represents fold difference in gene expression versus control that was not treated with hemin. Clusters of genes and samples were generated with MeV software.

As expected, hemin decreased expression of 5'-aminolevulinate synthase 1 in all tested cell types. Alas1 is heme synthesis rate limiting enzyme, whose levels are tightly regulated because of the presence of heme regulatory motif [293] in its promoter. Other enzymes involved in the heme synthesis were not changed and did not differ between *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ cells. With regard to the iron metabolism, hemin-treated *Hmox1*$^{+/+}$ MSCs up-regulated ferroportin. *Fpn* expression did not change in MSC *Hmox1*$^{-/-}$ cells but they showed trend towards higher ferroportin levels than wildtype controls. On the other hand *Hmox1*$^{-/-}$ but not *Hmox1*$^{+/+}$ MSCs or any of fibroblast cells increased ferritin that captures labile iron and therefore protects cells from oxidative stress (reviewed in [282]).

Importantly, *Hmox1*$^{-/-}$ mesenchymal stromal cells up-regulated in response to hemin a set of genes involved in antioxidant defence, namely *Sod2, Prdx3, Prdx6, Cat, Gclc, Gclm, Gss, Gsr, Gstp1* (Figure 53), which all can be regulated by the Nrf2 transcription factor. Especially, hemin induced expression of enzymes involved in both synthesis and metabolism of glutathione. Bilirubin, which is rapidly formed by biliverdin reductase from biliverdin, product of heme oxygenase activity, is a potent antioxidant [294] that have properties complementary to glutathione [295]. Although glutathione has much higher cellular concentration than bilirubin, it protects mainly hydrophilic proteins. On the other hand, lipophilic bilirubin can protect lipids. In our

hands, MSC *Hmox1*^{-/-} cells devoid of heme oxygenase-1 did not change expression of heme oxygenase-2, another source of cellular biliverdin.

High levels of glutathione were reported previously in both human fibroblast and mesenchymal stem cells when compared to INS-1 cells. Furthermore, glutathione depletion in MSC increased their sensitivity to H_2O_2, S-nitroso-*N*-acetylpenicillamine and 3-morpholinosyndonimine hydrochloride [285].
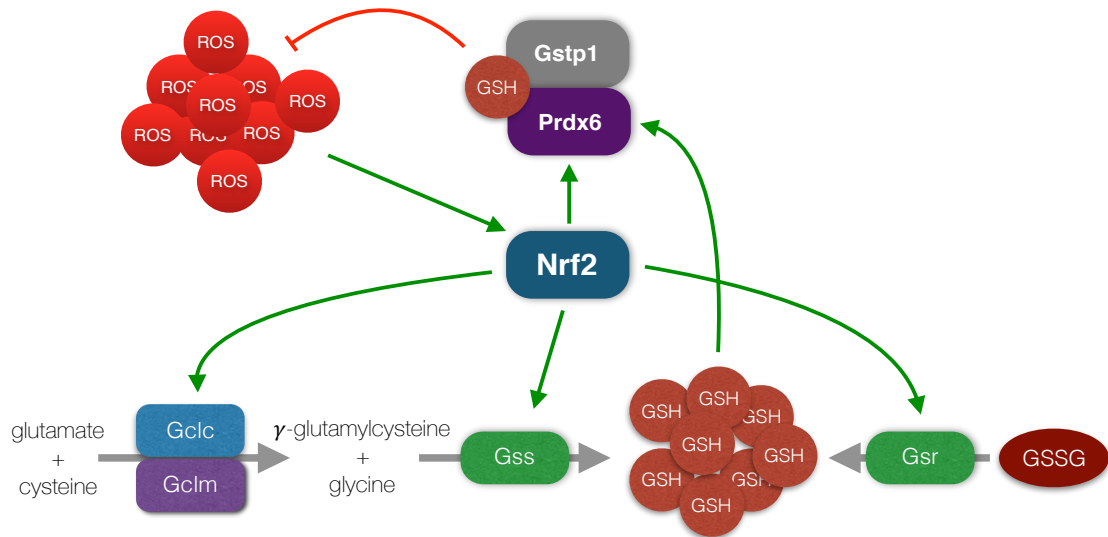
Figure 54. Nrf2 induces expression of enzymes involved in glutathione synthesis and metabolism as well as peroxiredoxin 6.

Increased expressions of γ-glytamylcysteine ligase and glutathione synthethase lead to enhanced production of glutathione while up-regulated glutathione reductase restores GSH from GSSG. In our experimental setting, GSH to GSSG ratio, which is an indication of cell redox status, decreased in all tested cells treated with hemin. However, the decrease was much stronger in *Hmox1*^{-/-} fibroblasts than in *Hmox1*^{-/-} MSCs.

Hmox1^{-/-} fibroblasts were characterized with higher total GSSG levels, while *Hmox1*^{-/-} MSCs were the only to increase total GSH. Of note, HUVEC cells with long alleles of *HMOX1* promoter, and thus lower levels of HMOX1, treated with H_2O_2 had higher concentrations of total glutathione and GSSG, but lower GSH/GSSG ratio than cells with short promoter [3]. Furthermore, hemin was shown to induce neuronal necroptosis, which was related to depletion of glutathione [296]. Cell death in neurons was preceded by increase of NFκB and induction of COX2, TNF-α and NOS2. What is more, hemin-induced necroptosis could be prevented with desferroxamine, which chelates iron, or with antioxidants such as tempol, trolox or ascorbic acid. Decrease in

GSH was related to the enhanced NFκB signaling and elevated levels of peroxinitrite [296].

Importantly, increased expression of glutathione metabolism genes in $Hmox1^{-/-}$ MSCs was accompanied with the up-regulation of peroxiredoxin 6. Prdx6 is the only 1-cys peroxiredoxin, which uses glutathione instead of thioredoxin, and works as a heterodimer with glutathione S transferase π (reviewed in [297]), which was also upregulated in hemin-treated MSC $Hmox1^{-/-}$. Furthermore, peroxiredoxin 6 has double activity: peroxidase and phospholipase A_2 [297]. Interestingly, slightly higher levels of peroxiredoxin 6 were reported in human mesenchymal stromal cells than in embryonic stem cells [298]. Furthermore, both peroxiredoxin 6 and glutathione S transferase π but also peroxiredoxins 1 and 2 were highly abundant in human MSCs [299]. Expression of peroxiredoxin 6 was not changed in late passage MSCs in comparison to early passage MSCs, although aged and more senescent cells showed increased H_2O_2 concentration [300]. In another study, aged MSCs were characterized rather with increased peroxiredoxin 5 expression [301]. Surprisingly, hemin treatment, which is used to induce erythroid differentiation of K562 erythroleukemia cells, decreased peroxiredoxin 6 levels in K562 cells [302].

In our hands, increased expression of peroxiredoxin 6 and other antioxidant genes should be rather considered as a protective mechanism that allows cells to deal better with oxidative stress than bone marrow-derived proangiogenic cells. Although levels of H_2O_2 in fibroblasts and MSCs were similar, only the latter cells were able to upregulate peroxiredoxin 6 and its partner – glutathione S transferase π and survive even in high glucose concentration.

Mesenchymal stromal cells isolated form $Hmox1^{-/-}$ mice were able to react to oxidative stress better than fibroblast and faster recovered glutathione. However, the mechanism of that resistance is still unknown and requires more studies. One may speculate that mesenchymal stromal cells can express lower levels of Keap1 or Nrf3 that can both decrease activity of Nrf2 transcription factor. Nevertheless, our data show that cells such as mesenchymal stromal cells are better equipped with the measures to deal with harsh conditions than other bone marrow-derived cells, especially proangiogenic cells. Furthermore, we can speculate that certain cell types are less dependent on heme oxygenase-1 which is considered as crucial cytoprotective enzyme. Finally, low levels of basal H_2O_2 and efficient antioxidant gene response observed in mesenchymal stromal cells $Hmox1^{-/-}$ can in part explain lack of differences in other

tested characteristics of MSCs.

10.   Summary and conclusions

The current study aimed to evaluate effects of various stress factors, especially diabetes and its complications or exercise or training in patients with intermittent claudications on circulating progenitor cells. We also carefully characterized effects of *Hmox1* knockout on bone marrow-derived mesenchymal stromal cells, an important component of bone marrow stem cell niche. Surprisingly, our data suggest that mesenchymal stromal cells are a rare case of cells that can deal with various stress factors even when they lack heme oxygenase-1 – cytoprotective enzyme that is considered crucial for stem cell survival and often exploited to improve results of stem cell therapies.

In summary, although our study included a limited number of subjects, the results indicate that patients with T2DM and different forms of DFS have an altered number of circulating stem cells. T2DM may also be associated with a changed serum growth factor and antioxidant gene expression profile, especially both tested isoforms of heme oxygenases. Altogether, these factors can contribute to the pathogenesis of different DFS forms [24]. Furthermore, numbers of circulating stem and progenitor cells in type 2 diabetes patients are not robust and specific enough to serve as markers of disease progression or to faster predict the occurrence of diabetes complictions.

Treadmill training improves walking performance of IC patients, attenuates the single exercise induced changes in gene expression profile or in the number of $CD45^{dim}CD34^+CD133^-KDR^+$ PACs, but may also result in a higher production of some inflammatory mediators [73]. Therefore one may consider increased number of circulating proangiogenic cells as a marker of general stress rather than a sign of improved regeneration. Furthermore, another marker of stress response in non-trained patients after single exercise was higher expression of heme oxygenase-1 in peripheral blood total nucleated cells.

Human peripheral blood $CD34^+$-derived cells mobilized with G-CSF have paracrine angiogenic activity. Therefore, such cells may serve as a source of conditioned media that could possibly be used as a treatment for diseases that result from impaired tissue regeneration. Furthermore, atorvastatin can enhance paracrine angiogenic activity of human $CD34^+$-derived cells *in vitro* but the observed effect was

not observed *in vivo*. Heme oxygenase-1 was not affected by atorvastatin treatment and *HMOX1* up-regulation with sulforaphane did not affect properties of tested cells.

Mesenchymal stromal cells lacking *Hmox1* gene show similar properties to wild type control cells. However, they did not inhibit SCC VII tumour growth *in vivo* and were characterized with enhanced antioxidant gene response. Therefore, one may speculate that expression of heme oxygenase-1 is crucial for the anti-tumour activity of mesenchymal stromal cells.

Lack of the effects of *Hmox1* knockout observed in murine bone marrow stromal cells can be in part explained with their relatively high resistance to hemin and oxidative stress. Mesenchymal stromal cells *Hmox1*$^{-/-}$ were able to survive better in presence of hemin and/or high glucose concentration than murine bone marrow-derived proangiogenic cells. MSC *Hmox1*$^{-/-}$ efficiently induced a vast panel of antioxidant response genes and were able to recover level of reduced glutathione.

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